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(54) Title: CONJUGATES COMPRISING HYDROXYALKYL STARCH AND A CYTOTOXIC AGENT AND PROCESS FOR THEIR PREPARATION

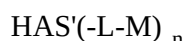
(57) Abstract: The present invention relates to hydroxyalkyl starch conjugates and a method for preparing the same, the hydroxyalkyl starch conjugate comprising a hydroxyalkyl starch derivative and a cytotoxic agent, the cytotoxic agent comprising at least one primary hydroxyl group, wherein the hydroxyalkyl starch is linked via said primary hydroxyl group to the cytotoxic agent. The conjugates according to the present invention have a structure according to the following formula $HAS'(-L-M)_n$ wherein M is a residue of the cytotoxic agent, L is a linking moiety, HAS' is the residue of the hydroxyalkyl starch derivative, and n is greater than or equal to 1, and wherein the hydroxyalkyl starch derivative has a mean molecular weight (MW) above the renal threshold and a molar substitution (MS) in the range of from 0.6 to 1.5.



WO 2012/004009 AI

Conjugates comprising Hydroxyalkyl Starch and a cytotoxic agent and process for their Preparation

The present invention relates to hydroxyalkyl starch conjugates comprising a hydroxyalkyl starch derivative and a cytotoxic agent, the cytotoxic agent comprising at least one primary hydroxyl group, wherein the hydroxyalkyl starch is linked via said primary hydroxyl group to the cytotoxic agent. The conjugates according to the present invention have a structure according to the following formula



wherein M is a residue of the cytotoxic agent, L is a linking moiety, HAS' is the residue of the hydroxyalkyl starch derivative, and n is greater than or equal to 1, and wherein the hydroxyalkyl starch derivative has a mean molecular weight (MW) above the renal threshold, preferably a mean molecular weight MW greater than or equal to 60 kDa, more preferably in the range of from 80 to 1200 kDa, and more preferably of from 90 to 800 kDa, and a molar substitution (MS) in the range of from 0.6 to 1.5. Moreover, besides the conjugate, the invention relates to the method for preparing said conjugate and conjugates obtained or obtainable by said method. Further, the invention relates to the HAS cytotoxic agent conjugates for the treatment of cancer as well as to pharmaceutical compositions comprising these conjugates for the treatment of cancer.

Hydroxyalkyl starch (HAS), in particular hydroxyethyl starch (HES), is a substituted derivative of the naturally occurring carbohydrate polymer amylopectin, which is present in corn starch at a concentration of up to 95 % by weight, and is degraded by other amylases in the body. HES in particular exhibits advantageous biological properties and is used as a blood volume replacement agent and in hemodilution therapy in clinics (Sommermeyer *et al*, 1987, Krankenhauspharmazie, 8(8): 271-278; Weidler *et al*, 1991, Arzneimittelforschung/Drug Research, 41: 494-498).

Cytotoxic agents are natural or synthetic substances which decrease the cell growth. A major drawback of many cytotoxic agents is their extreme low water solubility which renders the *in vivo* administration of the agent extremely complicated. Thus, this poor water solubility usually has to be overcome by complex formulation techniques including various excipients, wherein these excipients usually also show toxic side effects. As an example, the emulsifier Cremophor EL and ethanol, which are used to formulate taxol-based agents in order to deliver the required dosis of these taxol-based agents *in vivo*, shows toxic effects such as vasodilation, dyspnea, and hypotension. In particular, Cremophor EL has also been shown to cause severe anaphylactic hypersensitivity

reactions, hyperlipidaemia, abnormal lipoprotein patterns, aggregation of erythrocytes and peripheral neuropathy ("Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation", European Journal of Cancer", Volume 31, Issue 13, Pages 1590-1598). In fact, the maximum dose of, for example paclitaxel, a taxol-based cytotoxic agent that can be administered to mice by injection, is dictated by the acute lethal toxicity of said Cremophor EL vehicle.

This is one reason why the potential use of soluble prodrugs, in particular macromolecular prodrugs, as a means of administering biologically effective cytotoxic agents to mammals has been proposed. Such prodrugs include chemical derivatives of the cytotoxic agents which, upon administration, will eventually liberate the active parent compound *in vivo*.

Besides the enhancement of the water solubility of the drug, prodrugs have been proposed to provide an advantageous targeting and/or an enhancement of the stability of the therapeutic agent. Further, such prodrugs were suggested to prolong the circulation lifetime, to provide an extended duration of activity, or to achieve a reduction of side effects and drug toxicity. Thus, besides the preparation of prodrugs of water insoluble cytotoxic agents, providing prodrugs of water soluble cytotoxic agents is also of high interest in order to modify the onset and/or duration of action of the cytotoxic agent *in vivo*.

A typical example in the preparation of prodrugs of cytotoxic agents involves the conversion of alcohols or thioalcohols to either organic phosphates or esters (Remington's Pharmaceutical Science, 16th ed., A. Ozols (ed.), 1980).

Numerous reviews have described the potential application of macromolecules as high molecular weight carriers for cytotoxic agents yielding in polymeric prodrugs of said agents. It was proposed that by coupling the cytotoxic agents to polymers, it is possible to increase the molecular weight and size of the prodrug so that the weight and size of the prodrugs are too high to be quickly removed by glomerular filtration in the kidney and that, as consequence, the plasma residence time can be drastically increased.

Most modifications to date have been carried out with polyethylene glycol or similar polymers with polyethylene glycol (PEG) being generally preferred as polymer because of its easy availability and the possibility to give defined products upon reaction of limited available functional groups for coupling to a cytotoxic agent being present in PEG.

For example, WO 93/24476 discloses conjugates between taxane-based drugs, such as paclitaxel, to polyethylene glycol as macromolecule. In these conjugates, paclitaxel is linked to the polyethylene glycol using an ester linkage.

Similarly, US 5,977,163 describes the conjugation of taxane-based drugs, such as paclitaxel or docetaxel, to similar water soluble polymers such as polyglutamic acid or polyaspartic acid.

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Likewise, polyethylene glycol conjugates with cytotoxic agents, such as camptothecins, are disclosed in WO 98/07713. According to WO 98/07713, the polymer is linked via a linker to a hydroxyl function of the cytotoxic agent providing an ester linkage which allows for a rapid hydrolysis of the polymer drug linkage *in vivo* to generate the parent drug. This is achieved by using a linker comprising an electron-withdrawing group in close proximity to the ester bond. No polysaccharide-based conjugates were disclosed in WO 98/07713.

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In a similar way, the influence of sterically demanding groups on the release rate of cytotoxic agents being incorporated into polyethylene glycol conjugates has been described in WO 01/146291A1.

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US 6,395,266 B1 discloses branched PEG polymers linked to various cytotoxic agents. The branched polymers are considered to be advantageous compared to linear PEG conjugates since a higher loading of parent drug per unit of polymer can be achieved. The actual activity of these conjugates *in vivo* for the treatment of cancer was, however, not shown.

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Similar to US 6,395,266 B1, EP 1 496 076 A1 discloses Y-shaped branched hydrophilic polymer derivatives conjugated to cytotoxic agents such as camptothecin. Again, the actual activity of these conjugates *in vivo* was not shown.

In a similar way, the following patent and non-patent literature discloses PEG conjugates: Greenwald *et al.*, J. Med. Chem., 1996, 39: 424-431 and US 5,840,900.

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PEG, however, is known to have unpleasant or hazardous side effects such as induction of antibodies against PEG (N. J. Ganson, S. J. Kelly *et al.*, Arthritis Research & Therapie 2006, 8:R12) and nephrotoxicity (G. A. Laine, S. M. Hamid Hossain *et al.*, The Annals of Pharmacotherapy, 1995 November, Volume 29) on use of such PEG or PEG-related conjugates. In addition, the biological activity of the active ingredients is most often greatly reduced in some cases after the PEG coupling. Moreover, the metabolism of the degradation products of PEG conjugates is still substantially unknown and possibly represents a health risk. Further, the functional groups available for coupling to cytotoxic agents are limited, so a high loading of the polymer with the respective drug is not possible.

35

Thus, there is still a need for physiologically well tolerated alternatives to such PEG conjugates with which the residence time of low molecular weight substances in the plasma can be increased and/or the efficacy of these drugs can be increased and/or non-specific toxicity can be decreased. Further, there is the need for macromolecular prodrugs which provide an advantageous targeting of the tumor and/or which, upon administration, will eventually liberate the active parent compound *in vivo* with improved pharmacodynamic properties.

It would be particularly desirable to provide prodrugs which take advantage of the so-called Enhanced Permeability and Retention (EPR) effect. This EPR effect describes the property by which certain sizes of molecules, such as macromolecules or liposomes, tend to accumulate in tumor tissue much more than they do in normal tissue (reference is made to respective passages of US 6,624,142 B2; or to Vasey P. A., Kaye S. B., Morrison R., *et al.* (January 1999) "Phase I clinical and pharmacokinetic study of PK1 [N-(2-hydroxypropyl)methacrylamide copolymer doxorubicin]: first member of a new class of chemotherapeutic agents-drug-polymer conjugates. Cancer Research Campaign Phase I/II Committee". *Clinical Cancer Research* 5 (1): 83-94). The general explanation for that effect is that tumor vessels are usually abnormal in form and architecture. This is due to the fact that, in order for tumor cells to grow quickly, they must stimulate the production of blood vessels.

Without wanting to be bound to any hypothesis, it is contemplated that the EPR effect allows for an enhanced or even substantially selective delivery of macromolecules to the tumor cells and as consequence, enrichment of the macromolecules in the tumor cells, when compared to the delivery of these molecules to normal tissue.

WO 03/074088 describes hydroxyalkyl starch conjugates with, for example, cytotoxic agents such as daunorubicin, wherein the cytotoxic agent is usually directly coupled via an amine group to the hydroxyalkyl starch yielding in 1:1 conjugates. No use of these conjugates *in vivo* was shown. Further, in WO 03/074088 no cleavable linkage between the cytotoxic agent and hydroxyalkyl starch was described, which, upon administration, would be suitable to readily liberate the active drug *in vivo*.

Thus, there is still the need to provide new prodrugs of cytotoxic agents being bound to advantageous polymers for the treatment of cancer *in vivo*.

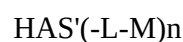
Thus, it is an object of the present invention to provide novel conjugates comprising a polymer linked to a cytotoxic agent. Further, it is an object of the present invention to provide a method for preparing such conjugates. Additionally, it is an object of the present

invention to provide pharmaceutical compositions comprising these novel conjugates as well as the use of the conjugates and the pharmaceutical composition, respectively, in the treatment of cancer.

5 Surprisingly, it was found that linking of a cytotoxic agents via a primary hydroxy! group to a hydroxyalkyl starch derivatives may lead to conjugates showing at least one of the desired beneficial properties, such as improved drug solubility, and/or optimized drug residence time *in vivo*, and/or reduced toxicity, and/or high efficiency, and/or effective targeting of tumor tissue *in vivo*. Without wanting to be bound to any theory, it is believed
10 that the specific biodegradable hydroxyalkyl starch polymers of the invention may exhibit an optimized size, characterized by specific values of MW, which is large enough to prevent the elimination of the intact conjugate - comprising the polymer and the cytotoxic agent - through the kidney prior to any release of the cytotoxic agent. Thus, rapid elimination of the cytotoxic agent through the kidney by filtration through pores may be
15 avoided. Further, the specific biodegradable hydroxyalkyl starch polymers of the invention comprised in the conjugate may exhibit an optimized molar substitution MS, and/or the conjugate as such may exhibit a preferred overall chemical constitution, so as to allow for a degradability of the hydroxyalkyl starch polymer comprised in the conjugate and release of the cytotoxic agent in favorable time range. Further, it is believed that in contrast to most
20 of the polymers described in the prior art, such as polyethylene glycol and derivatives thereof, the polymer fragments obtained from degradation of the conjugate of the present invention can be removed from the bloodstream by the kidneys or degraded via the lysosomal pathway without leaving any unknown degradation products of the polymer in the body.

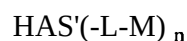
25 Without wanting to be bound to any theory as to how the conjugates of the invention might operate, it is further believed that at least some of the conjugates of the invention might be able to deliver the respective cytotoxic agent into extracellular tissue space, such as into tissue exhibiting an EPR effect. However, it has to be understood that it is not intended to
30 limit the scope of the invention only to such conjugates which take advantage of the EPR effect; also conjugates which show, possibly additionally, different advantageous characteristics, such as advantageous activity and/or low toxicity *in vivo* due to alternative mechanisms, are encompassed by the present invention.

35 Thus, the present invention relates to hydroxyalkyl starch (HAS) conjugate comprising a hydroxyalkyl starch derivative and a cytotoxic agent, said conjugate having a structure according to the following formula



wherein M is a residue of a cytotoxic agent, and wherein the cytotoxic agent comprises a primary hydroxyl group, L is a linking moiety (linking the HAS derivative and M), HAS' is a residue of the hydroxyalkyl starch derivative, n is greater than or equal to 1, and wherein the hydroxyalkyl starch derivative has a mean molecular weight MW above the renal threshold, preferably a MW greater than or equal to 60 kDa, and a molar substitution MS in the range of from 0.6 to 1.5, and wherein the linking moiety L is linked to a primary hydroxyl group of the cytotoxic agent.

Further, the present invention also relates to a method for preparing a hydroxyalkyl starch (HAS) conjugate comprising a hydroxyalkyl starch derivative and a cytotoxic agent, said conjugate having a structure according to the following formula



wherein

M is a residue of a cytotoxic agent, said cytotoxic agent comprising a primary hydroxyl group, L is a linking moiety, HAS' is a residue of the hydroxyalkyl starch derivative, and n is equal or greater than 1, said method comprising

- (a) providing a hydroxyalkyl starch (HAS) derivative having a mean molecular weight MW above the renal threshold, preferably a mean molecular weight MW greater than or equal to 60 kDa, and a molar substitution MS in the range of from 0.6 to 1.5, said HAS derivative comprising a functional group Z¹; and providing a cytotoxic agent comprising a primary hydroxyl group;
- (b) coupling the HAS derivative to the cytotoxic agent via an at least bifunctional crosslinking compound L comprising a functional group K¹ and a functional group K², wherein K² is capable of being reacted with Z¹ comprised in the HAS derivative and wherein K¹ is capable of being reacted with the primary hydroxyl group comprised in the cytotoxic agent.

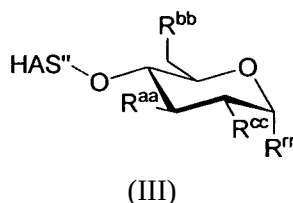
Moreover, the present invention relates to a hydroxyalkyl starch conjugate obtainable or obtained by the above-mentioned method.

The term "linked to the primary hydroxyl group of the cytotoxic agent" as used in the context of the present invention is denoted to mean that the cytotoxic agent is reacted via its primary group. The resulting conjugated residue of the cytotoxic agent M is thus linked via an -O- group to the linking moiety -L- wherein the oxygen of this -O- group corresponds to the oxygen of the reacted primary hydroxyl group cytotoxic agent.

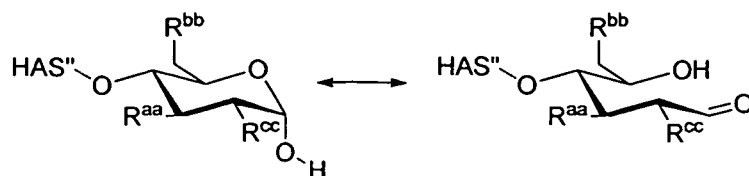
Further, the present invention relates to a pharmaceutical compound or composition comprising the hydroxyalkyl starch conjugate or the hydroxyalkyl starch conjugate obtainable or obtained by the above-mentioned method. In addition, the present invention relates to the hydroxyalkyl starch conjugate as described above, or the pharmaceutical composition as described above, for the use as a medicament, in particular for the treatment of cancer. Moreover, the present invention relates to the use of the hydroxyalkyl starch conjugate as described above, or the pharmaceutical composition as described above for the manufacture of a medicament for the treatment of cancer. Moreover, the present invention relates to a method of treating a patient suffering from cancer comprising administering a therapeutically effective amount of the hydroxyalkyl starch conjugate as described above, or the pharmaceutical composition as described above.

The hydroxyalkyl starch

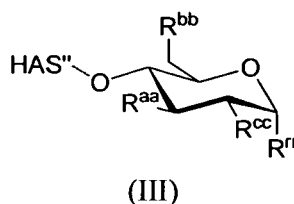
- 15 In the context of the present invention, the term "hydroxyalkyl starch" (HAS) refers to a starch derivative having a constitution according to the following formula (III)



- 20 wherein the explicitly shown ring structure is either a terminal or a non-terminal saccharide unit of the HAS molecule and wherein HAS'' is a remainder, i.e. a residual portion of the hydroxyalkyl starch molecule, said residual portion forming, together with the explicitly shown ring structure containing the residues R^{3a} , R^{bb} and R^{cc} and R^r the overall HAS molecule. In formula (III), R^{3a} , R^{bb} and R^{cc} are independently of each other hydroxyl, a linear or branched hydroxyalkyl group or -O-HAS'', in particular R^{3a} , R^{bb} and R^{cc} are independently of each other $-[O-(CR^wR^x)-(CR^yR^z)]_x-OH$ or -O-HAS'', wherein R^w , R^x , R^y and R^z are independently of each other selected from the group consisting of hydrogen and alkyl, x is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4. Preferably, R^{3a} , R^{bb} and R^{cc} are independently of each other -O-HAS'' or $-[O-CH_2-CH_2]_s-OH$ with s being in the range of from 0 to 4. In particular, R^{aa} , R^{bb} and R^{cc} are independently of each other -OH, $-O-CH_2-CH_2-OH$ (2-hydroxyethyl), or -O-HAS''. Residue R^r is -O-HAS'' in case the explicitly shown ring structure is a non-terminal saccharide unit of the HAS molecule. In case the explicitly shown ring structure is a terminal saccharide unit of the HAS molecule, R^r is -OH, and formula (III) shows this terminal saccharide unit in its hemiacetal form. This hemiacetal form, depending on e.g. the solvent, may be in equilibrium with the free aldehyde form as shown in the scheme below:

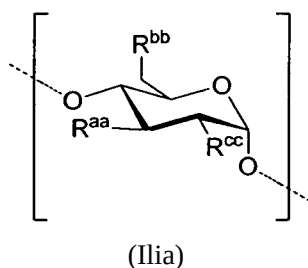


The term O-HAS" as used in the context of the residue R^{π} as described above is, in addition to the remainder HAS" shown at the left hand side of formula (111), a further remainder of the HAS molecule which is linked as residue R^{π} to the explicitly shown ring structure of formula (III)



and forms, together with the residue HAS" shown at the left hand side of formula (III) and the explicitly shown ring structure the overall HAS molecule.

Each remainder HAS" discussed above comprises, preferably essentially consists of - apart from terminal saccharide units - one or more repeating units according to formula (IIa)



According to the present invention, the HAS molecule shown in formula (III) is either linear or comprises at least one branching point, depending on whether at least one of the residues R^{33} , R^{bb} and R^{cc} of a given saccharide unit comprises yet a further remainder -O-HAS". If none of the R^{33} , R^{bb} and R^{cc} of a given saccharide unit comprises yet a further remainder -O-HAS", apart from the HAS" shown on the left hand side of formula (III), and optionally apart from HAS" contained in R^{π} , the HAS molecule is linear.

Hydroxyalkyl starch comprising two or more different hydroxyalkyl groups is also conceivable. The at least one hydroxyalkyl group comprised in the hydroxyalkyl starch may contain one or more, in particular two or more, hydroxyl groups. According to a

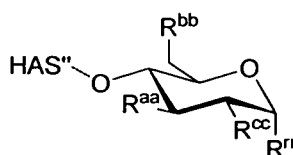
preferred embodiment, the at least one hydroxyalkyl group contains only one hydroxy! group.

5 The term "hydroxyalkyl starch" as used in the present invention also includes starch derivatives wherein the alkyl group is suitably mono- or polysubstituted. Such suitable substituents are preferably halogen, especially fluorine, and/or an aryl group. Yet further, instead of alkyl groups, HAS may comprise also linear or branched substituted or unsubstituted alkenyl groups.

10 Hydroxyalkyl starch may be an ether derivative of starch, as described above. However, besides of said ether derivatives, also other starch derivatives are comprised by the present invention, for example derivatives which comprise esterified hydroxyl groups. These derivatives may be, for example, derivatives of unsubstituted mono- or dicarboxylic acids with preferably 2 to 12 carbon atoms or of substituted derivatives thereof. Especially useful
15 are derivatives of unsubstituted monocarboxylic acids with 2 to 6 carbon atoms, especially derivatives of acetic acid. In this context, acetyl starch, butyryl starch and propynyl starch are preferred.

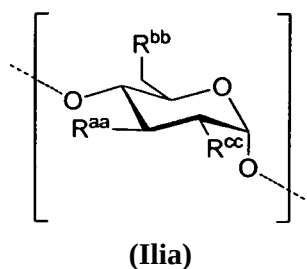
Furthermore, derivatives of unsubstituted dicarboxylic acids with 2 to 6 carbon atoms are
20 preferred. In the case of derivatives of dicarboxylic acids, it is useful that the second carboxy group of the dicarboxylic acid is also esterified. Furthermore, derivatives of monoalkyl esters of dicarboxylic acids are also suitable in the context of the present invention. For the substituted mono- or dicarboxylic acids, the substitute group may be preferably the same as mentioned above for substituted alkyl residues. Techniques for the
25 esterification of starch are known in the art (cf. for example Klemm, D. *etal*, Comprehensive Cellulose Chemistry, vol. 2, 1998, Wiley VCH, Weinheim, New York, especially Chapter 4.4, Esterification of Cellulose (ISBN 3-527-29489-9)).

According to a preferred embodiment of the present invention, a hydroxyalkyl starch
30 (HAS) according to the above-mentioned formula (III)



(III)

35 is employed. The saccharide units comprised in HAS'', apart from terminal saccharide units, may be the same or different, and preferably have the structure according to the formula (IIia)

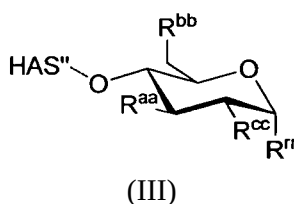


as shown above.

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According to the invention, the term "hydroxyalkyl starch" is preferably a hydroxyethyl starch, hydroxypropyl starch or hydroxybutyl starch, wherein hydroxyethyl starch is particularly preferred.

- 10 Thus, according to the present invention, the hydroxyalkyl starch (HAS) is preferably a hydroxyethyl starch (HES), the hydroxyethyl starch preferably having a structure according to the following formula (III)



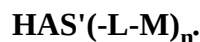
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- wherein R^{aa} , R^{bb} and R^{cc} are independently of each other selected from the group consisting of -O-HES", and $[-O-CH_2-CH_2]_s-OH$, wherein s is in the range of from 0 to 4 and wherein HAS", is, in case the hydroxyalkyl starch is hydroxyethyl starch, the remainder of the hydroxyethyl starch and could be abbreviated with HES". Residue R^{rr} is either -O-HAS" (which, in case the hydroxyalkyl starch is hydroxyethyl starch, could be abbreviated with -O-HES") or, in case the formula (III) shows the terminal saccharide unit of HES, R^{rr} is -OH. For the sake of consistency, the abbreviation "HAS" is used throughout all formulas in the context of the present invention, and if HAS is concretized as HES, it is explicitly mentioned in the corresponding portion of the text.
- 20
- 25

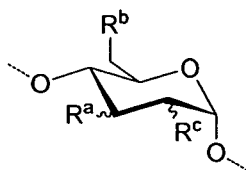
The term "Hydroxyalkyl Starch Derivative"

- In the context of the present invention, the term "hydroxyalkyl starch derivative" refers to a derivative of starch being functionalized with at least one functional group Z^1 , said group being a functional group capable of being linked to (reacted with) a further compound, in particular to the linking moiety L comprised in the structural unit-L-M which in turn is
- 30

comprised in above- defined conjugate having a structure according to the following formula



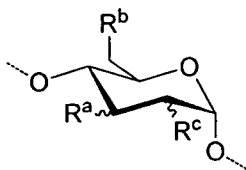
In accordance with the above-mentioned definition of HAS, the hydroxyalkyl starch derivative preferably comprises at least one structural unit according to the following formula (I)



(I),

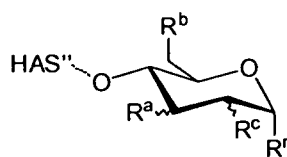
wherein at least one of R^a , R^b or R^c comprises the functional group Z^1 and wherein R^a , R^b and R^c are, independently of each other, selected from the group consisting of $-\text{O}-\text{HAS}''$, $-\text{[0-(CR}^w\text{R}^x\text{MCR}^y\text{R}^z)]_x\text{-OH}$, $-\text{[CHCR}^w\text{R}^x\text{MCR}^y\text{R}^z)]_y\text{-Z}^1$, $-\text{[0-(CR}^w\text{R}^x\text{)-(CR}^y\text{R}^z)]_y\text{-[F}^1]_p\text{-L}^1\text{-Z}^1$, wherein R^w , R^x , R^y and R^z are independently of each other selected from the group consisting of hydrogen and alkyl, y is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4, x is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4, F^1 is a functional group, p is 0 or 1, L^1 is a linking moiety and Z^1 is a functional group which is capable of being linked to a further compound, in particular to the linking moiety L comprised in the structural unit $-\text{L}-\text{M}$.

In particular, a hydroxyalkyl starch derivative which comprises at least one structural unit according to the following formula (I)



(I)

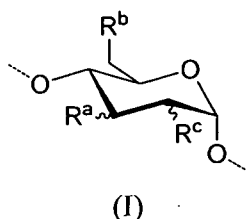
has preferably a structure according to the following formula (IV)



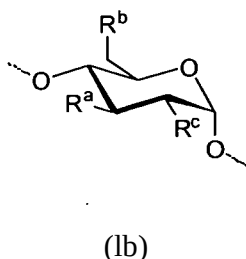
(IV)

wherein R^f is -O-HAS" or, in case the ring structure of formula (IV) shows the terminal saccharide unit of HAS, R^f is -OH, and wherein HAS" is a remainder of the hydroxyalkyl starch derivative.

- 5 Analogously to the above-discussed definition of the term HAS" in the context of the hydroxyalkyl starch as such, the term "remainder of the hydroxyalkyl starch derivative" is denoted to mean a linear or branched chain of the hydroxyalkyl starch derivative, being linked to the oxygen groups shown in formula (IV) or being comprised in the residues R^a , R^b or R^c of formula (I), wherein said linear or branched chains comprise at least one structural unit according to formula (I),

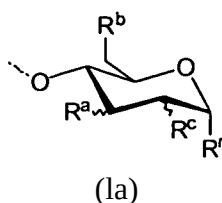


- wherein at least one of R^a , R^b or R^c comprises the functional group Z^1 and/or one or more structural units of the formula (Ib)



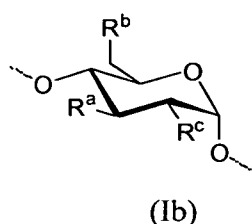
- 20 wherein R^a , R^b and R^c are, independently of each other, selected from the group consisting of -O-HAS" and $-[O-(CR^wR^x)-(CR^yR^z)]_x-OH$, wherein R^w , R^x , R^y , R^z are as described above.
- 25 In case the hydroxyalkyl starch derivative has a linear starch backbone, none of R^a , R^b or R^c comprises a further group -O-HAS". In case at least one of R^a , R^b or R^c is -O-HAS", the hydroxyalkyl starch derivative comprises at least one branching point.

- In particular, in case, the structural unit is the reducing sugar moiety of the hydroxyalkyl starch derivative, the terminal structural unit has a structure according to the following formula (Ia):

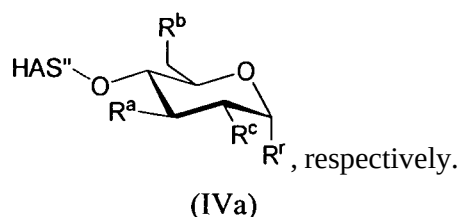


wherein R^r is -OH or a group comprising the functional group Z^1 . Residue R^r is preferably selected from the group consisting of -OH, $-Z^1$ and $-[F^1]_p-L^1-Z^1$, most preferably R^r is -OH, the reducing end of the hydroxyalkyl starch thus being present in unmodified form.

In the above-mentioned formula (Ia), the bond "~~~~" represents a bond with non-defined stereochemistry, i.e. this term represents a bond encompassing both possible stereochemistries. Preferably, the stereochemistry in most building blocks, preferably in all building blocks of the HAS derivative is defined according to the formulas (Ib) and (IVa)



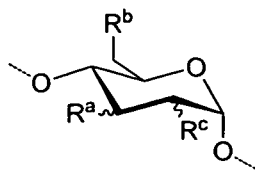
and



According to a preferred embodiment of the present invention, the hydroxyalkyl starch (HAS) derivative is a hydroxyethyl starch (HES) derivative.

Therefore, the present invention also describes a hydroxyalkyl starch derivative as described above, and a method for preparing said hydroxyalkyl starch derivative, and a conjugate comprising said hydroxyalkyl starch derivative and a cytotoxic agent, and a conjugate obtained or obtainable by the above-mentioned method wherein the conjugate comprises said hydroxyalkyl starch derivative and a cytotoxic agent, wherein the hydroxyalkyl starch derivative is a hydroxyethyl starch derivative.

Accordingly, in case the hydroxyalkyl starch (HAS) is hydroxyethyl starch (HES), the HAS derivative preferably comprises at least one structural unit, preferably 3 to 100 structural units, according to the following formula (I)



(I)

wherein R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS", $-[O-CH_2-CH_2]_s-OH$, $-[O-Ctb-CH^Z-Z^1]$ and $-[O-CH_2-CH_2]-[F^1]_p-L^1-Z^1$,
 5 wherein at least one of R^a , R^b and R^c is $-[O-CH^Z-CH^Z-Z^1]$ or $-[O-CH_2-CH_2]-[F^1]_p-L^1-Z^1$,
 wherein s is in the range of from 0 to 4, wherein t is in the range of from 0 to 4, and
 wherein p is 0 or 1.

The amount of functional groups Z^1 present in the hydroxyalkyl starch derivative

10

As regards the amount of functional groups Z^1 present in a given hydroxyalkyl starch derivative, preferably 0.15 % to 2 % of all residues R^a , R^b and R^c present in the hydroxyalkyl starch derivative contain the functional group Z^1 .

15 More preferably, 0.15 % to 2 % of all residues R^a , R^b and R^c present in the hydroxyalkyl starch derivative have the structure $-[O-(CR^{wR^x})-(CR^{yR^z})]_y-Z^1$ or $-[O-(CR^{wR^x})-(CR^{yR^z})]_y-[F^1]_p-L^1-Z^1$.

20 According to a particularly preferred embodiment, R^a , R^b and R^c are selected from the group consisting of -O-HAS", $-[O-(CR^{wR^x}MCR^{yR^z})]_x-OH$ and $-[O-(CR^{wR^x})-(CR^{yR^z})]_y-Z^1$, wherein 0.15 % to 2 % of all residues R^a , R^b and R^c present in the hydroxyalkyl starch derivative have the structure $-[O-(CR^{wR^x})-(CR^{yR^z})]_y-Z^1$.

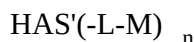
25 According to an alternative preferred embodiment, R^a , R^b and R^c are selected from the group consisting of -O-HAS", $-[O-(CR^{wR^x})-(CR^{yR^z})]_x-OH$ and $-[O-(CR^{wR^x})-(CR^{yR^z})]_y-[F^1]_p-L^1-Z^1$, wherein 0.15 % to 2 % of all residues R^a , R^b and R^c present in the hydroxyalkyl starch derivative have the structure $-[O-(CR^{wR^x})-(CR^{yR^z})]_y-[F^1]_p-L^1-Z^1$.

The term "Residue of the Hydroxyalkyl Starch Derivative"

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The term "residue of the hydroxyalkyl starch derivative" (HAS') refers to a hydroxyalkyl starch derivative being incorporated into a hydroxyalkyl starch conjugate. Within the meaning of the present invention the term "a conjugate comprising a hydroxyalkyl starch derivative" thus refers to a conjugate comprising a residue of a hydroxyalkyl starch

derivative being incorporated into the conjugate and thus being linked to the linking moiety L comprised in the conjugate having a structure according the following formula



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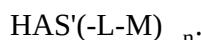
Upon incorporation into the conjugate, the hydroxyalkyl starch derivative is coupled via at least one of its functional groups Z^1 to the crosslinking compound L (which is further reacted with the cytotoxic agent) or to the derivative of the cytotoxic agent having the structure -L-M, as described hereinabove and hereinunder, thereby forming a covalent linkage between the residue of the hydroxyalkyl starch derivative and L or -L-M, wherein the functional group X is formed upon reaction of Z^1 with L or -L-M, respectively.

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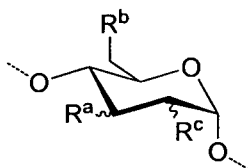
Analogously to the above-discussed definition of the term "hydroxyalkyl starch derivative", the term "residue of a hydroxyalkyl starch derivative" refers to a derivative of starch being linked via at least one functional group X via a linking moiety to a further compound, in particular via the linking moiety L comprised in the structural unit -L-M which in turn is comprised in above-defined conjugate having a structure according to the following formula

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In accordance with the above-mentioned definition of the hydroxyalkyl starch derivative, the residue of the hydroxyalkyl starch derivative preferably comprises at least one structural unit according to the following formula (I)



25

(I)

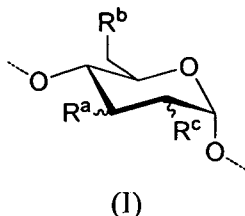
wherein R^a , R^b and R^c are, independently of each other, selected from the group consisting of -O-HAS", $-[O-(CR^wR^xHCR^yR^z)]_x-OH$, $-[O-(CR^wR^x)-(CR^yR^z)]_y-X$ and $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^1-X$, and wherein at least one of R^a , R^b or R^c comprises the functional group $-[O-(CR^wR^x)-(CR^yR^z)]_x-X$ or $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^1-X$, and wherein R^w , R^x , R^y and R^z are independently of each other selected from the group consisting of hydrogen and alkyl, y is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4, x is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4, F^1 is a functional group, p is 0 or 1, L^1 is a linking moiety and X is a

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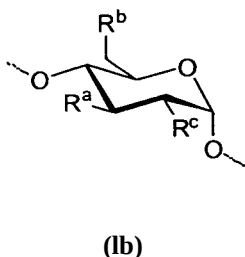
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functional group which is linked to a further compound, in particular to the linking moiety L comprised in the structural unit -L-M.

Besides the at least one structural unit according to formula (1),



wherein at least one of R^a , R^b or R^c comprises the functional group $-[0-(CR^wR^x)-(CR^yR^z)]_x-[F^1]_p-L^1-X-$ or $-[0-(CR^wR^x)-(CR^yR^z)]_y-X-$, the residue of the hydroxyalkyl starch preferably comprises one or more structural units of the formula (Ib)



wherein R^a , R^b and R^c are, independently of each other, selected from the group consisting of -O-HAS" and $-[0-(CR^wR^xMCR^yR^z)]_x-OH$.

As disclosed above, preferably 0.15 % to 2 % of all residues R^a , R^b and R^c present in the hydroxyalkyl starch derivative contain the functional group Z^1 . Further, preferably all functional groups Z^1 being present in a given hydroxyalkyl starch derivative are coupled according to the coupling reaction of step (b) as defined hereinabove, thereby forming the covalent linkage via functional group X. Consequently, preferably 0.15 % to 2 % of all residues R^a , R^b and R^c present in the residue of the hydroxyalkyl starch derivative contain the functional group X. Thus, preferably 0.15 % to 2 % of all residues R^a , R^b and R^c present in the residue of the conjugate of the present invention contain the functional group X.

However, in case the hydroxyalkyl starch derivative comprises at least two functional groups Z^1 , it may be possible that in step (b) not all of these functional groups Z^1 reacted with the crosslinking compound L, which in turn is reacted (either prior to, or after the reaction with the HAS derivative) with the cytotoxic agent, giving a conjugate in which the HAS derivative is linked via the linking moiety L to the residue of the cytotoxic agent M..

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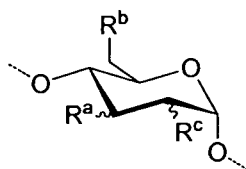
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more preferably at least 95 % most preferably at least 99 %, of all functional groups X present in the conjugate of the invention are linked to the functional moiety -L-M.

Alternatively, the conjugates of the present invention may also be described by the formula

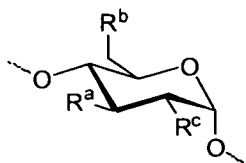


wherein beta is 0 or 1, preferably 0, and wherein generally $0 \leq \text{gamma} < n$, preferably wherein $0 \leq \text{gamma} \ll n$, especially preferably wherein gamma is 0, wherein the residue of the hydroxyalkyl starch derivative HAS* comprises at least one structural unit according to formula (I),



(I)

wherein at least one of R^a , R^b or R^c comprises the functional group X, and wherein the residue of the hydroxyalkyl starch HAS* preferably comprises one or more structural units of the formula (Ib)



(Ib)

wherein R^a , R^b and R^c are, independently of each other, selected from the group consisting of -O-HAS" and $-[0-(\text{CR}^w\text{R}^x)-(\text{CR}^y\text{R}^z)]_x\text{-OH}$, and wherein HAS* comprises no structural units $-[0-(\text{CR}^w\text{R}^x\text{HCR}^y\text{R}^z)]_y\text{-X-(L)}_{\beta^{\text{ct}_a}}\text{-D}$ or $-[0-(\text{CR}^w\text{R}^x\text{HCR}^y\text{R}^z)]_y\text{-[F]}_p\text{-L}^1\text{-X-(L)}_{\beta^{\text{ct}_a}}\text{-D}$.

Substitution Pattern: Molar Substitution (MS) and Degree of Substitution (DS)

HAS, in particular HES, is mainly characterized by the molecular weight distribution, the degree of substitution and the ratio of $C_2 : C_6$ substitution. There are two possibilities of describing the substitution degree.

The degree of substitution (DS) of HAS is described relatively to the portion of substituted glucose monomers with respect to all glucose moieties.

- 5 The substitution pattern of HAS can also be described as the molar substitution (MS), wherein the number of hydroxyethyl groups per glucose moiety is counted.

In the context of the present invention, the substitution pattern of the hydroxyalkyl starch (HAS), preferably HES, is referred to as MS, as described above, wherein the number of
10 hydroxyalkyl groups present per sugar moiety is counted (see also Sommermeyer *et al*, 1987, Krankenhauspharmazie, 8(8): 271-278, in particular page 273). The MS is determined by gaschromatography after total hydrolysis of the hydroxyalkyl starch molecule.

- 15 The MS values of the respective hydroxyalkyl starch, in particular hydroxyethyl starch starting materials, are given since it is assumed that the MS value is not affected during the derivatization procedures as well as during the coupling step of the present invention.

The MS value corresponds to the degradability of the hydroxyalkyl starch via alpha-amylase. The higher the MS value, the lower the degradability of the hydroxyalkyl starch.
20 It was surprisingly found that the MS of the hydroxyalkyl starch derivative present in the conjugates according to the invention should preferably be in the range of from 0.6 to 1.5 to provide conjugates with advantageous properties. Without wanting to be bound to any theory, it is believed that a MS in the above mentioned range combined with the specific
25 molecular weight range of the conjugates results in conjugates with an optimized enrichment of the cytotoxic agent in the tumor and/or residence time in the plasma allowing for a controlled release of the cytotoxic agent prior to the degradation of the polymer and the subsequent removal of polymer fragments through the kidney.

- 30 According to a preferred embodiment of the present invention, the molar substitution MS is in the range of from 0.70 to 1.45, more preferably in the range of 0.80 to 1.40, more preferably in the range of from 0.90 to 1.35, such as 0.90, 0.95, 1.0, 1.05, 1.1, 1.15, 1.2, 1.25, 1.3 or 1.35.

- 35 Thus, the present invention also relates to a method for preparing a conjugate comprising a hydroxyalkyl starch derivative and a cytotoxic agent, as described above, and a conjugate obtained or obtainable by said method, wherein the hydroxyalkyl starch derivative has a MS in the range of from 0.70 to 1.45, preferably in the range of 0.80 to 1.40, more preferably in the range of from 0.90 to 1.35.

Likewise, the present invention also relates to a hydroxyalkyl starch (HAS) conjugate comprising a hydroxyalkyl starch derivative and a cytotoxic agent, as described above, wherein the hydroxyalkyl starch derivative has a molar substitution MS in the range of
 5 from 0.70 to 1.45, preferably in the range of from 0.80 to 1.40, more preferably in the range of from 0.90 to 1.35. Likewise, the present invention relates to a pharmaceutical composition comprising a hydroxyalkyl starch conjugate, as described above, or a hydroxyalkyl starch conjugate obtained or obtainable by the above described method, wherein the hydroxyalkyl starch derivative has a molar substitution MS in the range of
 10 from 0.70 to 1.45, preferably in the range of from 0.80 to 1.40, more preferably in the range of from 0.90 to 1.35.

As far as the ratio of C₂ : C₆ substitution is concerned, i.e. the degree of substitution (DS) of HAS, said substitution is preferably in the range of from 2 to 20, more preferably in the
 15 range of from 2 to 15 and even more preferably in the range of from 3 to 12, with respect to the hydroxyalkyl groups.

Mean molecular weight MW (or Mw)

20 HAS and in particular HES compounds are present as polydisperse compositions, wherein each molecule differs from the other with respect to the polymerization degree, the number and pattern of branching sites, and the substitution pattern. HAS and in particular HES is therefore a mixture of compounds with different molecular weight. Consequently, a particular HAS and in particular a HES is determined by average molecular weight with
 25 the help of statistical means.

In this context the number average molecular weight is defined by equation 1:

$$\overline{M}_n = \frac{\sum_i n_i \cdot M_i}{\sum_i n_i}$$

(9)

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where n_i is the number of molecules of species i of molar mass M_i.

$\overline{M}_n$ indicates that the value is an average, but the line is normally omitted by convention.

M_w is the weight average molecular weight, defined by equation 2:

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$$\overline{M}_w = \frac{\sum_i n_i \cdot M_i^2}{\sum_i n_i M_i}$$

(2)

- 5 where n_i is the number of molecules of species i of molar mass M_i and $\overline{M}_w$ indicates that the value is an average, but the line is normally omitted by convention.

Preferably, the hydroxyalkyl starch derivative, in particular the hydroxyethyl starch derivative comprised in the conjugate, as described above, has a mean molecular weight
10 MW (weight mean) above the renal threshold.

The renal threshold is determined according to the method described by Waitzinger et al. (Clin. Drug Invest. 1998; 16: 151-160) and reviewed by Jungheinrich et al. (Clin. Pharmacokinet. 2006; 44(7): 681-699). Preferably, the renal threshold is denoted to mean
15 molecular weight MW above 40 kDa.

More preferably, the hydroxyalkyl starch derivative, in particular the hydroxyethyl starch derivative comprised in the conjugate, as described above, has a mean molecular weight
20 MW above 45 kDa, more preferably above 50 kDa, more preferably above 60 kDa.

More preferably the hydroxyalkyl starch derivative, in particular the hydroxyethyl starch derivative, according to the invention, has a mean molecular weight MW (weight mean) in the range of from 80 to 1200 kDa, preferably in the range of from 90 to 800 kDa.

- 25 The term "mean molecular weight" as used in the context of the present invention relates to the weight as determined according to MALLS (multiple angle laser light scattering) - GPC method as described in example 7.

Therefore, the present invention also relates to a method as described above, for preparing
30 a hydroxyalkyl starch derivative, as well as to a method for preparing a hydroxyalkyl starch conjugate, wherein the hydroxyalkyl starch derivative has a mean molecular weight MW above the renal threshold, preferably a MW greater than or equal to 60 kDa, more preferably in the range of from 80 to 1200 kDa, preferably in the range of from 90 to 800 kDa. Likewise, the present invention relates to a hydroxyalkyl starch conjugate, as
35 described above, comprising a hydroxyalkyl starch derivative, as well as to a hydroxyalkyl starch conjugate obtained or obtainable by the above-mentioned method, wherein the

hydroxyalkyl starch derivative has a mean molecular weight MW above the renal threshold, preferably a MW greater than or equal to 60 kDa, more preferably a mean molecular weight MW in the range of from 80 to 1200 kDa, more preferably in the range of from 90 to 800 kD.

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According to an especially preferred embodiment, the hydroxyalkyl starch derivative has a MS in the range of from 0.70 to 1.45 and a mean molecular weight MW in the range of from 80 to 1200 kDa, more preferably a molar substitution MS in the range of from 0.80 to 1.40 and a mean molecular weight MW in the range of from 90 to 800 kDa, more preferably a molar substitution in the range of from 0.90 to 1.35, more preferably a mean molecular weight MW in the range of from 90 to 800 kDa and a MS in the range of from 0.95 to 1.35.

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As regards integer n, as described above and below, according to a preferred embodiment of the present invention, n is in the range of from 2 to 300, preferably of from 2 to 100, more preferably of from 3 to 100.

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Drug loading

The amount of M, present in the conjugates of the invention, can further be described by the drug loading (also: drug content). The "drug loading" as used in the context of the present invention is calculated as the mean molecular weight of the cytotoxic agent measured in mg drug, i.e. cytotoxic agent, per 1 g of the conjugate.

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The drug loading is determined by measuring the absorbance of M (thus the cytotoxic agent bound to HAS) at a specific wavelength in a stock solution, and calculating the content using the following equation (Lambert Beer's law):

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$$c_{drug}[\mu\text{mol l cm}^3] = \frac{(A - A^0)}{\epsilon * d}$$

30

where ϵ is the extinction coefficient of the cytotoxic agent at the specific wavelength, which is obtained from a calibration curve of the cytotoxic agent dissolved in the same solvent which is used as in the stock solution (given in cm^2/mol), at the specific wavelength, A is the absorption at this specific wavelength, measured in a UV-VIS spectrometer, A^0 is the absorption of a blank sample and d the width of the cuvette (equals the slice of absorbing material in the path of the beam, usually 1 cm). The appropriate wavelength for the determination of drug loading is derived from a maximum in the UV-VIS-spectra, preferably at wavelengths above 230 nm.

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With a known concentration of conjugate in the sample ($c_{\text{conjugate}}$) and the concentration of drug in the sample determined by Lambert Beer's law, the loading in micromol/g can be calculated according to the following equation:

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$$\text{Loading}[\mu\text{mol/g}] = \frac{1000 * c_{\text{drug}} [\mu\text{mol/l}]}{c_{\text{conjugate}} [\text{mg/ml}]}$$

The loading (in mg/g) may finally be determined taking into account the molecular weight of the cytotoxic agent as shown in the following equation:

10

$$\text{Loading}[\text{mg/g}] = \text{Loading}[\mu\text{mol/g}] * MW_{\text{drug}} [\mu\text{g}/\mu\text{mol}] / 1000$$

As regards the drug loading, according to a preferred embodiment of the present invention, the drug loading of the conjugates is preferably in the range of from 40 to 1100 $\mu\text{mol drug/g}$, more preferably in the range of from 80 to 800 $\mu\text{mol drug/g}$, more preferably in the range of from 110 to 700 $\mu\text{mol drug/g}$ and most preferably in the range of from 150 to 600 $\mu\text{mol drug/g}$ (-L-M).

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The cytotoxic agent

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The term "cytotoxic agent" as used in the context of the present invention refers to natural or synthetic substances, which inhibit the cell growth or the cell division *in vivo*. The term is intended to include chemotherapeutic agents, antibiotics and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

25

The term "residue of the cytotoxic agent M" as used in the context of the present invention refers to the cytotoxic agent being linked to L via a group -O-, said group being derived from a primary hydroxyl group being present in the cytotoxic agent.

30

Preferably, the term "cytotoxic agent" is a natural or synthetic substance which inhibits the cell growth or the cell division of a tumor *in vivo*. Most preferably, the cytotoxic agent is a chemotherapeutic agent. The therapeutic use of these preferred cytotoxic agents, most preferably of the chemotherapeutic agents, is based on the difference in the rate of cell division and cell growth of tumor cells compared to normal cells. Among others, tumor cells differ from normal cells in that tumor cells are no longer subject to physiological growth control and therefore have an increased rate of cell division. Since the toxic activity of cytotoxic agents is usually primarily directed against proliferating cells, such cytotoxic

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agents can be used for inhibiting a development or progression of a neoplasm *in vivo*, particularly a malignant (cancerous) lesion, such as a carcinoma, sarcoma, lymphoma, or leukemia. Inhibition of metastasis is frequently also a property of the cytotoxic agents encompassed by the present invention.

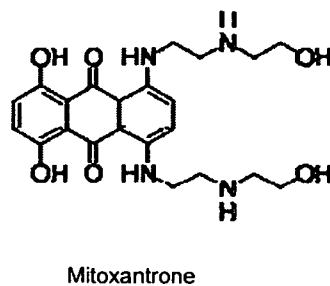
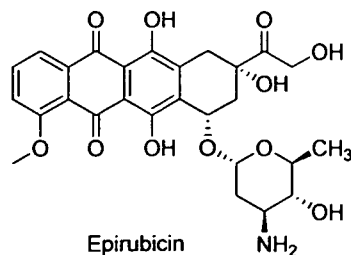
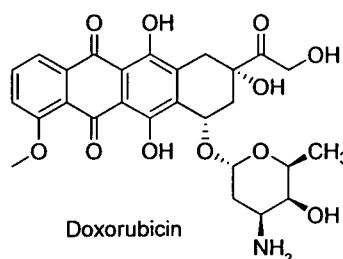
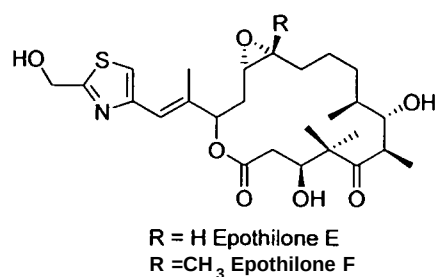
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With respect to the chemistry used in the context of the present invention, any cytotoxic agent, preferably any chemotherapeutic agent, known to those skilled in the art can be incorporated into the conjugates according to the present invention provided that this cytotoxic agent, preferably the chemotherapeutic agent, comprises a primary hydroxyl group. Preferably the cytotoxic agent is an agent for the treatment of cancer.

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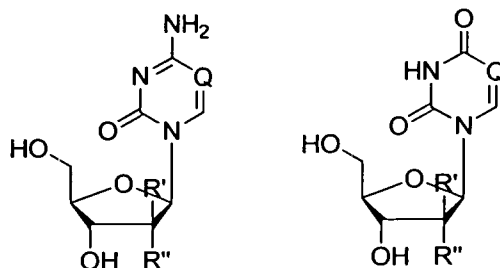
Preferably, the cytotoxic agent is selected from the group consisting of - a primary hydroxyl group containing - tubulin inhibitors, such as tubulin inhibitors or tubulin stabilizers, vinca alkaloids, topoisomerase I inhibitors (e.g. camptothecin analogues such as DRF-1042), topoisomerase II inhibitors, tubulin stabilizers such as peloruside A, dictyostatin, discondermolide, taxane derivatives or members of the epothilone family such as epothilone E and F, DNA intercalators such as mitoxantrone and the anthracycline family (doxorubicin, epirubicin), antimetabolites such as clofarabine, nelarabine, cytarabine, cladribine, decitabine, azacitidine, floxuridine, pentostatin or gemcitabine, mitotic inhibitors such as halichondrin B and eribulin, protein kinase inhibitors including rapamcyin analogues such as temsirolimus and everolimus, hormone analogues such as octreotide, alkylating agents such as streptozocin and DNA damaging agents such as bleomycin, vascular disrupting agents, colchicinol-derivatives and HSP-90 inhibitors. The following cytotoxic agents encompassed by the present invention are mentioned by way of example:

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According to a preferred embodiment of the invention, the cytotoxic agent is an antimetabolite, more preferably a nucleoside analogue, such as clofarabine, nelarabine, cytarabine, cladribine, decitabine, azacitidine, floxuridine, pentostatin or gemcitabine.

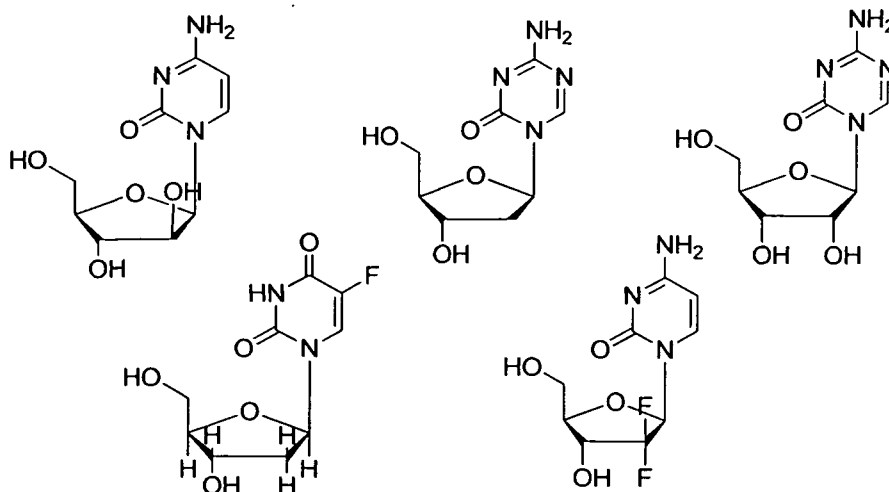
- 5 More preferably, the cytotoxic agent is a cytidine analogue having one of the following structures:



- wherein Q is selected from the group consisting of C-H, C-F, C-CH₃ and N, and wherein
 10 R' and R'' are independently of each other selected from the group consisting of OH, H and F.

Most preferably the cytotoxic agent is selected from the group consisting of cytarabine, decitabine, azacitidine, floxuridine and gemcitabine (see structures below):

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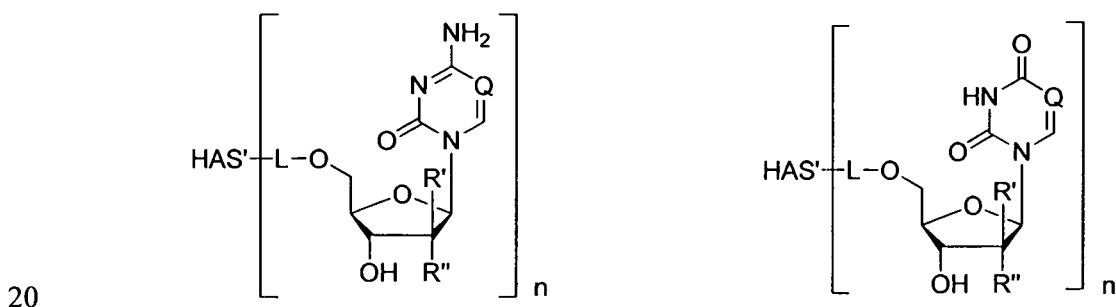
- Accordingly, the present invention preferably relates to a hydroxyalkyl starch conjugate as described above, as well as to a method for preparing a hydroxyalkyl starch conjugate and
 20 the respective conjugate obtained or obtainable by said method, the conjugate comprising a residue of a cytotoxic agent, said cytotoxic agent being selected from the group consisting of clofarabine, nelarabine, cytarabine, cladribine, decitabine, azacitidine, floxuridine, pentostatin or gemcitabine, most preferably of cytarabine, decitabine, azacitidine, floxuridine and gemcitabine.

Most preferably, the cytotoxic agent is gemcitabine.

According to a further preferred embodiment of the invention, the cytotoxic agent is a
 5 kinase inhibitor including rapamycin and rapamycin analogues, preferably the cytotoxic agent is a rapamycin analogue, in particular, temsirolimus or everolimus.

Antimetabolites, in particular nucleoside analogues have been found to be effective anti-
 cancer agents. However, to date, their use is limited due to their non specific toxicity and
 10 to their short residence time in the plasma. It is herein proposed that this drawback can be overcome by the conjugates according to the present invention, thus, by conjugates, wherein a hydroxyalkyl starch derivative, as described above, is linked via a linking moiety L to a group -O- derived from the primary hydroxyl group of a cytotoxic agent, preferably to a group -O- derived from the primary hydroxyl group of an antimetabolite.

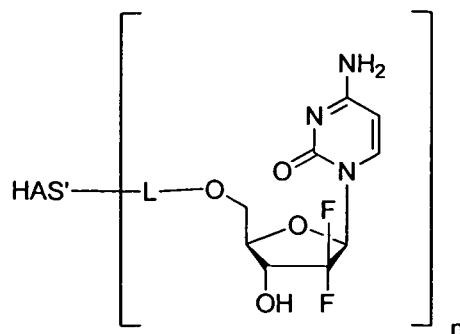
15 Thus, preferably, the present invention also relates to a conjugate, as described above, as well as to a conjugate obtained or obtainable by a method, as described above, the conjugate having a structure according to one of the following formulas:



wherein Q is selected from the group consisting of C-H, C-F, C-CH₃ and N, and wherein R' and R'' are independently of each other selected from the group consisting of OH, H and F.

25

The following particularly preferred structures shall be mentioned:



The linking moiety L

- 5 According to the invention, the cytotoxic agent is preferably linked via a cleavable linker to the hydroxyalkyl starch derivative.

The expression "cleavable linker" refers to any linker which can be cleaved physically or chemically and preferably releases the cytotoxic agent in unmodified form. Examples for physical cleavage may be cleavage by light, radioactive emission or heat, while examples
 10 for chemical cleavage include cleavage by redox-reactions, hydrolysis, pH-dependent cleavage or cleavage by enzymes.

According to a preferred embodiment of the present invention, the cleavable linker comprises one or more cleavable bonds, preferably hydrolytically cleavable bonds, the cleavage, in particular the hydrolysis, of which releases the cytotoxic agent *in vivo*.
 15 Preferably the bond between linker moiety L and the group -O- derived from the primary hydroxyl group of the cytotoxic agent is a cleavable linkage.

Thus, the present invention also relates to a conjugate as described above, as well as to a conjugate obtained or obtainable by the above described method, wherein the linking moiety L and the residue of the cytotoxic agent M are linked via the group -O- derived
 20 from the primary hydroxyl group of the cytotoxic agent, wherein said linkage between -O- and the linking moiety L is cleaved, preferably is hydrolyzed, *in vivo* and allows for the release of the cytotoxic agent, preferably in unmodified form.

Preferably, the linking moiety L has a structure -L'-F³-, wherein F³ is the functional group linking L' with M, and wherein the linkage between F³ and the group -O- derived from the
 25 primary hydroxyl group of M is cleaved *in vivo* and releases the (residue of the) cytotoxic agent. L' is a linking moiety linking the functional group F³ with the hydroxyalkyl starch derivative.

30 The functional group F³

There are in principle no restrictions as to the nature of the functional group F^3 provided that this group forms together with the primary hydroxyl group of the cytotoxic agent a functional moiety capable of being cleaved *in vivo*.

5

Thus, the present invention also relates to a conjugate as described above, as well as to a conjugate obtained or obtainable by the above described method, wherein the bond between the functional group F^3 and the functional group -O- of the residue of the cytotoxic agent M (said group being derived from the primary hydroxyl group of the cytotoxic agent) is a cleavable linkage, which is cleaved *in vivo* so as to release the cytotoxic agent.

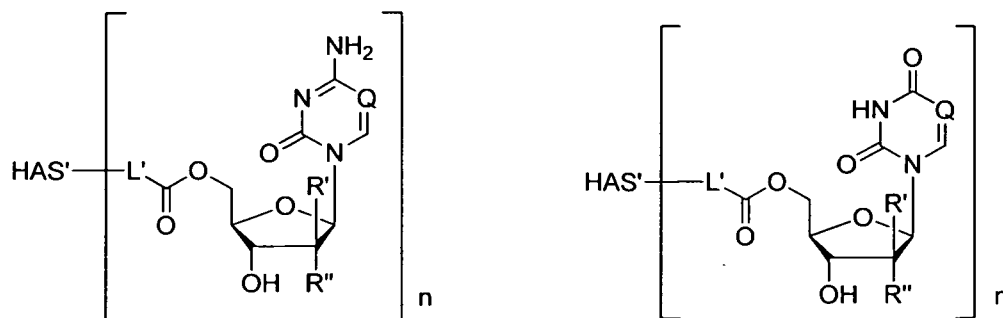
Beside the -C(=Y)- function, this accounts, inter alia, for groups F^3 which form together with the group -O- of the residue of the cytotoxic agent M (derived from the primary hydroxyl group of the cytotoxic agent) the structural unit - F^3 -O-, with - F^3 -O- being a carbonate, thiocarbonate, xanthogenate, carbamate, or thiocarbamate of the type - Y^Y -C(=Y)-O- with Y^Y being -O-, -S- or -NH- and Y being O, S or NH.

Preferably, the functional group F^3 is selected from the group consisting of -C(=Y)- and - Y^Y -C(=Y)-, with Y being O, NH or S and with Y^Y being -O-, -S- or -NH-. In particular, the functional group F^3 is -C(=Y)-, with Y being O, NH or S. Together with the the group -O- derived from the primary hydroxyl group of the cytotoxic agent, the functional group F^3 therefore preferably forms a -C(=Y)-O- bond with Y being O, NH or S, in particular with Y being O or S, more preferably with Y being O, and wherein L' is a linking moiety linking the functional group F^3 with the hydroxyalkyl starch derivative.

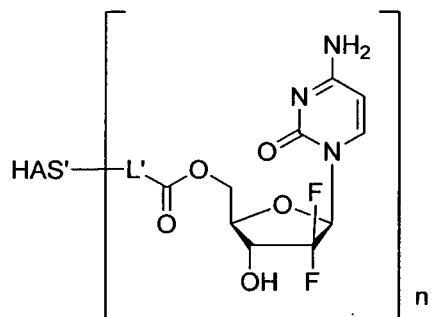
Therefore, the present invention also relates to a hydroxyalkyl starch conjugate comprising a hydroxyalkyl starch derivative and a cytotoxic agent, said conjugate having a structure according to the following formula $HAS'(-L-M)_n$, wherein the linking moiety L has a structure - $L'-F^3$ -, wherein F^3 is a functional group linking L' with the residue of the cytotoxic agent M, thereby forming a - F^3 -O- group, preferably wherein F^3 is a -C(=Y)-group, with Y being O, NH or S, and wherein F^3 is linked to the primary hydroxyl group of the cytotoxic agent, i.e. to the group -O- derived from the primary hydroxyl group of the cytotoxic agent, thereby forming a -C(=Y)-O- bond with Y being O, NH or S, in particular with Y being O or S, more preferably with Y being O, and wherein L' is a linking moiety. Likewise, the present invention relates to a method for preparing a conjugate having a structure $HAS'(-L-M)_n$, wherein L has a structure - $L'-F^3$ -, wherein F^3 is a functional group linking L' with M, preferably wherein F^3 is a -C(=Y)- group, with Y being O, NH or S, and wherein the structural unit - F^3 -O- is formed upon reaction of the crosslinking

compound L with the primary hydroxyl group of the cytotoxic agent. Likewise, the present invention relates to a conjugate obtained or obtainable by the method, as described above.

- Accordingly, the present invention also relates to a conjugate, as described above, as well as to a conjugate, obtained or obtainable by a method, as described above, the conjugate having a structure according to one of the following formulas:



- wherein Q is selected from the group consisting of C-H, C-F, C-CH₃ and N, and wherein R' and R'' are independently of each other selected from the group consisting of OH, H and F; more preferably according to the following formula



The linking moiety L'

- According to a preferred embodiment of the present invention, the functional group F³ and the hydroxyalkyl starch derivative are separated by a suitable linking moiety L', as described above. The term linking moiety L' as used in this context of the present invention relates to any suitable chemical moiety bridging F³ and the hydroxyalkyl starch derivative.

- In general, there are no particular restrictions as to the chemical nature of the linking moiety L' with the proviso that L' provides suitable chemical properties for the novel conjugates for their intended use.

Preferably, L' is a linking moiety such as an alkyl, alkenyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl group.

5 Within the meaning of the present invention, the term "alkyl" relates to non-branched alkyl residues, branched alkyl residues, cycloalkyl residues, as well as residues comprising one or more heteroatoms or functional groups, such as, by way of example, -O-, -S-, -NH-, -NH-C(=O)-, -C(=O)-NH-, and the like. The term also encompasses alkyl groups which are further substituted by one or more suitable substituents. The term "substituted alkyl" as
 10 used in this context of the present invention preferably refers to alkyl groups being substituted in any position by one or more substituents, preferably by 1, 2, 3, 4, 5 or 6 substituents, more preferably by 1, 2 or 3 substituents. If two or more substituents are present, each substituent may be the same or may be different from the at least one other substituent. There are in general no limitations as to the substituent. The substituents may
 15 be, for example, selected from the group consisting of aryl, alkenyl, alkynyl, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxy carbonyloxy, aryloxy carbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxy carbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxy, phosphate, phosphonate, phosphinato, amino, acylamino, including alkylcarbonylamino,
 20 arylcarbonylamino, carbamoyl and ureido, amidino, nitro, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonate, sulfamoyl, sulfonamido, trifluoromethyl, cyano, azido, cycloalkyl such as e.g. cyclopentyl or cyclohexyl, heterocycloalkyl such as e.g. morpholino, piperazinyl or piperidinyl, alkylaryl, arylalkyl and heteroaryl. Preferred substituents of such organic residues are, for example, halogens,
 25 such as fluorine, chlorine, bromine or iodine, amino groups, hydroxyl groups, carbonyl groups, thiol groups and carboxyl groups.

The term "alkenyl" as used in the context of the present invention refers to unsaturated alkyl groups having at least one double bond. The term also encompasses alkenyl groups
 30 which are substituted by one or more suitable substituents.

The term "alkynyl" refers to unsaturated alkyl groups having at least one triple bond. The term also encompasses alkynyl groups which are substituted by one or more suitable substituents.

35 Within the meaning of the present invention, the term "aryl" refers to, but is not limited to, optionally suitably substituted 5- and 6-membered single-ring aromatic groups as well as optionally suitably substituted polycyclic groups, for example bicyclic or tricyclic aryl groups. The term "aryl" thus includes, for example, optionally substituted phenyl groups or

optionally suitably substituted naphthyl groups. Aryl groups can also be fused or bridged with alicyclic or heterocycloalkyl rings which are not aromatic so as to form a polycycle, e.g., benzodioxolyl or tetraline.

- 5 The term "heteroaryl" as used within the meaning of the present invention includes optionally suitably substituted 5- and 6-membered single-ring aromatic groups as well as substituted or unsubstituted multicyclic aryl groups, for example bicyclic or tricyclic aryl groups, comprising one or more, preferably from 1 to 4 such as 1, 2, 3 or 4, heteroatoms, wherein in case the aryl residue comprises more than 1 heteroatom, the heteroatoms may
10 be the same or different. Such heteroaryl groups including from 1 to 4 heteroatoms are, for example, benzodioxolyl, pyrrolyl, furanyl, thiophenyl, thiazolyl, isothiazolyl, imidazolyl, triazolyl, tetrazolyl, pyrazolyl, oxazolyl, isoxazolyl, pyridinyl, pyrazinyl, pyridazinyl, benzoxazolyl, benzodioxazolyl, benzothiazolyl, benzoimidazolyl, benzothiophenyl, methylenedioxyphenyl, naphthyridinyl, quinolinyl, isoquinolinyl, indolyl, benzofuranyl,
15 purinyl, deazapurinyl, or indolizinyl.

- The term "substituted aryl" and the term "substituted heteroaryl" as used in the context of the present invention describes moieties having substituents replacing a hydrogen on one or more atoms, e.g. C or N, of an aryl or heteroaryl moiety. Again, there are in general no
20 limitations as to the substituent. The substituents may be, for example, selected from the group consisting of alkyl, alkenyl, alkynyl, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxy carbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxy, phosphate, phosphonato, phosphinato, amino, acylamino, including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido, amidino, nitro,
25 imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonate, sulfamoyl, sulfonamido, trifluoromethyl, cyano, azido, cycloalkyl such as e.g. cyclopentyl or cyclohexyl, heterocycloalkyl such as e.g. morpholino, piperazinyl or piperidinyl, alkylaryl, arylalkyl and heteroaryl. Preferred substituents of such organic residues are, for
30 example, halogens, such as fluorine, chlorine, bromine or iodine, amino groups, hydroxyl groups, carbonyl groups, thiol groups and carboxyl groups.

- The term "alkylaryl" as used in the context of any linking moiety described in the present invention is denoted to mean a linking moiety having the structure -alkyl-aryl-, thus being
35 linked on one side via the alkyl group and on the other side via the aryl group, wherein this term is meant to also encompass linking moieties such as -alkyl-aryl-alkyl- linking moieties. The term "alkylaryl group", when used in the context of any substituent described hereinunder and above, is denoted to mean a residue being linked via the alkyl portion, said alkyl portion being further substituted with an aryl moiety.

The term "arylalkyl" as used in the context of any linking moiety described in the present invention is denoted to mean a linking moiety having the structure -aryl-alkyl-, thus being linked on one side via the aryl group and on the other side via the alkyl group, wherein this
5 term is meant to also encompass linking moieties such as -aryl-alkyl-aryl- linking moieties. The term "arylalkyl group", when used in the context of any substituent described hereinunder and above, is denoted to mean a residue being linked via the aryl portion, said aryl portion being further substituted with an alkyl moiety.

10 The term "alkylheteroaryl" as used in the context of any linking moiety described in the present invention is denoted to mean a linking moiety having the structure -alkyl-heteroaryl-, thus being linked on one side via the alkyl group and on the other side via the heteroaryl group, wherein this term is meant to also encompass linking moieties such as -alkyl-heteroaryl-alkyl- linking moieties. The term "alkylheteroaryl group", when
15 used in the context of any substituent described hereinunder and above, is denoted to mean a residue being linked via the alkyl portion, said alkyl portion being further substituted with a heteroaryl moiety.

The term "heteroarylalkyl" as used in the context of any linking moiety described in the present invention is denoted to mean a linking moiety having the structure -heteroaryl-alkyl-, thus being linked on one side via the heteroaryl group and on the other side via the
20 alkyl group, wherein this term is meant to also encompass linking moieties such as -heteroaryl-alkyl-heteroaryl- linking moieties. The term "heteroarylalkyl group", when used in the context of any substituent described hereinunder and above, is denoted to mean
25 a residue being linked via the heteroaryl portion, said heteroaryl portion being further substituted with an alkyl moiety.

Without wanting to be bound to any theory, it is believed that the efficacy and/or unspecific toxicity of the conjugates of the invention can further be favorably controlled by
30 providing linking moieties L' which have an advantageous influence on the respective release rate of the cytotoxic agent *in vivo*.

The term "advantageous influence on the release rate" as used herein shall describe an influence allowing for a release rate which generates suitable amounts of the cytotoxic
35 agent in a suitable time period so that therapeutic levels of the cytotoxic agent are delivered prior to excretion of the conjugate or conjugate fragments through the kidney or inactivation of the cytotoxic agent comprised in the conjugate by alternative mechanisms in the body. The term "suitable amounts" as used in this context of the present invention shall describe an amount with which the desired therapeutic effect of the cytotoxic agent is

achieved, preferably together with an unspecific toxicity of the cytotoxic agent as low as possible.

5 In the context of the present invention, it is assumed that the release rates can, inter alia, be tailored to specific needs by choosing a suitable electron-withdrawing group and/or a suitable sterically demanding group and/or an unsubstituted linear alkyl group in close proximity to the functional group F³.

10 The term "present in close proximity to" as used in the context of the present invention is preferably denoted to mean a group which is present in alpha, beta, or gamma position to the functional group F³.

15 The term "electron-withdrawing group" is recognized in the art, and denotes the tendency of a functional group to attract valence electrons from neighboring atoms by means of a difference in electronegativity with respect to the neighboring atom (inductive effect) or by withdrawal of π -electrons via conjugation (mesomeric effect).

20 The term "sterically demanding group" is denoted to mean a group, being sterically more demanding than a hydrogen, preferably a substituent such as an alkyl, aryl or heteroaryl group, or a side chain of a natural or unnatural amino acid.

25 Without wanting to be bound to any theory, it is believed that the higher the tendency of the electron-withdrawing group to attract valence electrons, the faster the cytotoxic agent is released *in vivo*.

Further, it is believed that the more sterically demanding the group present in close proximity, more preferably in alpha position, the slower the release rate and the longer the residence time in the plasma allowing an accumulation in tumor tissue and preventing the premature clearance of the low molecular weight cytotoxic agent, through the kidney.

30

Accordingly, depending on the specific needs, the following embodiments are described:

35 (i) A hydroxyalkyl starch conjugate comprising an electron-withdrawing group in close proximity to the functional group F³. Preferably, the electron-withdrawing group is present in alpha, beta or gamma position to the functional group F³, more preferably in alpha or beta position.

(ii) A hydroxyalkyl starch conjugate comprising at least one sterically demanding group in close proximity to the functional group F³. Preferably, the sterically demanding group

is present in alpha, beta or gamma position to the functional group F^3 , more preferably in alpha position.

5 (iii) A hydroxyalkyl starch conjugate comprising at least one sterically demanding group and an electron-withdrawing group in close proximity to the functional group F^3 , more preferably at least one sterically demanding group in alpha position as well as an electron-withdrawing group in alpha position.

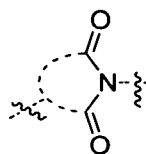
10 (iv) A hydroxyalkyl starch conjugate comprising an unsubstituted alkyl group in close proximity to the functional group F^3 , preferably a $-CH_2-$ group in alpha, beta and gamma position.

15 The electron-withdrawing group, if present, may be either part of the linking moiety L' or, according to an alternative embodiment, may be present in the hydroxyalkyl starch derivative, provided that the electron-withdrawing group is present in close proximity to the functional group F^3 , as described above.

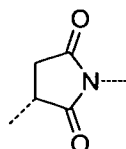
20 Preferably, the electron-withdrawing group is a moiety selected from the group consisting of $-O-$, $-S-$, $-SO-$, $-SO_2-$, $-NR^e-$, $-C(=Y^e)-$, $-NR^e-C(=Y^e)-$, $-C(=Y^e)-NR^e-$, $-NO_2$ comprising groups such as $-CH(NO_2)-$, $-CN$ comprising groups, such as $-CH(CN)-$, aryl groups, heteroaryl groups, cyclic imide groups and at least partially fluorinated alkyl moieties, wherein Y^e is either O, S or NR^e , and wherein R^e is one of hydrogen, alkyl, aryl, arylalkyl, heteroaryl, alkylaryl, alkylheteroaryl or heteroarylalkyl group, and the like.

25 Within the meaning of the present invention, the term "at least partially fluorinated alkyl moiety" refers to, optionally substituted, alkyl groups, such as non-branched alkyl residues, branched alkyl residues, cycloalkyl residues, as well as residues comprising one or more heteroatoms or functional groups, such as, by way of example, $-O-$, $-S-$, $-NH-$, $-NH-C(=O)-$, $-C(=O)-NH-$, and the like, having at least one of the hydrogen atoms replaced with a fluorine atom. In some fluorinated alkyl groups, all the hydrogen atoms are replaced with fluorine atoms, i.e., the fluorinated alkyl group is a perfluoroalkyl group. The following groups are mentioned, by way of example: $-CH_2F$, $-CF_3$, $-CHF_2$, $-CF_2-$, $-CFTF-$, $-CH_2-CF_3$, $-CH_2-CHF_2$ and $-CH_2-CH_2F$.

35 Within the context of the present invention, the term "cyclic imide groups" is denoted to mean a cyclic structural unit according to the general formula:



wherein the ring structure is preferably a 5-membered ring, 6-membered ring or 7-membered ring. Most preferably the cyclic imide is a -succinimide- having the following structure

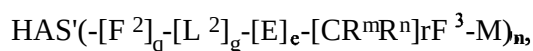


Preferably the electron-withdrawing group, if present, is selected from the group consisting of -NH-C(=O)-, -C(=O)-NH-, -NH-, -O-, -S-, -SO-, -SO₂- and -succinimide-. More preferably the electron-withdrawing group is selected from the group consisting of -C(=O)-NH-, -NH-, -O-, -S-, -SO₂- and -succinimide-.

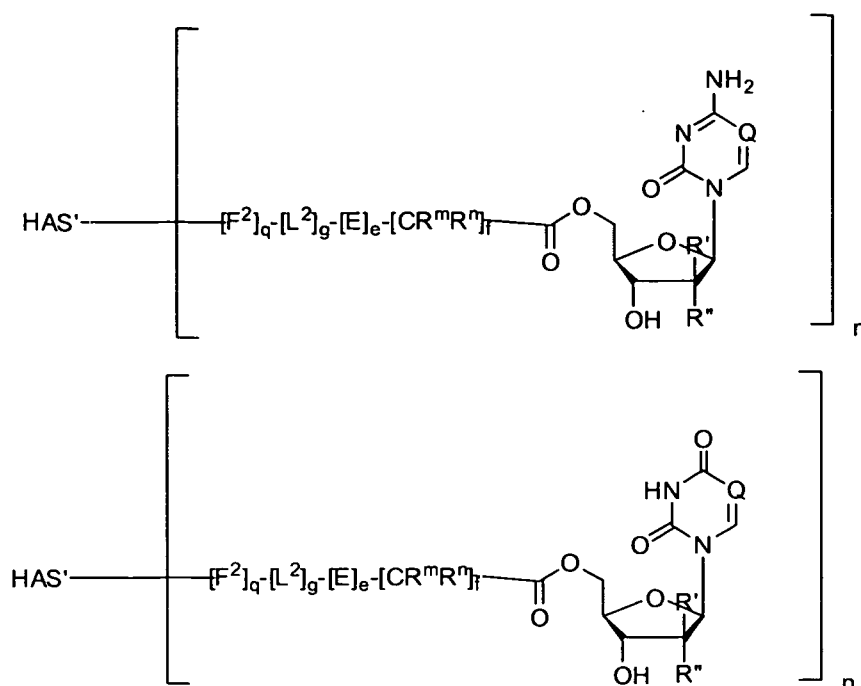
Thus, the present invention also relates to a conjugate, as described above, as well as a conjugate obtained or obtainable by the above-described method, wherein the conjugate comprises an electron-withdrawing group, preferably in alpha or beta position to each functional group F³, more particular in alpha position to each functional group F³, wherein the electron-withdrawing group is a group selected from the group consisting of -NH-C(=O)-, -C(=O)-NH-, -NH-, -O-, -S-, -SO-, -SO₂- and -succinimide-.

According to a particularly preferred embodiment of the present invention, the linking moiety L' has a structure according to the following formula -[F²]_q-[L²]_g-[E]_e-[CR^mRⁿ]_f, wherein E is an electron-withdrawing group, L² is a linking moiety, F² is a functional group, f is in the range of from 1 to 20, g is 0 or 1, q is 0 or 1, e is 0 or 1, and wherein R^m and Rⁿ are, independently of each other, H, aryl, alkyl or the side chain of a natural or unnatural amino acid, preferably H or alkyl.

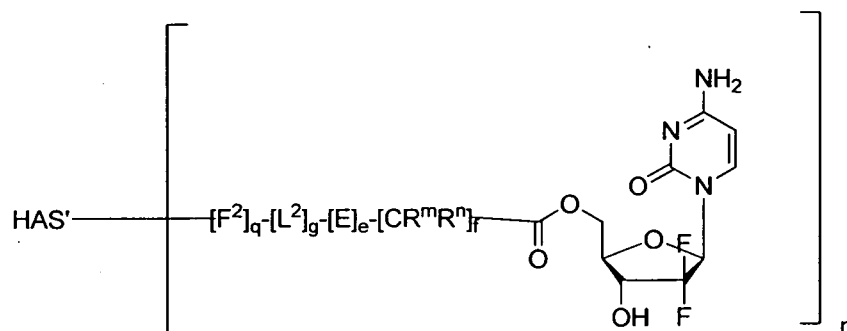
Thus, the conjugate, described above, has a structure according to the formula



more preferably according to one of the following formulas



most preferably according to the following formula



5

According to the first preferred embodiment of the invention, an electron-withdrawing group E is present in linking moiety L'. In this case, integer e is 1.

- 10 Preferably E, if present, is selected from the group consisting of -O-, -S-, -SO-, -SO₂-, -NR^e-, -C(=Y^e)-, -NR^e-C(=Y^e)-, -C(=Y^e)-NR^e-, -CH(NO₂)-, -CH(CN)-, aryl groups, heteroaryl groups, cyclic imide groups and at least partially fluorinated alkyl moieties, more preferably of the group consisting of -C(=O)-NH-, -NH-C(=O)-, -O-, -S-, -SO-, -SO₂- and -succinimide-, more preferably E, if present, is selected from the group
- 15 consisting of -NH-C(=O)-, -C(=O)-NH-, -succinimide-, -O- and -S-.

Accordingly, the following conjugate structures are thus preferred: HAS'(-[F²]_q-[L²]_g-C(=O)-NH-[CR^mRⁿ]_f-F³-M)_n, HAS'(-[F²]_q-[L²]_g-NH-C(=O)-[CR^mRⁿ]_f-F³-M)_n, HAS'(-

$[F^2]_q-[L^2]_g-0-[CR^mR^n]_fF^3-M)_n$, $HAS'(-[F^2]_q-[L^2]_g-S-[CR^mR^n]_fF^3-M)_n$, $HAS'(-[F^2]_q-[L^2]_g-succinimide-[CR^mR^n]_fF^3-M)_n$. More preferably, the electron-withdrawing group E is selected from the group consisting of -NH-C(=O)-, -C(=O)-NH-, -succinimide-, -O- and -S- and the functional group F^3 is a -C(=Y)- group, the hydroxyalkyl starch conjugate thus

5 having preferably a structure selected from the group consisting of $HAS'(-[F^2]_q-[L^2]_g-C(=O)-NH-[CR^mR^n]_fC(=Y)-M)_n$, $HAS'(-[F^2]_q-[L^2]_g-NH-(C=O)-[CR^mR^n]_fC(=Y)-M)_n$, $HAS'(-[F^2]_q-[L^2]_g-0-[CR^mR^n]_fC(=Y)-M)_n$, $HAS'(-[F^2]_q-[L^2]_g-S-[CR^mR^n]_fC(=Y)-M)_n$, $HAS'(-[F^2]_q-[L^2]_g-succinimide-[CR^mR^n]_fC(=Y)-M)_n$, wherein Y is preferably selected from O or S, in particular wherein Y is O.

10

According to an alternative preferred embodiment the functional group F^2 is an electron-withdrawing group present in close proximity to the functional group F^3 . In this case, F^2 may for example be a group such as -C(=O)-NH-, -NH-, -O-, -S- or -succinimide-. In case F^2 is an electron-withdrawing group present in close proximity to the functional group F^3 ,

15 which is in alpha, beta or gamma position to the functional group F^3 , F^2 may be present instead of E or in addition to E.

According to this embodiment, the following conjugate structures are thus particularly preferred: $HAS'(-C(=O)-NH-[L^2]_g-[E]_e-[CR^mR^n]_fF^3-M)_n$, $HAS'(-NH-[L^2]_g-[E]_e-[CR^mR^n]_fF^3-M)_n$, $HAS'(-O-[L^2]_g-[E]_e-[CR^mR^n]_fF^3-M)_n$, $HAS'(-S-[L^2]_g-[E]_e-[CR^mR^n]_fF^3-M)_n$ and $HAS'(-succinimide-[L^2]_g-[E]_e-[CR^mR^n]_fF^3-M)_n$, more preferably F^3 is -C(=Y)- and the conjugate structures are selected from the group consisting of $HAS'(-C(=O)-NH-[L^2]_g-[E]_e-[CR^mR^n]_fC(=Y)-M)_n$, $HAS'(-NH-[L^2]_g-[E]_e-[CR^mR^n]_fC(=Y)-M)_n$, $HAS'(-S-[L^2]_g-[E]_e-[CR^mR^n]_fC(=Y)-M)_n$, $HAS'(-O-[L^2]_g-[E]_e-[CR^mR^n]_fC(=Y)-M)_n$ and $HAS'(-succinimide-[L^2]_g-[E]_e-[CR^mR^n]_fC(=Y)-M)_n$, wherein Y is preferably selected from O or S, in particular wherein Y is O.

20
25

According to an alternative embodiment, the electron-withdrawing group, if present in the linking moiety L' , may also be present in the linking moiety L^2 .

30

Further, the electron-withdrawing group, if present, may also be present in the structural unit $[CR^mR^n]_f$. It is recalled that integer f of the structural unit $[CR^mR^n]_f$ is preferably in the range of from 1 to 3 and R^m and R^n are, independently of each other, H, aryl or alkyl or the side chain of a natural or unnatural amino acid, preferably H or alkyl. Since the term

35 "alkyl" as used in the context of the present invention also encompasses alkyl groups which are further substituted, the electron-withdrawing group may also be present in at least one of R^m or R^n , such as, e.g. in the form of a -CH₂F, -CHF₂ or -CF₃ group or the like.

According to a further preferred embodiment of the present invention, the electron-withdrawing group, if present, is not present in the linking moiety L' but is instead part of the hydroxyalkyl starch derivative (HAS'). in this case e is 0 and the integers q, g and f are chosen so that the electron-withdrawing group is preferably present in the hydroxyalkyl starch derivative in a position being in close proximity to the functional group F³, as described above, preferably in alpha or beta position to the functional group F³.

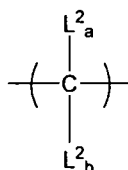
The sterically demanding group, if present, is preferably present in the structural unit $-\text{[CR}^m\text{R}^n\text{]}_f$, as described in detail hereinunder.

Linking moiety L²

In general, there are no particular restrictions as to the chemical nature of the linking moiety L². The term "linking moiety L²" as used in the context of the present application, relates to any suitable chemical moiety bridging F² and E, in case q and e are 1, or bridging F² and the structural unit $-\text{[CR}^m\text{R}^n\text{]}_f$ in case q is 1, e is 0 and f is in the range of from 1 to 10, or bridging E and the hydroxyalkyl starch derivative in case q is 0 and e is 1. Thus L² may, inter alia, be alkyl, alkenyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl group. The respective residues may comprise one or more substituents as described above.

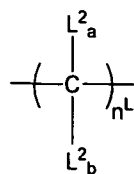
Preferably, L² is an alkyl group comprising 1 to 20, preferably 1 to 10, more preferably 1 to 8, more preferably, 1 to 6, such as 1, 2, 3, 4, 5 or 6, more preferably 1 to 4, more preferably from 1 to 3, and most preferably from 2 to 3 carbon atoms. According to the definition of the term "alkyl", the above mentioned alkyl groups may be substituted.

In particular, L² comprises at least one structural unit according to the following formula

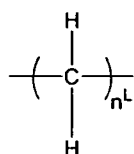


wherein L²_a and L²_b are independently of each other H or an organic residue selected from the group consisting of alkyl, alkenyl, aryl, arylalkyl, alkylaryl, heteroaryl, heteroarylalkyl, alkylheteroaryl, hydroxyl, and halogen, such as fluorine, chlorine, bromine, or iodine.

More preferably, L² has a structure according to the following formula



with L^2_a and L^2_b being selected from the group consisting of H, methyl or hydroxyl, with n^L being preferably in the range of from 1 to 8, more preferably of from 1 to 6, more preferably of from 1 to 4, more preferably of from 1 to 3, and most preferably of from 1 to 2. According to an even more preferred embodiment, the spacer L^2 consists of the structural unit according to the following formula



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wherein integer n^L is from 1 to 8, more preferably in the range of from 1 to 6, more preferably in the range of from 1 to 4, more preferably from 1 to 3, and most preferably from 1 to 2. Therefore, according to a preferred embodiment of the present invention, L^2 has a structure selected from the group consisting of $-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-$, $-CH_2-$, more preferably L^2 is selected from the group consisting of $-CH_2-$, $-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-$.

According to one preferred embodiment of the present invention, the present invention also relates to a conjugate, as described above, as well as a conjugate obtained or obtainable by the above-mentioned method, wherein the conjugate has a structure selected from the group consisting of the following formulas $HAS'(-[F^2]_q[CH_2]_g[E]_e-[CR^mR^n]_rF^3-M)_n$, $HAS'(-[F^2]_q[CH_2-CH_2]_g[E]_e-[CR^mR^n]_rF^3-M)_n$, $HAS'(-[F^2]_q[CH_2-CH_2-CH_2]_g[E]_e-[CR^mR^n]_rF^3-M)_n$, $HAS'(-[F^2]_q[CH_2-CH_2-CH_2-CH_2]_g[E]_e-[CR^mR^n]_rF^3-M)_n$, $HAS'(-[F^2]_q[CH_2-CH_2-CH_2-CH_2-CH_2]_g[E]_e-[CR^mR^n]_rF^3-M)_n$, $HAS'(-[F^2]_q[CH_2-CH_2-CH_2-CH_2-CH_2-CH_2]_g[E]_e-[CR^mR^n]_rF^3-M)_n$, more preferably the conjugate is selected from the following structures: $HAS'(-[F^2]_q[CH_2]_g[E]_e-[CR^mR^n]_rF^3-M)_n$, $HAS'(-[F^2]_q[CH_2-CH_2]_g[E]_e-[CR^mR^n]_rF^3-M)_n$ and $HAS'(-[F^2]_q[CH_2-CH_2-CH_2]_g[E]_e-[CR^mR^n]_rF^3-M)_n$, more preferably from the group consisting of $HAS'(-[F^2]_q[CH_2-CH_2-CH_2]_g[E]_e-[CR^mR^n]_rF^3-M)_n$, $HAS'(-[F^2]_q[CH_2-CH_2-CH_2-CH_2]_g[E]_e-[CR^mR^n]_rF^3-M)_n$ and $HAS'(-[F^2]_q[CH_2-CH_2-CH_2-CH_2-CH_2]_g[E]_e-[CR^mR^n]_rF^3-M)_n$.

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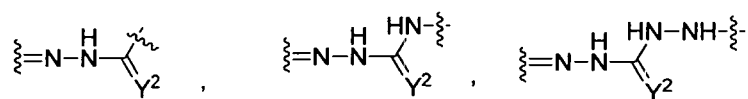
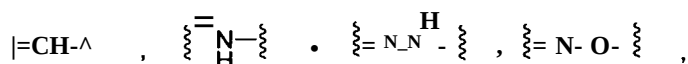
Most preferably g is 1, i.e. L^2 is present, and L^2 is $-CH_2-$, $-CH_2-CH_2-$ or $-CH_2-CH_2-CH_2-$.

The functional group F²

The functional group F², if present, is a functional group linking the hydroxyalkyl starch derivative with the linking moiety L², in case g is 1, or with the electron-withdrawing group E in case g is 0 and e is 1, or with the structural unit [CR^mRⁿ]_f in case g and e are 0.

There are, in general, no particular restrictions as regards the chemical nature of the functional group F² provided that a stable bond is formed linking the hydroxyalkyl starch derivative with L², E or the structural unit [CR^mRⁿ]_f, respectively. The stable bond may also be a bond which is eventually cleaved *in vivo*. As described above, the functional group F² may serve as electron-withdrawing group in close proximity to the functional group F³ to provide an optimized hydrolysis rate of the linkage between F³ and the cytotoxic agent.

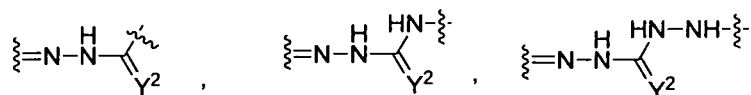
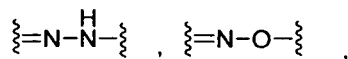
Preferably, F² is a group consisting of -Y¹-, -C(=Y²)-, -C(=Y²)-NR^{F2}-,



and -CH₂-CH₂-C(=Y²)-NR^{F2}-,

wherein Y¹ is selected from the group consisting of -S-, -O-, -NH-, -NH-NH-, -CH₂-CH₂-SO₂-NR^{F2}-, -CH₂-CHOH-, and cyclic imides, such as succinimide, and wherein Y² is selected from the group consisting of NH, S and O, and wherein R^{F2} is selected from the group consisting of hydrogen, alkyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl group.

More preferably, F² is a group consisting of -Y¹-, -C(=Y²)-, -C(=Y²)-NR^{F2}-;



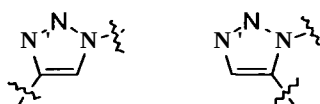
and -CH₂-CH₂-C(=Y²)-NR^{F2}-.

Preferably, F² is selected from the group consisting of -S-, -NH-NH-, and -succinimide-, more preferably F² is -succinimide- or -S-, most preferably -succinimide-.

The functional group F^2 is suitably chosen depending on the functional group -X- being present in the hydroxyalkyl starch derivative.

Thus, according to one preferred embodiment of the invention, the present invention also relates to the conjugate as described above, wherein in the structural unit $[F^2]_q$, q is 1 and F^2 is -S- or -succinimide-, the conjugate having a structure $HAS'(-succinimide-[L^2]_g-[E]_e-[CR^mR^n]_f-F^3-M)_n$ or $HAS'(-S-[L^2]_g-[E]_e-[CR^mR^n]_f-F^3-M)_n$, more preferably $HAS'(-succinimide-[L^2]_g-[E]_e-[CR^mR^n]_f-F^3-M)_n$.

Furthermore, the functional group F^2 may form together with a functional group of the hydroxyalkyl starch a 1,2,3-triazole ring. In the event that the functional group F^2 forms together with a functional group of the hydroxyalkyl starch derivative a 1,2,3-triazole, inter alia, the following structures are conceivable for this structural building block.



In case the conjugate comprises a triazole linking group, preferably the functional group F^2 forms together with the functional group X present in the residue of the hydroxyalkyl starch derivative a 1,2,3-triazole. Preferably such a triazole group is formed via a 1,3-dipolar cycloaddition between an azide and a terminal or internal alkynyl group to give a 1,2,3-triazole. For example in case Z^1 is an alkynyl group or azide and the crosslinking compound L bears a functional group K^2 being the respective azide or alkynyl, a triazole linkage may be formed upon reaction of the crosslinking compound L with the hydroxyalkyl starch derivative.

The structural unit $[CR^mR^n]_f$

As regards the structural unit $[CR^mR^n]_f$, integer f is in the range of from 1 to 20, preferably in the range of from 1 to 10, such 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10, more preferably in the range of from 1 to 5, and R^m and R^n are, independently of each other, H, alkyl, aryl or a side chain of a natural or unnatural amino acid, preferably H or alkyl. In case integer f is greater than 1, each repeating unit $[CR^mR^n]$ may be the same or may be different from each other.

Preferably, R^m and R^n are, independently of each other, selected from H or branched or linear alkyl chains, comprising 1 to 10, preferably 1 to 8, more preferably 1 to 5, most preferably 1 to 3 carbon atoms. More preferably R^m and R^n are, independently of each

other, selected from the group consisting of H, methyl, ethyl, propyl, butyl, sec-butyl and tert-butyl, more preferably R^m and R^n are, independently of each other, H or methyl.

By way of example, the following preferred structures for the structural unit $[CR^mR^n]_f$ are mentioned:

5 $-CH_2-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-$, $-CH_2-$, $-CH(CH_3)-$, $-CH(CH_2CH_3)-$, $-CH(CH(CH_3)_2)-$, $-CH(CH_3)-CH_2-$, $-CH_2-CH(CH_3)-$, $-CH(CH_3)-CH_2-CH_2-$, $-CH_2-CH(CH_3)-CH_2-$, $-CH_2-CH_2-CH(CH_3)-$, $-CH_2-CH_2-CH_2-CH(CH_3)-$, $-C(CH_3)_2-$, $-CH(CH_3)-CH(CH_3)-$, $-CH(CH_3)-CH(CH_3)-CH_2-$, $-CH_2-CH(CH_3)-CH(CH_3)-$, $-CH(CH_3)-CH_2-CH(CH_3)-$.

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According to one preferred embodiment of the present invention, R^m and R^n are both H. The structural unit $[CR^mR^n]_f$ is thus preferably $-CH_2-CH_2-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-$ or $-CH_2-$. Thus, the present invention also relates to the conjugate as described above, the conjugate having a structure selected from the

15 group consisting of $HAS'(-[F^2]_q-[L^2]_g-[E]_e-CH_2-CH_2-CH_2-CH_2-CH_2-F^3-M)_n$, $HAS'(-[F^2]_q-[L^2]_g-[E]_e-CH_2-CH_2-CH_2-CH_2-F^3-M)_n$, $HAS'(-[F^2]_q-[L^2]_g-[E]_e-CH_2-CH_2-F^3-M)_n$ and $HAS'(-[F^2]_q-[L^2]_g-[E]_e-CH_2-F^3-M)_n$.

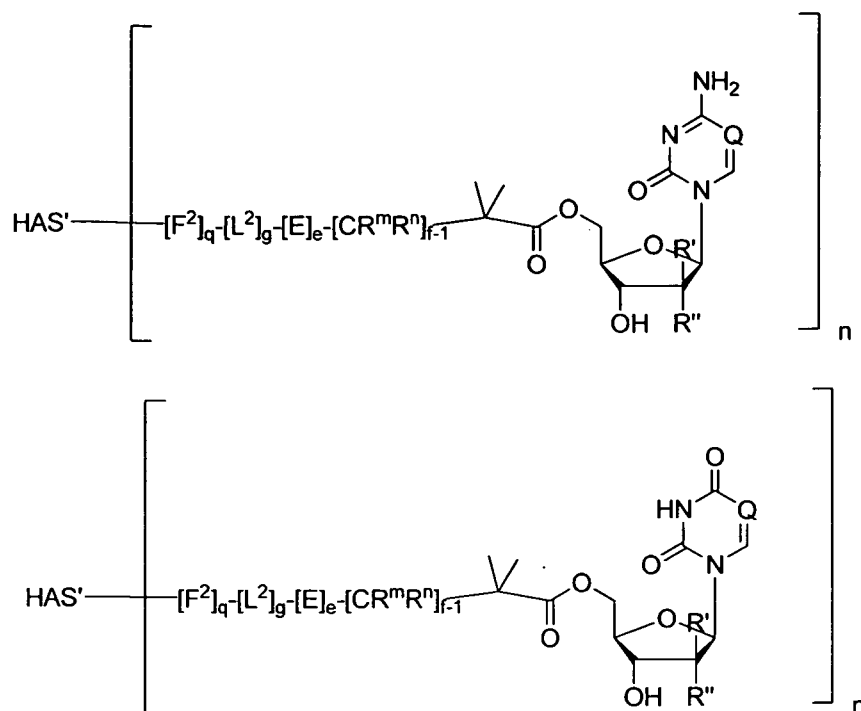
According to another preferred embodiment, at least one of R^m or R^n of at least one repeating unit of the structural unit $[CR^mR^n]_f$ is a sterically demanding group, more preferably an alkyl group, most preferably at least one of R^m or R^n is present in alpha, beta or gamma position, more preferably in alpha position. Most preferably, at least one of R^m or R^n is a methyl group. Preferably, the structural unit $[CR^mR^n]_f$ is a group having the structure

25 $-[CR^mR^n]_{f-1}-CH(CH_3)-$ or $-[CR^mR^n]_{f-1}-C(CH_3)_2-$.

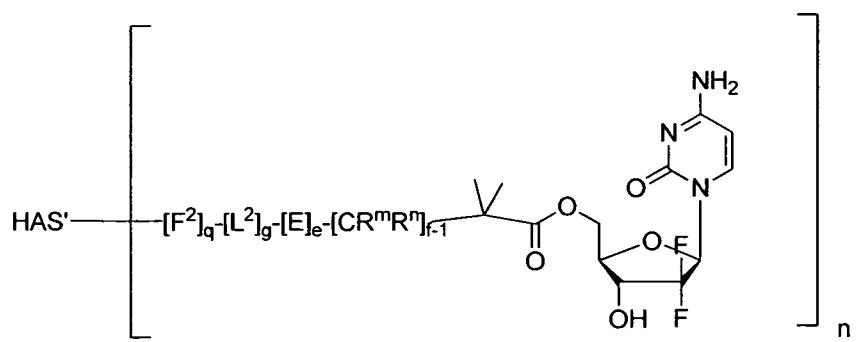
Thus, the present invention also relates to a conjugate, as described above, as well as to a conjugate obtained or obtainable by the above described method, the conjugate having a structure according to the formula

30 $HAS'(-[F^2]_q-[L^2]_g-[E]_e-[CR^mR^n]_{f-1}-C(CH_3)_2-F^3-M)_{n-1}$,
or
 $HAS'(-[F^2]_q-[L^2]_g-[E]_e-[CR^mR^n]_{f-1}-CH(CH_3)-F^3-M)_n$

more preferably according to one of the following formulas



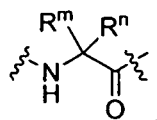
most preferably according to the following formula



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According to an alternative embodiment, the structural unit $-[E]_e-[CR^mR^n]_f-F^3-$ is a residue derived from a natural or unnatural amino acid being linked to the linking moiety L^2 , wherein e is 1 and E is $-C(=O)-NH-$ and wherein one of R^m and R^n is the side chain of a natural or unnatural amino acid. Alternatively, e is 0 and g is 0 and the structural unit $-[F^2]_q-$
 10 $[CR^mR^n]_f-F^3-$ is a residue of a natural or unnatural amino acid, wherein one of R^m and R^n is the side chain of a natural or unnatural amino acid.

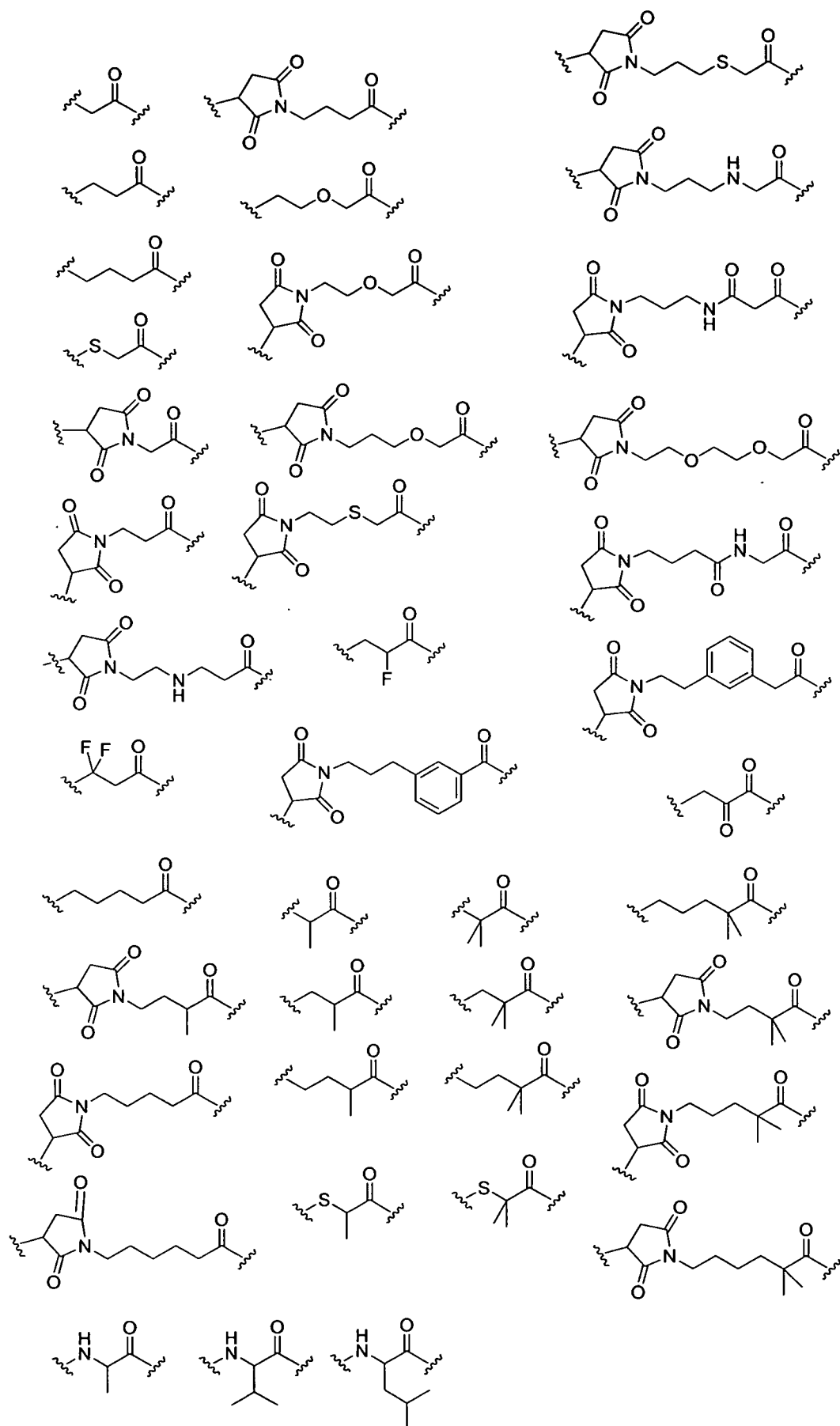
The term "residue of an amino acid" is denoted to mean an amino acid being incorporated into the linker L or an amino acid being the linker L and being incorporated into the conjugate of the invention, respectively, wherein the residue of an amino acid has a
 15 structure according to the following formula:



The term "side chain of a natural or unnatural amino acid" refers to the residue being linked to the alpha carbon atom of a natural or unnatural amino acid, in this case the C atom of the structural unit $-\text{[CR}^m\text{R}^n\text{]}_r$. The term "natural amino acid" refers to naturally occurring amino acids or residues which typically occur in proteins including their stereoisomeric forms. Natural amino acids include alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) and valine (Val). The term unnatural amino acid includes any conceivable amino acid. This term includes amino acids bearing a side chains comprising acidic, basic, neutral and/or aromatic moieties. Conceivable amino acids to be mentioned are, for example, azetidine carboxylic acid, 2-aminoadipic acid, 3-aminoadipic acid, beta-alanine, aminopropionic acid, 2-aminobutyric acid, 4-aminobutyric acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2-aminoisobutyric acid, 3-aminoisobutyric acid, 2-aminopimelic acid, 2,4-diaminoisobutyric acid, desmosine, 2,2'-diaminopimelic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-ethylasparagine, hydroxylysine, allo-hydroxylysine, 3-hydroxyproline, 4-hydroxyproline, isodesmosine, allo-isoleucine, N-methylglycine, N-methylisoleucine, N-methylvaline, norvaline, norleucine, ornithine, naphthylalanine, diaminopropionic acid, N-(fluoropropionyl)-diaminobutyric acid, N-fluorobenzoyl-diaminobutyric acid, N-fluorobenzoyl-diaminopropionic acid, citrulline and pipercolic acid.

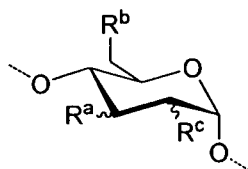
Examples of preferred linking moieties L

By way of example, the following preferred linking moieties L are mentioned:



The Residue of the Hydroxyalkyl Starch Derivative comprised in the Conjugate

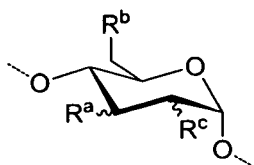
- In accordance with the above-mentioned definition of HAS, the residue of the hydroxyalkyl starch derivative preferably comprises at least one structural unit according to the following formula (I)



(I)

- wherein at least one of R^a , R^b or R^c comprises the functional group $-X-$ and wherein R^a , R^b and R^c are, independently of each other, selected from the group consisting of $-O-HAS''$, $-[O-(CR^wR^x)-(CR^yR^z)]_x-OH$, $-[O-(CR^wR^x)-(CR^yR^z)]_y-X-$, $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^1-X-$, wherein R^w , R^x , R^y and R^z are independently of each other selected from the group consisting of hydrogen and alkyl, y is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4, F^1 is a functional group, p is 0 or 1, L^1 is a linking moiety and $-X-$ is a functional group linking the hydroxyalkyl starch derivative and the linking moiety L . Preferably X is formed upon reaction of Z^1 with the crosslinking compound L . HAS'' is a remainder of the hydroxyalkyl starch derivative, as described above.
- The amount of functional groups X present in the residue of the hydroxyalkyl starch derivative being incorporated into the conjugate of the invention corresponds to the amount of functional groups Z^1 present in the corresponding hydroxyalkyl starch derivative prior to the conjugation of said derivative to the crosslinking compound L or the structural unit $-L-M$. Thus, preferably 0.15 % to 2 % of all residues R^a , R^b and R^c present in the hydroxyalkyl starch derivative contain the functional group Z^1 . More preferably, 0.15 % to 2 % of all residues R^a , R^b and R^c present in the hydroxyalkyl starch derivative have the structure $-[O-(CR^wR^x)-(CR^yR^z)]_y-X-$ or $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^1-X-$. According to a particularly preferred embodiment, R^a , R^b and R^c are selected from the group consisting of $-O-HAS''$, $-[O-(CR^wR^x)-(CR^yR^z)]_x-OH$ and $-[O-(CR^wR^x)-(CR^yR^z)]_y-X-$, wherein 0.15 % to 2 % of all residues R^a , R^b and R^c present in the hydroxyalkyl starch derivative have the structure $-[O-(CR^wR^x)-(CR^yR^z)]_y-X-$. According to an alternative preferred embodiment, R^a , R^b and R^c are selected from the group consisting of $-O-HAS''$, $-[O-(CR^wR^x)-(CR^yR^z)]_x-OH$ and $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^1-X-$, wherein 0.15 % to 2 % of all residues R^a , R^b and R^c present in the hydroxyalkyl starch derivative have the structure $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^1-X-$.

According to a preferred embodiment of the present invention, the hydroxyalkyl starch derivative is a hydroxyethyl starch derivative. Therefore, the present invention also describes a conjugate, comprising a residue of a hydroxyalkyl starch derivative, as described above, as well as a conjugate obtained or obtainable by the above-mentioned method, wherein the conjugate comprises a residue of a hydroxyethyl starch derivative and a cytotoxic agent, the residue of HES derivative preferably comprises at least one structural unit, according to the following formula (I)



(I)

wherein R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS', $-[O-CH_2-CH_2]_s-OH$, $-[O-CH_2-CH_2]_t-X-$ and $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X-$, wherein t is in the range of from 0 to 4, and wherein s is in the range of from 0 to 4, p being 0 or 1, and wherein at least one of R^a , R^b and R^c comprises the functional group X , and wherein X is linked to the linking moiety L comprised in the conjugate of the present invention.

According to a preferred embodiment of the present invention, this linkage between X and L is obtained by coupling a hydroxyalkyl starch derivative being functionalized with at least one functional group Z^1 , as described above, to the crosslinking compound L comprising the functional group K^2 or a derivative of a cytotoxic agent $-L-M$ comprising the functional group K^2 , thereby obtaining a covalent linkage between HAS' and L , wherein, as result, the residue of the hydroxyalkyl starch is linked via the functional group X to the linking moiety L . Further preferred embodiments as to this method are described below.

Preferably all functional groups X present in a given hydroxyalkyl starch derivative comprised in a conjugate according to the invention, are linked to the linking moiety L , most preferably to the structural unit $-L-M$.

The Functional Group X

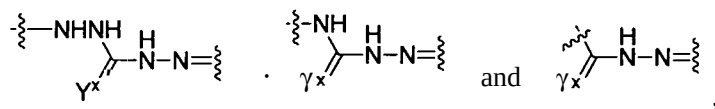
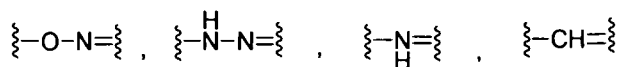
X is a functional group linking the hydroxyalkyl starch derivative with the linking moiety L , wherein L is preferably $-L^1-F^3-$, and wherein more preferably L^1 is $-[F^2]_q-[L^2]_g-[E]_e-[CR^mR^n]_f-$. Thus, X is a linking group preferably linking the hydroxyalkyl starch derivative

with the functional group F^2 in case q is 1, or with the linking moiety L^2 in case q is 0 and g is 1, or with the electron-withdrawing group E in case q and g are 0 and e is 1, or with the structural unit $[CR^mR'']^f$ in case q, g, e are 0 and f is in the range of from 1 to 20, preferably in the range of from 1 to 10.

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In general, there exists no limitation regarding the functional group X provided that the functional group X is able to link the hydroxyalkyl starch derivative with the linking moiety L. According to a preferred embodiment of the present invention, and depending on the respective group of the linking moiety L being linked to X, X is selected from the group consisting of $-Y^{**}-$, $-C(=Y^X)-$, $-C(=Y^X)-NR^{xx}-$, $-CH_2-CH_2-C(=Y^X)-NR^{xx}-$

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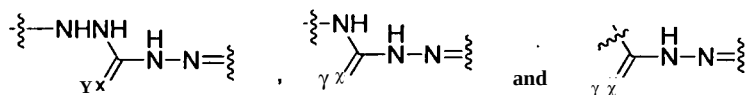
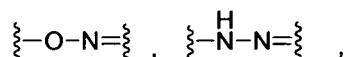
wherein Y^{**} is selected from the group consisting of $-S-$, $-O-$, $-NH-$, $-NH-NH-$, $-CH_2-CH_2-SO_2-NR^{xx}-$, and cyclic imides, such as succinimide, and wherein Y^X is selected from the group consisting of NH, S and O, and wherein R^{**} is selected from the group consisting of hydrogen, alkyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl group.

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Furthermore, the functional group X may form together with a functional group of the linking moiety L, such as with the functional group F^2 , a 1,2,3-triazole ring, as described hereinabove.

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More preferably X is selected from the group consisting of $-Y^{**}-$, $-C(=Y^X)-$, $-C(=Y^X)-NR^{xx}-$, $-CH_2-CH_2-C(=Y^X)-NR^{xx}-$



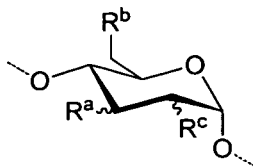
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More preferably X is selected from the group consisting of $-O-$, $-S-$, $-NH-$ and $-NH-NH-$. Most preferably X is $-S-$.

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Therefore, the present invention also describes a conjugate, comprising a residue of a hydroxyalkyl starch derivative, as described above, as well as a conjugate obtained or

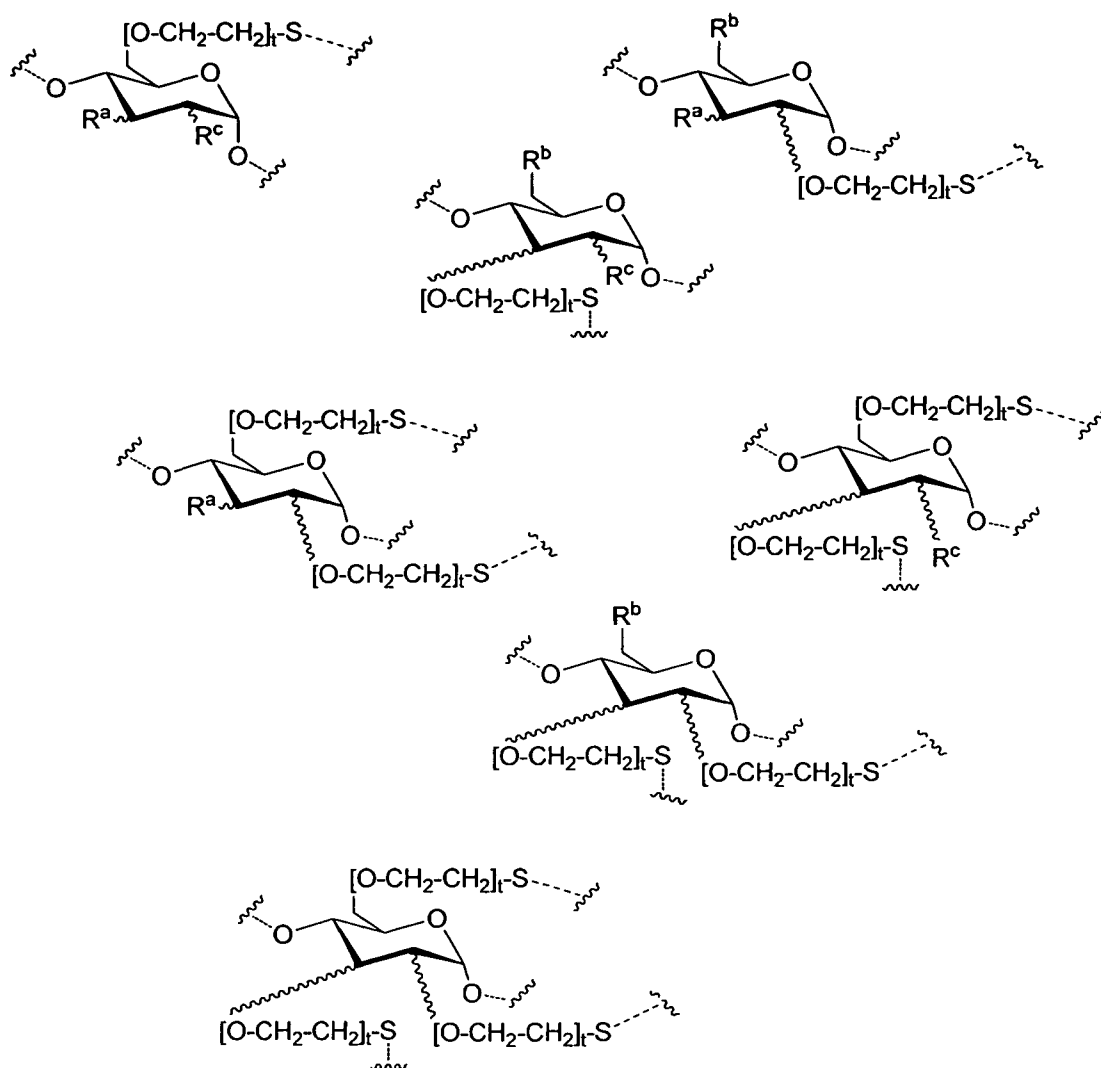
obtainable by the above-mentioned method, wherein the conjugate comprises a residue of a hydroxyalkyl starch derivative and a cytotoxic agent, the residue of the hydroxyalkyl starch derivative preferably comprises at least one structural unit according to the following formula (I)



(I)

wherein at least one of R^a , R^b and R^c is $-[O-(CR^wR^x)-(CR^yR^z)]_y-S-$ or $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F]_p-L^1-S-$, preferably wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-S-$ or $-[O-CH_2-CH_2]_t-[F]_p-L^1-S-$.

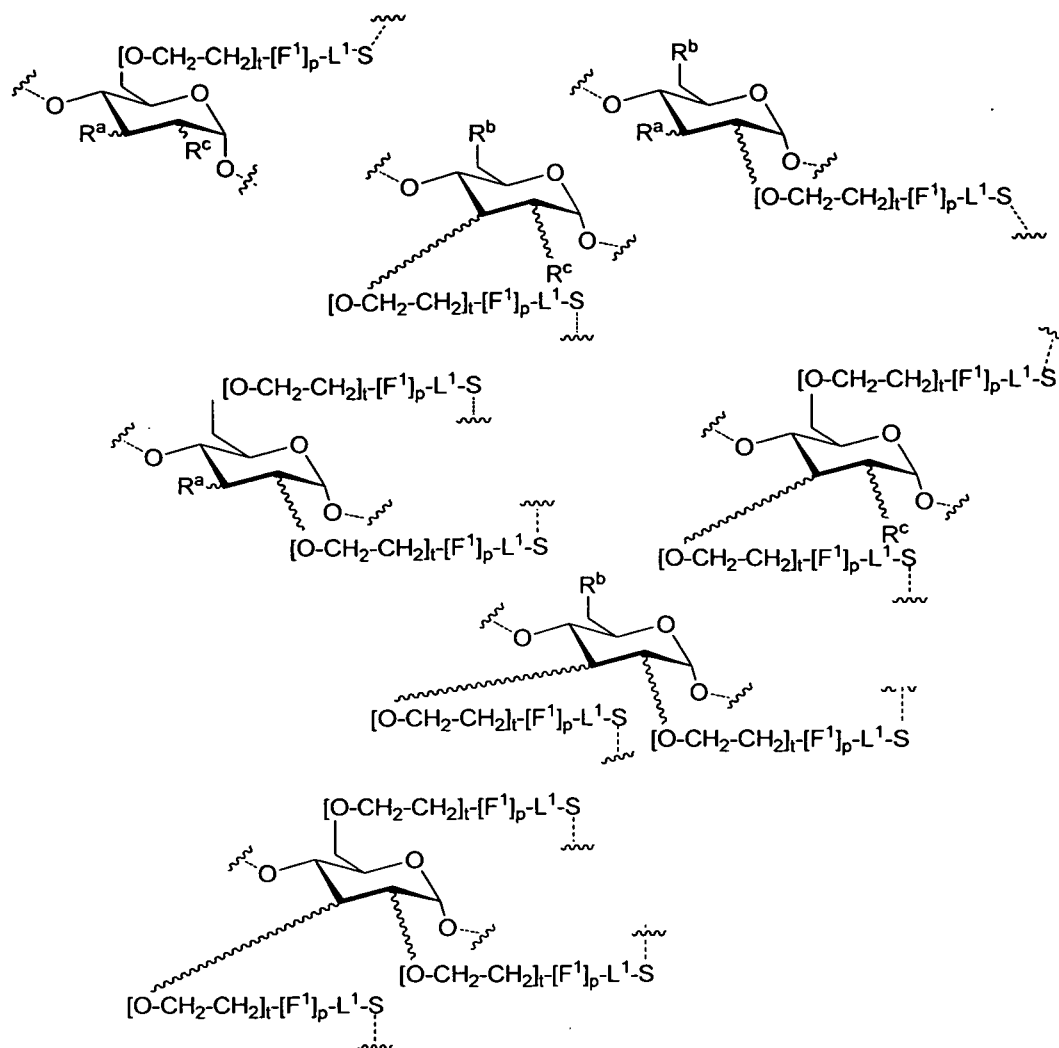
According to one preferred embodiment of the present invention, at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-S-$. Thus, the following hydroxyalkyl starch derivatives may be mentioned as preferred embodiments of the invention:



According to another preferred embodiment of the present invention, at least one of R^a , R^b and R^c is $-\text{O}-\text{CH}_2-\text{CH}_2-[\text{F}^1]_p-\text{L}^1-\text{S}-$. Thus, the following hydroxyalkyl starch derivatives

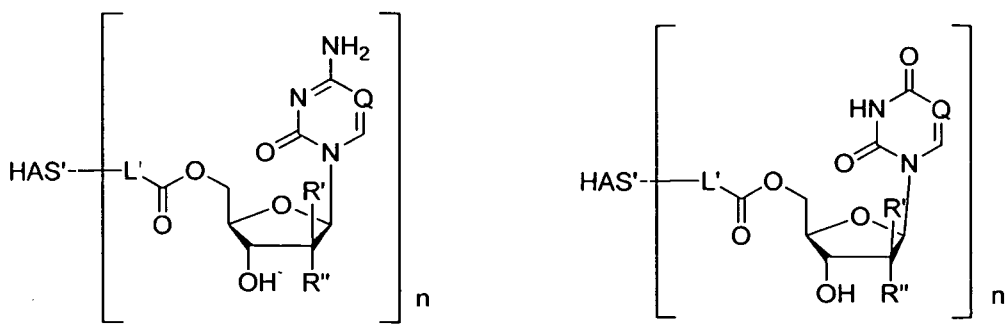
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may be mentioned as preferred embodiments of the invention:



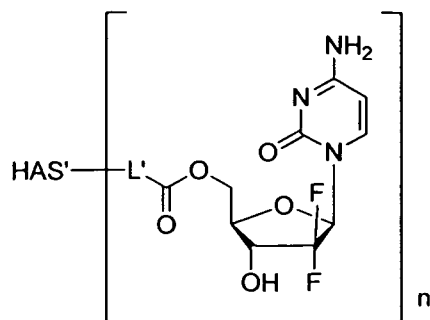
According to a preferred embodiment of the invention, the linking moiety L is directly linked to the functional group X of the hydroxyalkyl starch derivative and, on the other side, directly linked to a the group -O- derived from the primary hydroxyl group of the cytotoxic agent.

According to a more preferred embodiment of the invention, the linking moiety L is -L'-C(=O)- and the conjugate has a structure according to the following formula:



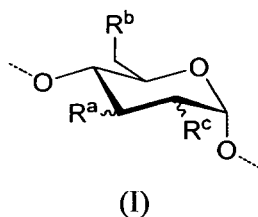
wherein Q is selected from the group consisting of C-H, C-F, C-CH₃ and N, and R' and R'' are independently of each other selected from the group consisting of OH, H and F, more preferably according to the following formula

5



wherein the hydroxyalkyl starch comprises at least one structure according to the following formula (I)

10



wherein at least one of R^a, R^b and R^c is -[O-(CR^wR^x)-(CR^yR^z)]_y-S- or -[O-(CR^wR^x)-(CR^yR^z)]_y-[F¹]_p-L¹-S-, preferably wherein at least one of R^a, R^b and R^c is -[O-CH₂-CH₂]-S- or -[O-CH₂-CH₂]_t-[F¹]_p-L¹-S- and wherein the linking moiety L' is linked to the functional group -S-.

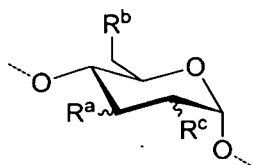
20 The Functional Group F¹

F¹ is a functional group, which, if present, is preferably selected from the group consisting of -Y⁷-, -Y⁷-C(=Y⁶)-, -C^{Y⁶}-, -Y⁷-C(=Y⁶)-Y⁸-, -C(=Y⁶)-Y⁸-, wherein -Y⁷- is selected from the group consisting of -NR^{Y⁷}-, -O-, -S-, -succinimide-, -NH-NH-, -NH-O-,
 25 -CH=N-O-, -O-N=CH-, -CH=N-, -N=CH-, -Y⁸- is selected from the group consisting of -NR^{Y⁸}-, -S-, -O-, -NH-NH- and Y⁶ is selected from the group consisting of NR^{Y⁶}, O and S, wherein R^{Y⁶} is H or alkyl, preferably H, and wherein R^{Y⁷} is H or alkyl, preferably H, and wherein R^{Y⁸} is H or alkyl, preferably H.

According to a preferred embodiment of the present invention the functional group F^1 is, if present, selected from the group consisting of -NH-, -O-, -S-, -NH-C(=O)-, -O-C(=O)-NH-, -NH-C(=O)-O-C(=O)-, -C(=O)-, -NH-C(=O)-NH-, -NH-NH-C(=O)-, -C(=O)-NH-NH-, -NH-C(=O)-NH-NH-, more preferably F^1 is, if present, -O- or -O-C(=O)-NH-.

5

Therefore, the present invention also describes a conjugate, comprising a hydroxyalkyl starch derivative, as described above, as well as a conjugate obtained or obtainable by the above-mentioned method, the hydroxyalkyl starch derivative preferably comprising at least one structural unit according to the following formula (I)



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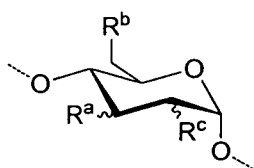
(I)

wherein at least one of R^a , R^b and R^c is $-[O-(CR^wR^x)-(CR^yR^z)]_y-X-$ or $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^1-X-$, preferably wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-X-$ or $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X-$, more preferably wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-S-$ or $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-S-$, wherein F^1 , if present, is preferably -O- or -O-C(=O)-NH-.

15

Thus, the following preferred conjugates are described, comprising a hydroxyalkyl starch derivative, as described above, wherein the hydroxyalkyl starch derivative comprises at least one structural unit according to the following formula (I)

20



(1)

25

wherein in each unit, independently of each other unit, at least one of R^a , R^b and R^c is

(i) $-[O-CH_2-CH_2]_t-X-$ or

30

(ii) $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X-$, preferably with p being 1 and F^1 being -O-, or

(iii) $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X-$, preferably with p being 1 and F^1 being -O-C(=O)-NH-,

wherein X is -S-, and wherein t is in the range of from 0 to 4, and wherein the linking moiety L of the structural unit -L-M is directly linked to at least one functional group X, preferably wherein all groups X present in the hydroxyalkyl starch derivative are linked to the structural unit -L-M, and wherein the linking moiety L is being attached to the group -O- derived from the the primary hydroxyl group of the cytotoxic agent.

The Linking Moiety L¹

The term "linking moiety L¹" as used in this context of the present invention relates to any suitable chemical moiety bridging X with the functional group F¹ or the building block - [0-(CR^wR^x)-{CR^yR^z}]_y- or the sugar backbone of the hydroxyalkyl starch derivative.

In general, there are no particular restrictions as to the chemical nature of the spacer L¹ with the proviso that L¹ provides for a stable linkage between the functional group -X- and the hydroxyalkyl starch building block. Preferably, L¹ is an alkyl, alkenyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl group. As described above, the terms alkyl, alkenyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl group, also encompass groups which are substituted by one or more suitable substituent.

According to a preferred embodiment of the present invention, the linking moiety L¹ is a spacer comprising at least one structural unit according to the following formula -{[CR^dR^f]_h-[F⁴]_u-[CR^{dd}R^{ff}]_z}_{alpha}-, wherein F⁴ is a functional group, preferably selected from the group consisting of -S-, -O- and -NH-, preferably wherein F⁴ is -O- or -S-, more preferably wherein F⁴ is -S-. The integer h is preferably in the range of from 1 to 20, more preferably of from 1 to 10, such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10, more preferably of from 1 to 5, most preferably of from 1 to 3. Integer z is in the range of from 0 to 20, more preferably of from 0 to 10, such as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10, more preferably of from 0 to 3, most preferably of from 0 to 2, such as 0, 1 or 2. Integer u is 0 or 1. Integer alpha is in the range of from 1 to 10, preferably of from 1 to 5, such as 1, 2, 3, 4, 5, more preferably 1 or 2. As regards residues R^d, R^f, R^{dd} and R^{ff}, these residues are, independently of each other, preferably selected from the group consisting of halogens, alkyl groups, H or hydroxyl groups. The repeating units of -[CR^dR^f]_h- may be the same or may be different. Likewise, the repeating units of -[CR^{dd}R^{ff}]_z- may be the same or may be different. Likewise in case integer alpha is greater than 1, the groups F⁴ in each repeating unit may be the same or may be different. Further, in case alpha is greater than 1, integer h in each repeating unit may be the same or may be different, integer z in each repeating unit may be the same or may be different and integer u in each repeating unit may be the same or may be different.

Thus, in case α is greater than 1, each repeating unit of $[\text{CR}^{\text{d}}\text{R}^{\text{f}}]_{\text{h}}-[\text{F}^4]_{\text{u}}-[\text{CR}^{\text{dd}}\text{R}^{\text{ff}}]_{\text{z}}$ may be the same or may be different. Most preferably, R^{d} , R^{f} , R^{dd} and R^{ff} are independently of each other H, alkyl or hydroxyl.

- 5 According to one embodiment of the present invention, u and z are 0, the linking moiety L^1 thus corresponds to the structural unit $-\text{[CR}^{\text{d}}\text{R}^{\text{f}}]_{\text{h}}-$.

According to an alternative embodiment of the present invention u is 1. According to this embodiment z is preferably greater than 0, preferably 1 or 2.

10

Thus, the following preferred structures for the linking moiety L^1 are mentioned, by way of example: $-\text{[CR}^{\text{d}}\text{R}^{\text{f}}]_{\text{h}}-\text{F}^4-\text{[CR}^{\text{dd}}\text{R}^{\text{ff}}]_{\text{z}}$ and $-\text{[CR}^{\text{d}}\text{R}^{\text{f}}]_{\text{h}}-$.

15

Thus, by way of the example, the following especially preferred linking moieties L^1 are mentioned:

- CH₂-,
 -CH₂-CH₂-,
 -CH₂-CH₂-CH₂-,
 -CH₂-CH₂-CH₂-CH₂-,
 20 -CH₂-CH₂-CH₂-CH₂-CH₂-,
 -CH₂-CH₂-CH₂-S-CH₂-CH₂-,
 -CH₂-CH₂-S-CH₂-CH₂-,
 -CH₂-CH₂-O-CH₂-CH₂-,
 -CH₂-CH₂-O-CH₂-CH₂-O-CH₂-CH₂-,
 25 -CH₂-CHOH-CH₂-,
 -CH₂-CHOH-CH₂-S-CH₂-CH₂-,
 -CH₂-CHOH-CH₂-S-CH₂-CH₂-CH₂-,
 -CH₂-CHOH-CH₂-NH-CH₂-CH₂-,
 -CH₂-CHOH-CH₂-NH-CH₂-CH₂-CH₂-,
 30 -CH₂-CHOH-CH₂-O-CH₂-CHOH-CH₂-,
 -CH₂-CHOH-CH₂-O-CH₂-CHOH-CH₂-S-CH₂-CH₂-,
 -CH₂-CH(CH₂OH)- and
 -CH₂-CH(CH₂OH)-S-CH₂-CH₂-.

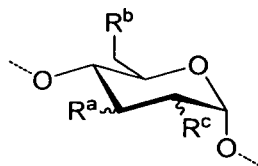
- 35 According to one preferred embodiment, R^{d} , R^{f} and, if present, R^{dd} and R^{ff} are preferably H or hydroxyl, more preferably at least one of R^{d} and R^{f} of at least one repeating unit of $-\text{[CR}^{\text{d}}\text{R}^{\text{f}}]_{\text{h}}-$ is -OH, wherein even more preferably, in this case, both R^{dd} and R^{ff} are H, if present. In particular, in this case, L^1 is selected from the group consisting of -CH₂-CHOH-CH₂-, -CH₂-CHOH-CH₂-S-CH₂-CH₂-, -CH₂-CHOH-CH₂-S-CH₂-CH₂-

CH_2^- , $-\text{CH}_2\text{-CHOH-CH}_2\text{-NH-CH}_2\text{-CH}_2^-$ and $-\text{CH}_2\text{-CHOH-CH}_2\text{-NH-CH}_2\text{-CH}_2\text{-CH}_2^-$, more preferably from the group consisting of $-\text{CH}_2\text{-CHOH-CH}_2^-$, $-\text{CH}_2\text{-CHOH-CH}_2\text{-S-CH}_2\text{-CH}_2^-$ and $-\text{CH}_2\text{-CHOH-CH}_2\text{-S-CH}_2\text{-CH}_2\text{-CH}_2^-$.

- 5 According to an alternative preferred embodiment, both residues R^d and R^f are H, and R^{dd} and R^f are, if present, H as well. In particular, in this case, L^1 is selected from the group consisting of: $-\text{CH}_2^-$, $-\text{CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-O-CH}_2\text{-CH}_2^-$ and $-\text{CH}_2\text{-CH}_2\text{-O-CH}_2\text{-CH}_2\text{-CH}_2^-$.

10

Therefore, the present invention also describes a hydroxyalkyl starch derivative, comprising at least one structural unit according to the following formula (I)



(I)

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wherein at least one of R^a , R^b and R^c has a structure according to the following formula $-\text{[O-CH}_2\text{-CH}_2\text{]}_l\text{-[F}^1\text{]}_p\text{-L}^1\text{-X-}$, wherein the linking moiety L^1 is selected from the group consisting of $-\text{CH}_2^-$, $-\text{CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CH}_2\text{-O-CH}_2\text{-CH}_2^-$.

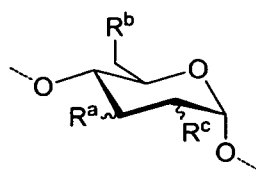
20

CH_2^- , $-\text{CH}_2\text{-CH}_2\text{-O-CH}_2\text{-CH}_2\text{-O-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CHOH-CH}_2^-$, $-\text{CH}_2\text{-CHOH-CH}_2\text{-S-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CHOH-CH}_2\text{-S-CH}_2\text{-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CHOH-CH}_2\text{-NH-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CHOH-CH}_2\text{-NH-CH}_2\text{-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CHOH-CH}_2\text{-O-CH}_2\text{-CHOH-CH}_2^-$, $-\text{CH}_2\text{-CHOH-CH}_2\text{-O-CH}_2\text{-CHOH-CH}_2\text{-S-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CH(CH}_2\text{OH)-}$ and $-\text{CH}_2\text{-CH(CH}_2\text{OH)-S-CH}_2\text{-CH}_2^-$, more preferably from the group consisting of $-\text{CH}_2\text{-CHOH-CH}_2^-$, $-\text{CH}_2\text{-CHOH-CH}_2\text{-S-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CHOH-CH}_2\text{-S-CH}_2\text{-CH}_2\text{-CH}_2^-$, $-\text{CH}_2\text{-CHOH-CH}_2\text{-NH-CH}_2\text{-CH}_2^-$ and $-\text{CH}_2\text{-CHOH-CH}_2\text{-NH-CH}_2\text{-CH}_2\text{-CH}_2^-$, more preferably from the group consisting of $-\text{CH}_2\text{-CHOH-CH}_2^-$, $-\text{CH}_2\text{-CHOH-CH}_2\text{-S-CH}_2\text{-CH}_2\text{-CH}_2^-$ and $-\text{CH}_2\text{-CHOH-CH}_2\text{-S-CH}_2\text{-CH}_2\text{-CH}_2^-$.

25

30

Further, the present invention also relates to a conjugate, comprising a hydroxyalkyl starch derivative, as described above, as well as a conjugate obtained or obtainable by the above-mentioned method, wherein the conjugate comprises a hydroxyalkyl starch derivative and a cytotoxic agent, wherein the hydroxyalkyl starch derivative preferably comprises at least one structural unit according to the following formula (I)



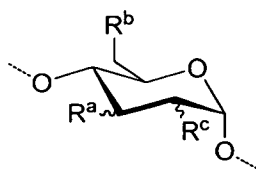
(I)

- wherein at least one of R^a , R^b and R^c has a structure according to the following
- 5 formula $[-O-C^A-CH^A-{}_tF^1]_p-L^1-X-$, wherein L^1 is selected from the group consisting of $-CH_2-$, $-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-S-CH_2-CH_2-$, $-CH_2-CH_2-S-CH_2-CH_2-$, $-CH_2-CH_2-O-CH_2-CH_2-$, $-CH_2-CH_2-O-CH_2-CH_2-O-CH_2-CH_2-$, $-CH_2-CHOH-CH_2-$, $-CH_2-CHOH-CH_2-S-CH_2-CH_2-$, $-CH_2-CHOH-CH_2-S-CH_2-CH_2-CH_2-$, $-CH_2-CHOH-CH_2-NH-CH_2-CH_2-$, $-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-$, $-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-S-CH_2-CH_2-$, $-CH_2-CH(CH_2OH)-$ and $-CH_2-CH(CH_2OH)-S-CH_2-CH_2-$, more preferably from the group consisting of $-CH_2-CHOH-CH_2-$, $-CH_2-CHOH-CH_2-S-CH_2-CH_2-$, $-CH_2-CHOH-CH_2-S-CH_2-CH_2-CH_2-$, $-CH_2-CHOH-CH_2-NH-CH_2-CH_2-$ and $-CH_2-CHOH-CH_2-NH-CH_2-CH_2-CH_2-$, more
- 10 preferably from the group consisting of $-CH_2-CHOH-CH_2-$, $-CH_2-CHOH-CH_2-S-CH_2-CH_2-$, $-CH_2-CHOH-CH_2-S-CH_2-CH_2-CH_2-$ and $-CH_2-CHOH-CH_2-S-CH_2-CH_2-CH_2-$.

Especially preferred conjugates according to the present invention

- 20 In the following, conjugate structures are mentioned, which comprise a particularly preferred combination of HAS' and different structural units $-L-M$.

- According to a first especially preferred embodiment of the present invention, the hydroxyalkyl starch conjugate comprises a residue of hydroxyalkyl starch derivative
- 25 comprising at least one structural unit according to the following formula (I)

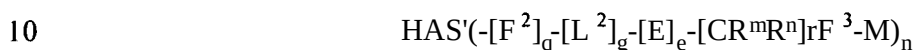


(I)

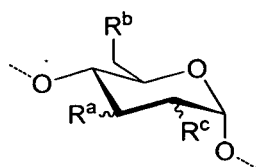
- 30 wherein in each unit, independently of each other unit, at least one of R^a , R^b and R^c is $[-O-CH_2-CH_2]_t-X-$ and X is $-S-$. The hydroxyalkyl starch conjugate further comprises the structural unit $-L-M$ having the structure $-[F^2]_q-[L^2]_g-[E]_e-[CR^mR^n]_f-F^3-M$, wherein q is 0, g is 0 and e is 0.

Accordingly, in this preferred embodiment, the functional group X is directly linked to the structural unit $-\text{[CR}^m\text{R}^n]\text{f}$. Integer f is preferably in the range of from 1 to 5.

- 5 Thus, the present invention also relates to a conjugate, comprising a hydroxyalkyl starch derivative, as described above, as well as a conjugate obtained or obtainable by the above-mentioned method, wherein the conjugate comprises a hydroxyalkyl starch derivative and a cytotoxic agent, the conjugate having a structure according to the following formula



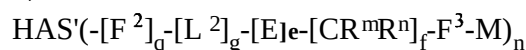
wherein q is 0, g is 0, e is 0, and wherein HAS' preferably comprises at least one structural unit according to the following formula (I)



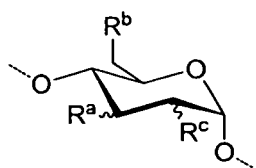
15 (I)

wherein at least one of R^a , R^b and R^c is $-\text{[O-CH}_2\text{-CH}_2\text{]}_t\text{-X-}$ and X is -S- and the functional group X is directly linked to the $-\text{[CR}^m\text{R}^n]\text{f}$ -group, and wherein integer f is 1, 2, 3, 4 or 5.

- 20 According to one preferred embodiment, integer f is 1, so that X is present in alpha position to the functional group F^3 . Accordingly, the present invention also relates to a conjugate, comprising a hydroxyalkyl starch derivative, as described above, as well as a conjugate obtained or obtainable by the above-mentioned method, wherein the conjugate comprises a hydroxyalkyl starch derivative and a cytotoxic agent, the conjugate having a structure according to the following formula
- 25



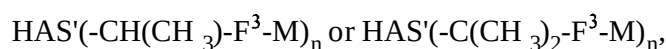
- 30 wherein q is 0, g is 0, e is 0, wherein HAS' preferably comprises at least one structural unit according to formula (I)



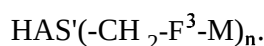
(I)

wherein in each unit, independently of each other unit, at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-X-$ and X is $-S-$ and the functional group X is directly linked to the $-[CR^mR^n]_r$ group, and wherein the hydroxyalkyl starch derivative comprises at least n functional groups X , and wherein f is 1. R^m and R^n are, independently of each other, H or alkyl, most preferably R^m and R^n are H or methyl.

Thus, according to this embodiment, the conjugate, or the conjugate obtained or obtainable by the above-mentioned method, preferably has a structure according to one of the following formulas

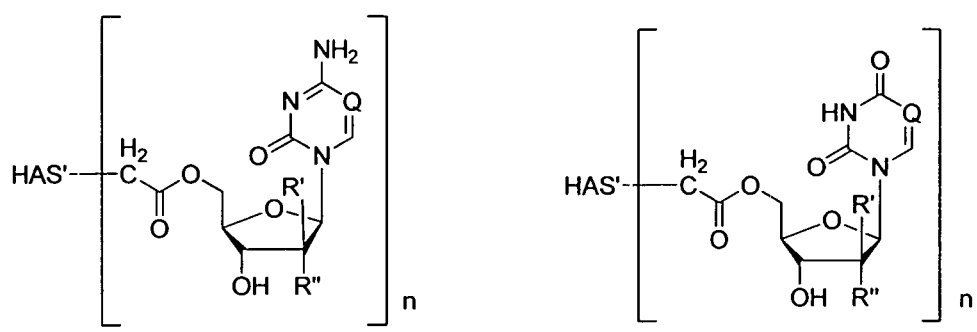


or according to the following formula

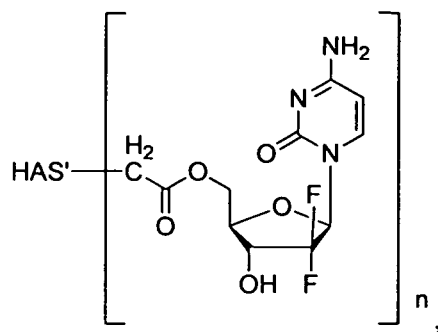


Particularly preferably F^3 in the above mentioned formula is $-C(=O)-$, as described above.

The present invention thus also relates to a conjugate, comprising a hydroxyalkyl starch derivative, as described above, as well as a conjugate obtained or obtainable by the above-mentioned method, the conjugate having a structure according to one of the following formulas

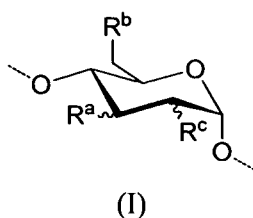


more preferably according to the following formula



wherein HAS' comprises at least one structural unit according to the following formula (I)

5

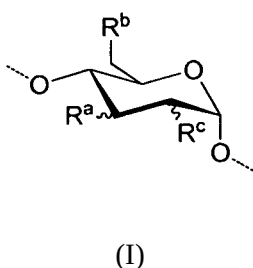


wherein in each unit, independently of each other unit, at least one of R^a , R^b and R^c is $-[O - CH_2 - CH_2]_t - X -$ and $-X -$ is $-S -$ and the functional group $-X -$ is directly linked to the $-CH_2 - C(=O) -$ group, as shown in the formulas above.

10

According to an alternative embodiment, the hydroxyalkyl starch conjugate comprises a hydroxyalkyl starch derivative comprising at least one structural unit according to the following formula (I)

15



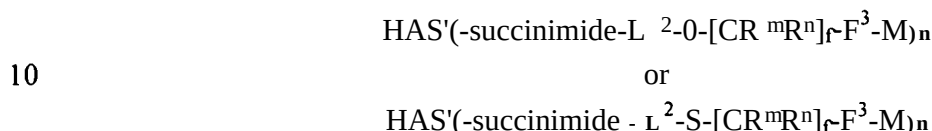
20

wherein at least one of R^a , R^b and R^c is $-[O - CH_2 - CH_2]_t - X -$ and X is $-S -$, thus at least one of R^a , R^b and R^c is $-[O - CH_2 - CH_2]_t - S -$, and wherein the conjugate further comprises the moiety $-L - M$, wherein $-L - M$ has the structure $(-[F^2]_q - [L^2]_g - [E]_e - [CR^m R^n]_r F^3 - M)_n$, as described above, and wherein e is 1 and E is preferably $-S -$ or $-O -$.

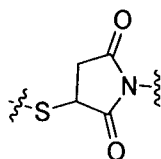
25

According to this embodiment, X is directly linked to the functional group F^2 with q and g preferably both being 1. As described above, the functional group F^2 is, if present, preferably selected from -S- and -succinimide-, preferably -succinimide-.

- 5 Thus, according to this embodiment, the conjugate, or the conjugate obtained or obtainable by the above-mentioned method, has in particular a structure according to one of the following formulas



- wherein HAS' comprises at least one structural unit according to the following formula (I), wherein in each unit, independently of each other unit, at least one of R^a , R^b and R^c is $-\text{[O-CH}_2\text{-CH}_2\text{]}_t\text{-X-}$ and X is -S- and wherein the succinimide is directly linked to X, thereby forming a



bond.

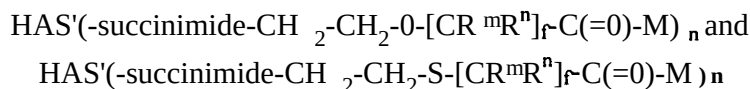
- 20 Particularly preferably F^3 in the above mentioned formula is $-\text{C(=O)-}$.

- As regards, the linking moiety L^2 according to this preferred embodiment, L^2 is preferably an alkyl group, as described above. More preferably L^2 is selected from the group consisting of $-\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-}$, $-\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-}$, $-\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-}$, $-\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-}$, $-\text{CH}_2\text{-CH}_2\text{-}$, $-\text{CH}_2\text{-}$, more preferably L^2 is selected from the group consisting of $-\text{CH}_2\text{-}$, $-\text{CH}_2\text{-CH}_2\text{-}$, $-\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-}$, most preferably L^2 is $-\text{CH}_2\text{-CH}_2\text{-}$.

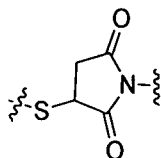
- Accordingly, the present invention also relates to a conjugate, comprising a residue of a hydroxyalkyl starch derivative, as described above, as well as a conjugate obtained or obtainable by the above-mentioned method, wherein the conjugate comprises a hydroxyalkyl starch derivative and a cytotoxic agent, the conjugate having a structure according to the following formula



- 35 more preferably a structure according to one of the following formulas

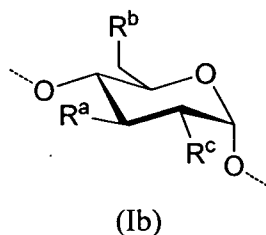


- wherein HAS' preferably comprises at least one structural unit according to the formula (1), wherein at least one of R^a, R^b and R^c is -[O-CH₂-CH₂],-X- and X is -S- and wherein the functional group X is directly linked to the succinimide group, thereby forming a



- bond and wherein most preferably all functional groups X present in a given hydroxyalkyl starch derivative comprised in a conjugate according to the invention, are directly linked to the succinimide group.

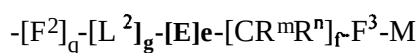
- According to a further especially preferred embodiment of the present invention, the hydroxyalkyl starch conjugate comprises a residue of a hydroxyalkyl starch derivative which comprises at least one structural unit according to the following formula (Ib)



- wherein at least one of R^a, R^b and R^c is -[O-CH₂-CH₂]_t-[F¹]_p-L¹-X- with X being -S-, preferably with p being 1 and F¹ being -O-, thus at least one of R^a, R^b and R^c has preferably the structure -[O-CH₂-CH₂]_t-O-L¹-S-, and wherein t is in the range of from 0 to 4, and wherein L¹ is a group, as described above, preferably an alkyl group. Most preferably the linking moiety L¹ is a spacer comprising at least one structural unit according to the formula -{[CR^dR^f]_h-[F⁴]_u-[CR^{dd}R^{ff}]_z]_a)-, as described above, wherein F⁴, if present, is preferably selected from the group consisting of -S-, -O- and -NH-, more preferably wherein F⁴, if present, is -O- or -S-, more preferably wherein F⁴ is -S-. According to this fourth especially preferred embodiment of the present invention, preferably at least one of R^d and R^f of at least one repeating unit of -[CR^dR^f]_h- is -OH. More preferably, R^d and R^f are either H or OH, wherein at least one of R^d and R^f of at least one repeating unit of -[CR^dR^f]_h- is -OH, wherein the repeating units may be the same or may be different. Most preferably R^{dd} and R^{ff} are, if present, H as well.

Particularly preferably, L^1 has a structure selected from the group consisting of $-\text{CH}_2-\text{CHOH}-\text{CH}_2-$, $-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-$, $-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{CH}_2-$, $-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{NH}-\text{CH}_2-\text{CH}_2-$, $-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{NH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-$, $-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2-$, $-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-$, more preferably from the group consisting of $-\text{CH}_2-\text{CHOH}-\text{CH}_2-$, $-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-$ and $-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{CH}_2-$, most preferably L^1 is $-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-$.

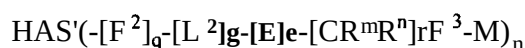
The hydroxyalkyl starch conjugate according to this fourth preferred embodiment, preferably further comprises the structural unit $-\text{L}-\text{M}$ having the structure



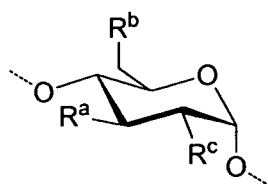
wherein q and g and e are 0.

In this preferred embodiment, $-\text{X}-$ is directly linked to the structural unit $-\text{[CR}^m\text{R}^n]_f-$, and integer f is preferably in the range of from 1 to 5.

Accordingly, the present invention also relates to a conjugate, comprising a hydroxyalkyl starch derivative, as described above, as well as a conjugate obtained or obtainable by the above-mentioned method, wherein the conjugate comprises a hydroxyalkyl starch derivative and a cytotoxic agent, the conjugate having a structure according to the following formula



wherein q is 0, g is 0, e is 0, and wherein HAS' preferably comprises at least one structural unit according to the following formula (Ib)

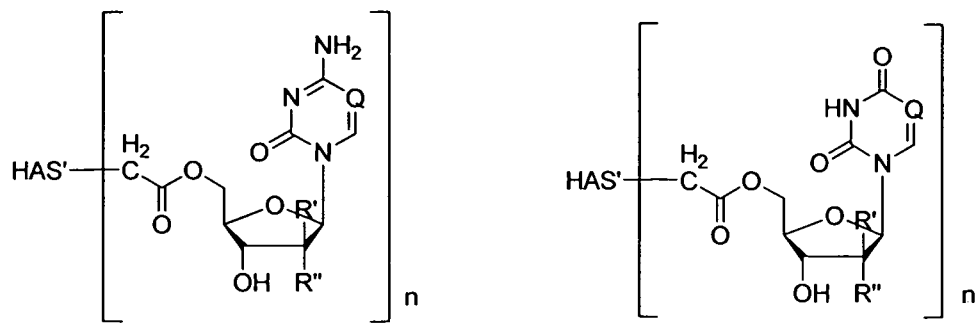


(Ib)

wherein at least one of R^a , R^b and R^c is $-\text{[O}-\text{CH}_2-\text{CH}_2]_t-[\text{F}^1]_p-\text{L}^1-\text{X}-$ with X being $-\text{S}-$, preferably with p being 1 and F^1 being $-\text{O}-$, thus at least one of R^a , R^b and R^c has preferably the structure $-\text{[O}-\text{CH}_2-\text{CH}_2]_t-\text{O}-\text{L}^1-\text{S}-$, wherein t is in the range of from 0 to 4, and wherein L^1 is preferably $-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-$, and wherein the functional group X is directly linked to the $-\text{[CR}^m\text{R}^n]_f-$ group, and wherein integer f is 1, 2, 3, 4 or 5. According to one preferred embodiment, f is 1 and R^m and R^n are, independently of each other, H or alkyl, most preferably R^m and R^n are H or methyl.

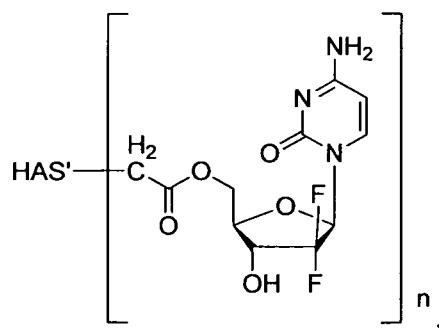
The present invention thus also relates to a conjugate, comprising a hydroxyalkyl starch derivative, as described above, as well as a conjugate obtained or obtainable by the above-mentioned method, the conjugate having a structure according to one of the following

5 formulas

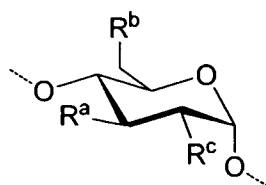


wherein Q is selected from the group consisting of C-H, C-F, C-CH₃ and N, and R' and R'' are independently of each other selected from the group consisting of OH, H and F,

10 more preferably according to the following formula



and wherein HAS' preferably comprises at least one structural unit according to the following formula (Ib)



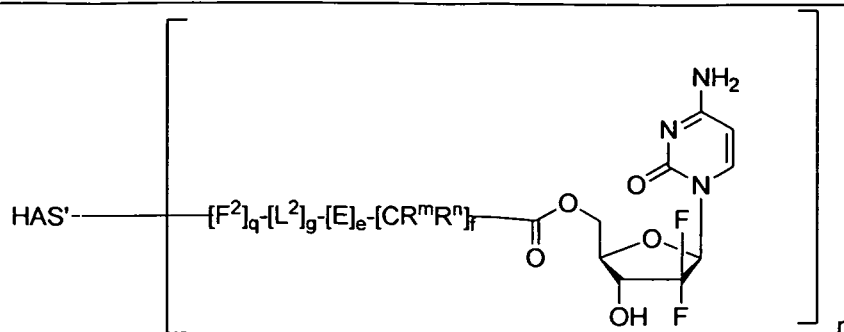
(Ib)

wherein at least one of R^a, R^b and R^c is $-[0-\text{CH}_2-\text{CH}_2]_t-[F^1]_p-L^1-X-$ with X being -S-, preferably with p being 1 and F¹ being -O-, thus at least one of R^a, R^b and R^c has preferably the structure $-[0-\text{CH}_2-\text{CH}_2]_t-0-L^1-S-$, wherein t is in the range of from 0 to 4, and wherein

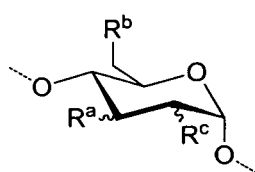
20 L¹ is preferably $-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-$.

Further preferred embodiments of the present invention are described in table 1:

Table 1: Preferred conjugates



and wherein HAS' comprises at least one structural unit according to formula (I)



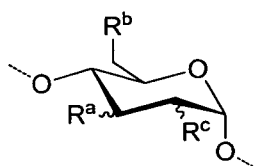
(I)

	$[F^2]_q$	$[L^2]_g$	$[E]_e$	$[CR^mR^n]_f$	at least one of R^a , R^b and R^c is
1	-succinimide- q is 1	g is 0	e is 0	selected from the group consisting of: -CH ₂ -CH ₂ -CH ₂ -CH ₂ -, -CH ₂ -CH ₂ -CH ₂ -CH ₂ -CH ₂ -, -CH ₂ -CH ₂ -CH ₂ -, -CH ₂ -CH ₂ -, -CH ₂ -, -CH(CH ₃)-, -CH(CH ₃)-CH ₂ -, -CH ₂ -CH(CH ₃)-, -CH(CH ₃)-CH ₂ -CH ₂ -, -CH ₂ -CH(CH ₃)-CH ₂ -,	-[O-CH ₂ -CH ₂] _i - X- and X is -S-
2	q is 0	g is 0	e is 0		
3	-succinimide- q is 1	g is 1 L^2 is -propyl-	e is 1 E is -S-		
4	-succinimide- q is 1	g is 1 L^2 is -ethyl-	e is 1 E is -S-		
5	-succinimide- q is 1	g is 1 L^2 is -butyl-	e is 1 E is -S-		
6	-succinimide- q is 1	g is 1 L^2 is -propyl-	e is 1 E is -O-		
7	-succinimide- q is 1	g is 1 L^2 is -ethyl-	e is 1 E is -O-		

8	-succinimide- q is 1	g is 1 L ² is -butyl-	e is 1 E is -O-	$\text{-CH}_2\text{-CH}_2\text{-CH(CH}_3\text{)-,}$ $\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH(CH}_3\text{)-,}$ $\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH(CH}_3\text{)-,}$ $\text{-C(CH}_3\text{)}_2\text{-,}$ $\text{-CH(CH}_3\text{)-CH(CH}_3\text{)-,}$ $\text{-CH(CH}_3\text{)-CH(CH}_3\text{)-CH}_2\text{-,}$ $\text{-CH}_2\text{-CH(CH}_3\text{)-CH(CH}_3\text{)-,}$ $\text{-CH(CH}_3\text{)-CH}_2\text{-CH(CH}_3\text{)-,}$ $\text{-CH(CH}_2\text{CH}_3\text{)-,}$ $\text{-CH(CH(CH}_3\text{)}_2\text{)-}$	$\text{-[O -CH}_2\text{-CH}_2\text{]}_n\text{-}$ [F'VL'-X- X is -S-, p is 1 $\text{F}^1\text{ is -O-,}$ $\text{L}^1\text{ is}$ $\text{-CH}_2\text{-CHOH-}$ $\text{CH}_2\text{-S-CH}_2\text{-CH}_2\text{-}$
9	-succinimide- q is 1	g is O	e is 0		
10	q is 0	g is O	e is 0		
11	-succinimide- q is 1	g is 1 L ² is -propyl-	e is 1 E is -S-		
12	-succinimide- q is 1	g is 1 L ² is -ethyl-	e is 1 E is -S-		
13	-succinimide- q is 1	g is 1 L ² is -butyl-	e is 1 E is -S-		
14	-succinimide- q is 1	g is 1 L ² is -propyl-	e is 1 E is -O-		
15	-succinimide- q is 1	g is 1 L ² is -ethyl-	e is 1 E is -O-		
16	-succinimide- q is 1	g is 1 L ² is -butyl-	e is 1 E is -O-		

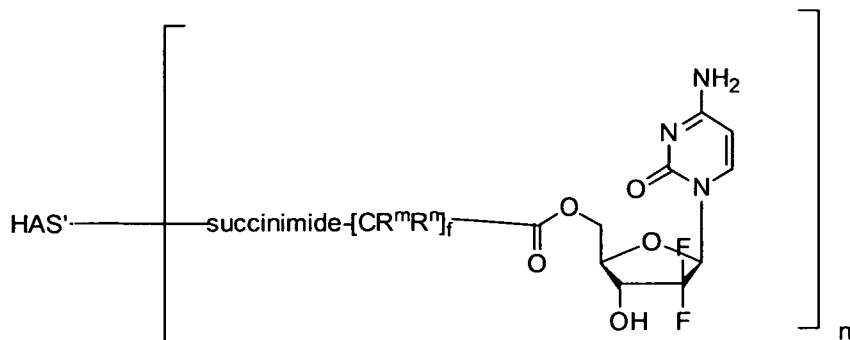
The way how to read this table is illustrated on the basis of entry 1:

The conjugate according to entry 1, comprises a hydroxyalkyl starch comprising at least one structural unit according to formula (I)



(I)

wherein at least one of R^a , R^b or R^c is $-[O-CH_2-CH_2]_t-S-$, and wherein in the above mentioned structure, $[F^2]_q$ is -succinimide- and q is 1, integer g is 0, thus L^2 is absent, and integer e is 0, thus E is absent. Thus, the following structure results:



with the structural unit $-[CR^mR^n]_f-$ being selected from the group consisting of
 $-CH_2-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-$, $-CH_2-$,
 $-CH(CH_3)-$, $-CH(CH_3)-CH_2-$, $-CH_2-CH(CH_3)-$, $-CH(CH_3)-CH_2-CH_2-$,
 10 $-CH_2-CH(CH_3)-CH_2-$, $-CH_2-CH_2-CH(CH_3)-$, $-CH_2-CH_2-CH_2-CH(CH_3)-$,
 $-CH_2-CH_2-CH_2-CH_2-CH(CH_3)-$, $-C(CH_3)_2-$, $-CH(CH_3)-CH(CH_3)-$,
 $-CH(CH_3)-CH(CH_3)-CH_2-$, $-CH_2-CH(CH_3)-CH(CH_3)-$,
 $-CH(CH_3)-CH_2-CH(CH_3)-$, $-CH(CH_2CH_3)-$, $-CH(CH_2CH_3)_2-$.

15 Synthesis of HAS Conjugates

As described above, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate comprising a hydroxyalkyl starch derivative and a cytotoxic agent, said conjugate having a structure according to the following formula $HAS'(-L-M)_n$,
 20 wherein M is a residue of a cytotoxic agent, wherein the cytotoxic agent comprises a primary hydroxyl group, L is a linking moiety, HAS' is a residue of the hydroxyalkyl starch derivative, and n is greater than or equal to 1,
 said method comprising the steps

25 (a) providing a hydroxyalkyl starch (HAS) derivative having a mean molecular weight MW above the renal threshold, preferably a molecular weight greater than or equal to 60 kDa, and a molar substitution MS in the range of from 0.6 to 1.5, said HAS derivative comprising a functional group Z^1 ; and providing a cytotoxic agent comprising a primary hydroxyl group,

30 (b) coupling the HAS derivative to the cytotoxic agent via an at least bifunctional crosslinking compound L comprising a functional group K^1 and a functional group K^2 , wherein K^2 is capable of being reacted with Z^1 comprised in the HAS derivative

and wherein K^1 is capable of being reacted with the primary hydroxyl group comprised in the cytotoxic agent.

The at least bifunctional crosslinking compound L

5

The term "at least bifunctional crosslinking compound L" as used in the context of the present invention refers to an at least bifunctional compound comprising the functional groups K^1 and K^2 .

10 Besides the functional group K^1 and the functional group K^2 the at least bifunctional crosslinking compound may optionally contain further functional groups, which may be used, for example, for the attachment of radiolabels, or the like. Hereinunder and above, the "at least bifunctional crosslinking compound L" is also referred to as "crosslinking compound L".

15

The crosslinking compound L is reacted via its functional group K^1 with the primary hydroxyl group of the cytotoxic agent, thereby forming a covalent linkage. On the other side, the crosslinking compound L is reacted via its functional group K^2 with the functional group Z^1 of the hydroxyalkyl starch derivative, thereby forming a covalent linkage as well.

20

The crosslinking compound L can be reacted with a cytotoxic agent either prior to or subsequent to the reaction with the hydroxyalkyl starch derivative. Preferably the crosslinking compound L is coupled to the cytotoxic agent prior to the reaction with the hydroxyalkyl starch derivative.

25

Thus, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate comprising a hydroxyalkyl starch derivative and a cytotoxic agent, said conjugate having a structure according to the following formula $HAS'(-L-M)_n$, wherein M is a residue of a cytotoxic agent, wherein the cytotoxic agent comprises a primary hydroxyl group, L is a linking moiety, HAS' is a residue of the hydroxyalkyl starch derivative, and n is greater than or equal to 1, preferably wherein n is in the range of from 3 to 100, said method comprising the steps

30

(a) providing a hydroxyalkyl starch derivative having a mean molecular weight MW above the renal threshold, preferably above 60 kDa, more preferably in the range of from 80 to 1200 kDa, more preferably in the range of from 90 to 800 kDa, and a molar substitution in the range of from 0.6 to 1.5, said hydroxyalkyl starch derivative comprising a functional group Z^1 ; and providing a cytotoxic agent comprising a primary hydroxyl group,

35

(b) coupling the HAS derivative to the cytotoxic agent via an at least bifunctional crosslinking compound L comprising a functional group K^1 and a functional group K^2 , wherein K^2 is capable of being reacted with Z^1 comprised in the HAS derivative and wherein K^1 is capable of being reacted with the primary hydroxyl group comprised in the cytotoxic agent, wherein L is coupled to Z^1 via the functional group K^2 comprised in L, and wherein each cytotoxic agent is coupled via the primary hydroxyl group to the HAS derivative via the functional group K^1 comprised in L, and wherein the cytotoxic agent is reacted with the at least one crosslinking compound L prior to the reaction with the hydroxyalkyl starch derivative, thereby forming a cytotoxic agent derivative comprising the functional group K^2 , and wherein said cytotoxic agent derivative is coupled in a subsequent step to the hydroxyalkyl starch derivative according to step (a).

Further, the present invention relates to a hydroxyalkyl starch conjugate obtained or obtainable by said method.

Upon reaction of the crosslinking compound L with the hydroxyalkyl starch derivative and the cytotoxic agent the hydroxyalkyl starch conjugate $HAS'(-L-M)_n$ is formed. In said conjugate, HAS' and M are linked via the linking moiety L, wherein said linking moiety L is the linking moiety derived from the at least bifunctional crosslinking compound L.

Preferably, the at least bifunctional crosslinking compound L has a structure according to the following formula



wherein L' is a linking moiety, K^2 is the functional group capable of being reacted with the functional group Z^1 of the hydroxyalkyl starch derivative and K^1 is the group capable of being reacted with the cytotoxic agent, as described above.

The functional group K^1

Accordingly, the functional group K^1 is a group capable of being coupled to a primary hydroxyl group of the cytotoxic agent. Upon reaction of the functional group K^1 with the primary hydroxyl group, preferably the linking unit -F³-O-, as described above, is formed. Preferably, K^1 is a functional group with which (upon reaction with the hydroxyl group) a covalent linkage between L, preferably L' and M, is formed which is cleavable *in vivo* as described above.

The crosslinking compound L may be reacted with either the cytotoxic agent or the hydroxyalkyl starch in an initial step. Preferably, the crosslinking compound L is reacted with the primary hydroxyl group of the cytotoxic agent prior to the reaction with the hydroxyalkyl starch derivative, thereby forming a derivative of the cytotoxic agent, the
 5 derivative of the cytotoxic agent preferably having the structure $K^2-L'-F^3-M$.

Thus, the present invention also describes a method for preparing a hydroxyalkyl starch conjugate, as described above, wherein step (b) comprises the steps

- 10 (b1) coupling the cytotoxic agent via its primary hydroxyl group to the crosslinking compound $K^2-L'-K^*$, thereby forming a derivative of the cytotoxic agent having the structure $K^2-L'-F^3-M$, wherein M is the residue of the cytotoxic agent,
- (b2) coupling the derivative of the cytotoxic agent having the structure $K^2-L'-F^3-M$ to the
 15 hydroxyalkyl starch derivative according to step (a), thereby forming the hydroxyalkyl starch conjugate.

Further, the present invention relates to a hydroxyalkyl starch conjugate obtained or obtainable by said method.

20

Preferably K^1 comprises the structural unit $-C(=Y)-$, with Y being O, NH or S. Thus, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate, as described above, wherein the cytotoxic agent is reacted with the at least one crosslinking compound L via the functional group K^1 comprised in the crosslinking compound L,
 25 wherein said functional group K^1 comprises the structural unit $-C(=Y)-$, with Y being O, NH or S, more preferably Y being O. Further, the present invention relates to a hydroxyalkyl starch conjugate obtained or obtainable by said method.

According to a particular preferred embodiment K^1 is a carboxylic acid group or a reactive
 30 carboxy group.

The term "reactive carboxy group" as used in this context of the present invention is intended to mean an activated carboxylic acid derivative that reacts readily with electrophilic groups, such as the -OH group of the cytotoxic agent, optionally in the presence of a suitable base, in contrast to those groups that require a further catalyst, such
 35 as a coupling reagent, in order to react. The term "activated carboxylic acid derivative" as used herein preferably refers to acid halides such as acid chlorides and also refers to activated ester derivatives including, but not limited to, formic and acetic acid derived anhydrides, anhydrides derived from alkoxycarbonyl halides such as

isobutyloxycarbonylchloride and the like, isothiocyanates or isocyanates, anhydrides derived from reaction of the carboxylic acid with N,N'-carbonyldiimidazole and the like, and esters derived from activation of the corresponding carboxylic acid with a coupling reagent. Such coupling reagents include, but are not limited to, HATU (0-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate); HOAt, HBTU (0-benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate); TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate); TFFH (N,N',N'',N''-tetramethyluronium-2-fluoro-hexafluorophosphate); BOP (benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate); PyBOP (benzotriazol-1-yloxy-trispyrrolidino-phosphonium hexafluorophosphate); EEDQ (2-ethoxy-1-ethoxycarbonyl-1,2-dihydro-quinoline); DCC (dicyclohexylcarbodiimide); DIPCDI (diisopropylcarbodiimide); HOBt (1-hydroxybenzotriazole); NHS (N-hydroxysuccinimide); MSNT (1-(mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazole); aryl sulfonyl halides, e.g. triisopropylbenzenesulfonyl chloride, EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, CDC (1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide), Pyclop, T3P, CDI, Mukayama's reagent, HODhbt, HAPyU, TAPipU, TPTU, TSTU, TNTU, TOTU, BroP, PyBroP, BOI, TOO, NEPIs, BBC, BDMP, BOMI, AOP, BDP, PyAOP, TDBTU, BOP-C1, CIP, DEPBT, Dpp-Cl, EEDQ, FDPP, HOTT, TOTT, PyCloP.

In case, K¹ is a carboxylic acid group, the coupling between the cytotoxic agent and the crosslinking compound L is preferably carried out in the presence of at least one coupling reagent, wherein the coupling reagent is preferably selected from the group of coupling reagents mentioned above. In case a coupling reagent is used, most preferably EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) is used. Additionally additives promoting the activation of the carboxylic acid, such as DMAP (4-(dimethylamino)-pyridine), may be used.

The coupling between the cytotoxic agent and the crosslinking compound L is preferably carried out in the presence of a suitable base, preferably an organic base, most preferably an amino group comprising base, most preferably a base selected from the group consisting of diisopropylamine (DIEA), triethylamine (TEA), N-methylmorpholine, N-methylimidazole, 1,4-diazabicyclo[2.2.2]octane (DABCO), N-methylpiperidine, N-methylpyrrolidine, 2,6-lutidine, collidine, pyridine, 4-dimethylaminopyridine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU).

As regards the reaction conditions used in this coupling step, preferably, the reaction is carried out in an organic solvent, such as N-methylpyrrolidone (NMP), dimethyl sulfoxide (DMSO), acetonitrile, acetone, dimethyl acetamide (DMA), dimethyl formamide (DMF),

formamide, tetrahydrofuran (THF), 1,4-dioxane, diethyl ether, tert.-butyl methyl ether (MTBE), dichloromethane (DCM), chloroform, tetrachloromethane and mixtures of two or more thereof. More preferably, the reaction is carried out in dichloromethane.

5 The temperature of the coupling reaction is preferably in the range of from 0 to 100 °C, more preferably of from 5 to 50 °C, and especially preferably of from 15 to 30 °C. During the course of the reaction, the temperature may be varied, preferably in the above given ranges, or held essentially constant.

10 The derivative of the cytotoxic agent, which in particular has the following structure



may be subjected to at least one isolation and/or purification step prior to the reaction with
15 the hydroxyalkyl starch derivative.

The functional group K^2 and the functional group Z^1

20 In the context of the present invention, K^2 is a functional group capable of being reacted with a functional group Z^1 of the hydroxyalkyl starch derivative, and Z^1 is the respective functional group capable of being reacted with the functional group K^2 . Upon reaction of K^2 with Z^1 the functional unit $-X-[F^2]_q-$ is formed, with X and $-[F^2]_q-$ being as , described above in the context of the conjugate structures.

25 Such functional groups Z^1 and K^2 may be suitably chosen. By way of example, one of the groups Z^1 and K^2 , i.e. Z^1 or K^2 , may be chosen from the group consisting of the functional groups according to the following list while the other group, K^2 or Z^1 , is suitably selected and capable of forming a chemical linkage with Z^1 or K^2 , wherein K^2 or Z^1 is also preferably selected from the following list:

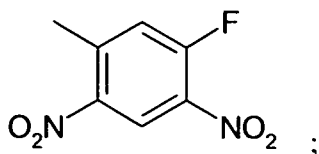
30

- C-C-double bonds or C-C-triple bonds, such as alkenyl groups, alkynyl groups or aromatic C-C-bonds, in particular alkynyl groups, most preferably $-C\equiv C-H$;
- alkyl sulfonic acid hydrazides, aryl sulfonic acid hydrazides;
- the thiol group or the hydroxy group;

35

- thiol reactive groups such as
 - a disulfide group comprising the structure $-S-S-$; such as pyridyl disulfides,
 - maleimide group,
 - haloacetyl groups,
 - haloacetamides,

- vinyl sulfones,
- vinyl pyridines,
- haloalkanes;



- the group
- 5 - dienes or dienophiles;
- azides;
- 1,2-aminoalcohols;
- amino groups comprising the structure $-NR^{\#}R^{\#\#}$, wherein $R^{\#}$ and $R^{\#\#}$ are independently of each other selected from the group consisting of H, alkyl groups, aryl groups, arylalkyl groups and alkylaryl groups; preferably $-NH_2$;
- 10 - hydroxylamino groups comprising the structure $-O-NR^{\#}R^{\#\#}$, wherein $R^{\#}$ and $R^{\#\#}$ are independently of each other selected from the group consisting of H, alkyl groups, aryl groups, arylalkyl groups and alkylaryl groups; preferably $-O-NH_2$;
- oxyamino groups comprising the structure unit $-NR^{\#}-O-$, with $R^{\#}$ being selected
- 15 from the group consisting of alkyl groups, aryl groups, arylalkyl groups and alkylaryl groups; preferably $-NH-O-$;
- residues having a carbonyl group, $-Q-C(=G)-M'$, wherein G is O or S, and M' is, for example,
 - $-OH$ or $-SH$;
- 20 -- an alkoxy group, an aryloxy group, an arylalkyloxy group, or an alkylaryloxy group;
- an alkylthio group, an arylthio group, an arylalkylthio group, or an alkylarylthio group;
- an alkylcarbonyloxy group, an arylcarbonyloxy group, an arylalkylcarbonyloxy group, an alkylarylcarbonyloxy group;
- 25 -- activated esters such as esters of hydroxylamines having an imide structure such as N-hydroxysuccinimide;
- $-NR^{\#}-NH_2$, wherein $R^{\#}$ is selected from the group consisting of H, alkyl, aryl, arylalkyl and alkylaryl groups; preferably wherein $R^{\#}$ is H;
- 30 - carbonyl groups such as aldehyde groups, keto groups; hemiacetal groups or acetal groups;
- the carboxy groups;
- the $-N=C=O$ group or the $-N=C=S$ group;
- vinyl halide groups such as the vinyl iodide group or the vinyl bromide group, or
- 35 triflate;
- $-(C=NH_2Cl)-OAlkyl$;

- epoxide;
- residues comprising a leaving group such as e.g. halogens or sulfonates.

Preferably, Z^1 is selected from the group consisting of aldehyde, keto, hemiacetal, acetal, alkynyl, azide, carboxy groups, alkenyl, thiol reactive groups, such as maleimide, halogen acetyl, pyridyl disulfides, haloacetamides, vinyl sulfones and vinyl pyridines, -SH, -NH₂, -O-NH₂, -NH-O-alkyl, -(C=G)-NH-NH₂, -G-(C=G)-NH-NH₂, -NH-(C=G)-NH-NH₂, and -SO₂-NH-NH₂, where G is O or S and, if G is present twice, it is independently O or S.

- Thus, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate, as described above, wherein K^2 is reacted with the functional group Z^1 , wherein Z^1 is selected from the group consisting of aldehyde groups, keto groups, hemiacetal groups, acetal groups, alkynyl groups, azide groups, carboxy groups, alkenyl groups, thiol reactive groups, -SH, -NH₂, -O-NH₂, -NH-O-alkyl, -(C=G)-NH-NH₂, -G-(C=G)-NH-NH₂, -NH-(C=G)-NH-NH₂, and -SO₂-NH-NH₂, where G is O or S and, if G is present twice, it is independently O or S. Further, the present invention also relates to the conjugate obtained or obtainable by said method.

By way of example, in the following Table 2, suitable combinations of Z^1 and K^2 are mentioned:

Table 2: Examples for K^2 and Z^1

K^2	Z^1
-SH	thiol reactive group
-NH ₂	aldehyde group, keto group, hemiacetal group, acetal group or carboxy group
-O-NH ₂	aldehyde group, keto group, hemiacetal group, acetal group or carboxy group
-(C=G)-NH-NH ₂	aldehyde group, keto group, hemiacetal group, acetal group or carboxy group
-G-(C=G)-NH-NH ₂	aldehyde group, keto group, hemiacetal group, acetal group or

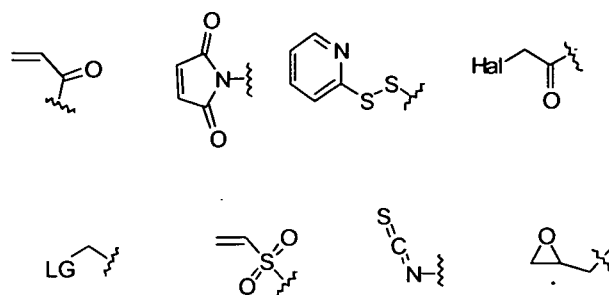
	carboxy group
-SO ₂ -NH-NH ₂	aldehyde group, keto group, hemiacetal group, acetal group or carboxy group
alkynyl or diphenylphosphinomethylthioester	azide
azide	alkynyl or diphenylphosphinomethylthioester
aldehyde group, keto group, hemiacetal group, acetal group or carboxy group	-NH ₂
aldehyde group, keto group, hemiacetal group, acetal group or carboxy group	-O-NH ₂
aldehyde group, keto group, hemiacetal group, acetal group or carboxy group	-(C=G)-NH-NH ₂
aldehyde group, keto group, hemiacetal group, acetal group or carboxy group	-G-(C=G)-NH-NH ₂
aldehyde group, keto group, hemiacetal group, acetal group or carboxy group	-SO ₂ -NH-NH ₂
thiol reactive group	-SH
thioester	alpha-thiol-beta-amino group
alpha-thiol-beta-amino group	thioester

According to a preferred embodiment of the present invention, the functional group Z¹ is a thiol group. Thus, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate, as described above, wherein in step (a) a derivative is

5 formed comprising at least one thiol group, preferably comprising multiple thiol groups, the derivative having a mean molecular weight MW above the renal threshold, preferably a MW greater than or equal to 60 kDa, more preferably in the range of from 80 to 1200 kDa, preferably more of from 90 to 800 kD, and a molar substitution MS in the range of from

0.6 to 1.5. The present invention further relates to the conjugate obtained or obtainable by said method.

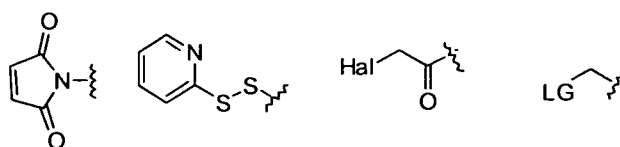
- 5 In case Z^1 is a thiol group, K^2 is preferably a thiol reactive group, preferably a group selected from the group consisting of pyridyl disulfides, maleimide group, haloacetyl groups, haloacetamides, vinyl sulfones and vinyl pyridines. Preferably, K^2 is a thiol-reactive group selected from the group consisting of the following structures:



10

wherein Hal is a halogen, such as Cl, Br, or I, and LG is a leaving group (or nucleofuge). The term "leaving group", as used in this context of the present invention, is denoted to mean a molecular fragment that departs with a pair of electrons in heterolytic bond
15 cleavage upon reaction with the functional group Z^1 . Examples are, inter alia, halogens or sulfonic esters. Examples for sulfonic esters are, inter alia, the mesyl and tosyl group.

More preferably, K^2 is a thiol-reactive group selected from the group consisting of the following structures:



20

more preferably from the following structures



- 25 Thus, the present invention also describes a method for preparing a hydroxyalkyl starch conjugate comprising a hydroxyalkyl starch derivative and a cytotoxic agent said conjugate having a structure according to the following formula $HAS'(-L-M)_n$, wherein M is a residue of a cytotoxic agent, and wherein the cytotoxic agent comprises a primary hydroxyl group,

L is a linking moiety, HAS' is a residue of the hydroxyalkyl starch derivative, and n is greater than or equal to 1,

said method comprising the steps

5

(a) providing a hydroxyalkyl starch derivative having a mean molecular weight MW above the renal threshold, preferably above 60 kDa, more preferably in the range of from 80 to 1200 kDa, more preferably of from 90 to 800 kDa, and a molar substitution MS in the range of from 0.6 to 1.5, said hydroxyalkyl starch derivative comprising a functional group Z¹; and providing a cytotoxic agent comprising a primary hydroxyl group,

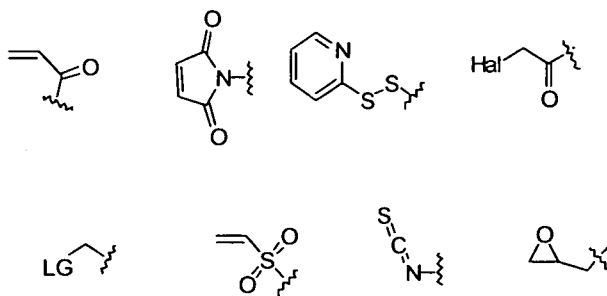
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(b) coupling the HAS derivative to the cytotoxic agent via an at least bifunctional crosslinking compound L comprising a functional group K¹ and a functional group K², wherein K² is capable of being reacted with Z¹ comprised in the HAS derivative and wherein K¹ is capable of being reacted with the primary hydroxyl group comprised in the cytotoxic agent, and wherein L is coupled to Z¹ via the functional group K² comprised in L, and wherein each cytotoxic agent is coupled via the primary hydroxyl group to the hydroxyalkyl starch derivative via the functional group K¹ comprised in L,

15

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and wherein Z¹ is -SH, and wherein K² is a thiol reactive group, preferably a group selected from the group consisting of the following structures:

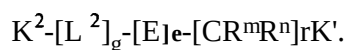


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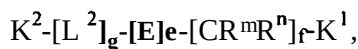
and wherein K¹ comprises the structural unit -C(=Y)-, with Y being O, NH or S, more preferably Y is O, preferably, wherein K¹ is a carboxylic acid group or a reactive carboxy group. Further, the present invention also relates to the respective conjugate obtained or obtainable by said method.

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Preferably, the at least bifunctional crosslinking compound L has a structure according to the following formula,



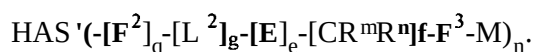
Thus, in step (b) of the present invention, the hydroxyalkyl starch derivative according to step (a) is preferably reacted with a crosslinking compound L, with L having the structure



5

wherein the crosslinking compound L is coupled to Z¹ comprised in the hydroxyalkyl starch derivative via the functional group K², and wherein each cytotoxic agent is coupled via the primary hydroxyl group to the hydroxyalkyl starch derivative via the functional group K¹ thereby forming a conjugate having the structure

10



with F², L², E, q, g, e and -[CR^mRⁿ]_f being as described hereinabove, preferably wherein E is an electron-withdrawing group selected from the group consisting of -O-, -S-, -SO-, -SO₂-, -NR^e-, -succinimide-, -C(=Y^e)-, -NR^e-C(=Y^e)-, -C(=Y^e)-NR^e-, -CH(NO₂)-, -CH(CN)-, aryl moieties or an at least partially fluorinated alkyl moiety, wherein Y^e is either O, S or NR^e, and R^e is hydrogen or alkyl, more preferably wherein E is selected from the group consisting of -NH-C(=O)-, -C(=O)-NH-, -NH-, -O-, -S-, -SO-, -SO₂- and -succinimide-, L² is a linking moiety, preferably an alkyl, alkenyl, alkylaryl, arylalkyl, heteroaryl, alkylheteroaryl, heteroarylalkyl or aryl group, f is in the range of from 1 to 20, g is 0 or 1, e is 0 or 1, and wherein R^m and Rⁿ are, independently of each other, H or alkyl or a side chain of a natural or unnatural amino acid, preferably H or alkyl, more preferably H or methyl.

By way of example, the following preferred crosslinking compounds L are mentioned in table 3:

Table 3: Preferred crosslinking compounds L, by way of example

$K^2-[L^2]_g-[E]_e-[CR^mR^n]_f-K^1$					
	K ²	[L ²] _g	[E] _e	[CR ^m R ⁿ] _f	K ¹
1	maleimide-	g is 0	e is 0	Selected from the group consisting of: -CH ₂ -CH ₂ -CH ₂ -CH ₂ -, -CH ₂ -CH ₂ -CH ₂ -CH ₂ -CH ₂ -, -CH ₂ -CH ₂ -CH ₂ -, -CH ₂ -CH ₂ -, -CH ₂ -, -CH(CH ₃)-,	-COOH
2	Hal-	g is 0	e is 0		-COOH
3	maleimide-	g is 1 L ² is -propyl-	e is 1 E is -S-		-COOH

4	maleimide-	g is 1 L ² is -ethyl-	e is 1 E is -S-	-CH(CH ₃)-CH ₂ -, -CH ₂ -CH(CH ₃)-, -CH(CH ₃)-CH ₂ -CH ₂ -, -CH ₂ -CH(CH ₃)-CH ₂ -, -CH ₂ -CH ₂ -CH(CH ₃)-, -CH ₂ -CH ₂ -CH ₂ -CH(CH ₃)-, -CH ₂ -CH ₂ -CH ₂ -CH ₂ -CH(CH ₃)-, -C(CH ₃) ₂ -, -CH(CH ₃)-CH(CH ₃)-, -CH(CH ₃)-CH(CH ₃)-CH ₂ -, -CH ₂ -CH(CH ₃)-CH(CH ₃)-, -CH(CH ₃)-CH ₂ -CH(CH ₃)-, -CH(CH ₂ CH ₃)-, -CH(CH(CH ₃) ₂)-	-COOH
5	maleimide-	g is 1 L ² is -butyl-	e is 1 E is -S-		-COOH
6	maleimide-	g is 1 L ² is -propyl-	e is 1 E is -O-		-COOH
7	maleimide-	g is 1 L ² is -ethyl-	e is 1 E is -O-		-COOH
8	maleimide-	g is 1 L ² is -butyl-	e is 1 E is -O-		-COOH
9	maleimide-	g is O	e is 0		-COOH

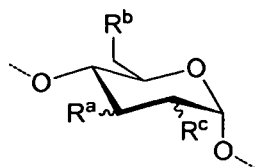
Step (a)

- 5 As regards, the provision of the hydroxyalkyl starch derivative according to step (a), preferably step (a) comprises the introduction of at least one functional group Z¹ into the hydroxyalkyl starch by
- (i) coupling hydroxyalkyl starch via at least one hydroxyl group to at least one suitable linker comprising the functional group Z¹ or a precursor of the functional group Z¹, or
- 10 (ii) displacing a hydroxyl group present in the hydroxyalkyl starch in a substitution reaction with a precursor of the functional group Z¹ or with a bifunctional linker comprising the functional group Z¹ or a precursor of the functional group Z¹.

The term "at least one suitable linker comprising a precursor of the functional group Z¹" as used in context of the present invention is denoted to mean a linker comprising a functional group which is capable of being transformed in at least one further step to give the functional group Z¹. The term "precursor" used in the context of "displacing the hydroxyl group of hydroxyalkyl starch with a precursor", is denoted to mean a reagent, which is capable of displacing the hydroxyl group, thereby forming a functional group Z¹ or a

20 group, which can be modified in at least one further step to give the functional group Z¹.

According to a preferred embodiment of the present invention, the present invention relates to a method for preparing a hydroxyalkyl starch conjugate, as described above, wherein the hydroxyalkyl starch derivative comprises at least one structural unit according to the following formula (I)

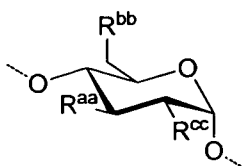


(I)

wherein at least one of R^a , R^b or R^c comprises the functional group Z^1 , wherein R^a , R^b and R^c are, independently of each other, selected from the group consisting of $-O-HAS''$, $-[O-(CR^wR^xHCR^yR^z)]_x-OH$, $-[O-(CR^wR^x)-(CR^yR^z)]_y-Z^1$, $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^1-Z^1$, wherein R^w , R^x , R^y and R^z are independently of each other selected from the group consisting of hydrogen and alkyl, y is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4, x is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4, F^1 is a functional group, p is 1 or 0, and L^1 is a linking moiety,

and wherein step (a) comprises

(a1) providing a hydroxyalkyl starch having a mean molecular weight MW above the renal threshold, preferably above 60 kDa, more preferably in the range of from 80 to 1200 kDa, more preferably of from 90 to 800 kDa, and a molar substitution MS in the range of from 0.6 to 1.5, comprising the structural unit according to the following formula (II)



(II)

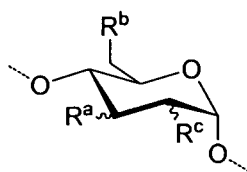
wherein R^{aa} , R^{bb} and R^{cc} are independently of each other selected from the group consisting of $-[O-(CR^wR^x)-(CR^yR^z)]_x-OH$ and $-O-HAS''$, wherein HAS'' is a remainder of the hydroxyalkyl starch,

(a2) introducing at least one functional group Z^1 by

- (i) coupling the hydroxyalkyl starch via at least one hydroxyl group to at least one suitable linker comprising the functional group Z^1 or a precursor of the functional group Z^1 , or
- 5 (ii) displacing a hydroxyl group present in the hydroxyalkyl starch in a substitution reaction with a precursor of the functional group Z^1 or with a bifunctional linker comprising the functional group Z^1 or a precursor of the functional group Z^1 .

Furthermore, the present invention relates to a conjugate obtained or obtainable by said method.

According to a preferred embodiment of the present invention, the present invention relates to a method for preparing a hydroxyalkyl starch conjugate, as described above, as well as to a conjugate obtained or obtainable by said method, wherein the hydroxyalkyl starch derivative provided in step (a2) comprises at least one structural unit according to the following formula (I)



(I)

wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS^n$, $-[O-CH_2-CH_2]_s-OH$, $-[O-CH_2-CH_2]_t-Z^1$ and $-[O-CH_2-CH_2]_t[F^{1p}]_p-L-Z^1$, with p being 0 or 1, and wherein at least one of R^a , R^b and R^c comprises the functional group Z^1 , and wherein t is in the range of from 0 to 4, wherein s is in the range of from 0 to 4.

Hydroxyalkyl starches having the desired properties are preferably produced from waxy maize starch or potato starch by acidic hydrolysis and reaction with ethylene oxide and purification by ultrafiltration.

Step (a2)(i)

According to a first preferred embodiment of the present invention, the functional group Z^1 is introduced by coupling the hydroxyalkyl starch via at least one hydroxyl group to at least one suitable linker comprising the functional group Z^1 or a precursor of the functional group Z^1 .

Organic chemistry offers a wide range of reactions to modify hydroxyl groups with linker constructs bearing functionalities such as aldehyde, keto, hemiacetal, acetal, alkynyl, azide, carboxy, alkenyl, and thiol reactive groups, such as maleimide, halogen, acetal, pyridyl, disulfides, haloacetamides, vinyl sulfones, vinyl pyridines, -SH, -NH₂, -O-NH₂, -NH-O-alkyl, -(C=G)-NH-NH₂, -G-(C=G)-NH-NH₂, -NH-(C=G)-NH-NH₂, and -SO₂-NH-NH₂, wherein G is O, NH or S, preferably O or S, and if present twice may be the same or may be different from each other. However, the hydroxyalkyl starch's polymeric nature and the abundance of hydroxyl groups present in the hydroxyalkyl starch usually strongly promote the number of possible side reactions such as inter- and intramolecular crosslinking. Therefore, a method was needed to functionalize the polymer under maximum retention of its molecular characteristics such as solubility, molecular weight and polydispersity. It was surprisingly found that when using the method according to this preferred embodiment, possible side reactions such as inter- and intramolecular crosslinking can be significantly diminished.

According to a preferred embodiment of the present invention, in step (a2)(i), the hydroxyalkyl starch is coupled to a linker comprising a functional group Z², said functional group Z² being capable of being coupled to a hydroxyl group of the hydroxyalkyl starch, thereby forming a covalent linkage between the first linker and the hydroxyalkyl starch. Further, the linker preferably comprises the functional group Z¹ or a precursor thereof. According to a particularly preferred embodiment, the linker comprises a precursor of the functional group Z¹ which is transformed in at least one further step to give the functional group Z¹.

The Functional Group Z²

The functional group Z² is a functional group capable of being coupled to at least one hydroxyl function of the hydroxyalkyl starch or to an activated hydroxyl function of hydroxyalkyl starch, thereby forming a covalent linkage F¹.

According to a preferred embodiment, the functional group Z² is a leaving group or a nucleophilic group. According to an alternative embodiment the functional group Z² is an epoxide.

According to a first preferred embodiment, Z² is a leaving group, preferably a leaving group being attached to a CH₂-group comprised in the at least one suitable linker which is reacted in step (a2)(ii) with the hydroxyalkyl starch. The term "leaving group" as used in this context of the present invention is denoted to mean a molecular fragment that departs with a pair of electrons in heterolytic bond cleavage upon reaction with the hydroxyl group

of the hydroxyalkyl starch, thereby forming a covalent bond between the oxygen atom of the hydroxyl group and the carbon atom formerly bearing the leaving group. Common leaving groups are, for example, halides such as chloride, bromide and iodide, and sulfonates such as tosylates, mesylates, fluorosulfonates, triflates and the like. According to a preferred embodiment of the present invention, the functional group Z^2 is a halide leaving group. Thus, upon reaction of the hydroxyl group with the functional group Z^2 , preferably a functional group F^1 is formed, which is preferably -O-.

Alternatively, Z^2 may also be an epoxide group, which reacts with a hydroxyl group of HAS in a ring opening reaction, thereby forming a covalent bond.

According to another embodiment, Z^2 is a nucleophile, thus a group capable of forming a covalent bond with an electrophile by donating both bonding electrons. In case Z^2 is a nucleophile, the method preferably comprises an initial step, in which at least one hydroxyl function of hydroxyalkyl starch is activated, thereby forming an electrophilic group. For example, the hydroxyl group may be activated by reacting at least one hydroxyl function with a reactive carbonyl compound, as described in detail below.

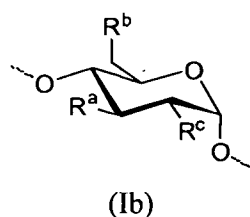
Thus, the present invention also describes a method, as described above, wherein the functional group Z^2 is a nucleophile, said nucleophile being capable of being reacted with at least one activated hydroxyl function of hydroxyalkyl starch, as described above, wherein the hydroxyl group is initially activated with a reactive carbonyl compound prior to coupling the hydroxyalkyl starch in step (a2)(ii) to the at least one suitable linker comprising the functional group Z^2 and the functional group Z^1 or a precursor of the functional group Z^1 .

The term "reactive carbonyl compound" as used in this context of the present invention, refers to carbonyl dication synthons having a structure $R^{**}-(C=O)-R^*$, wherein R^* and R^{**} may be the same or different, and wherein R^* and R^{**} are both leaving groups. As leaving groups halides, such as chloride, and/or residues derived from alcohols, may be used. The term "residue derived from alcohols", refers to R^* and/or R^{**} being a unit -O- R^f or -O- R^{88} , with -O- R^f and -O- R^{88} preferably being residues derived from alcohols such as N-hydroxy succinimide or sulfo-N-hydroxy succinimide, suitably substituted phenols such as p-nitrophenol, o,p-dinitrophenol, o,o'-dinitrophenol, trichlorophenol such as 2,4,6-trichlorophenol or 2,4,5-trichlorophenol, trifluorophenol such as 2,4,6-trifluorophenol or 2,4,5-trifluorophenol, pentachlorophenol, pentafluorophenol, heterocycles such as imidazol or hydroxyazoles such as hydroxy benzotriazole may be mentioned. Reactive carbonyl compounds containing halides are phosgene, related compounds such as diphosgene or triphosgene, chloroformic esters and other phosgene substitutes known in the art.

Especially preferred are carbonyldiimidazol (CDI), N,N'-disuccinimidyl carbonate and sulfo-N,N'-disuccinimidyl carbonate, or mixed compounds such as p-nitrophenyl chloroformate.

- 5 Preferably, the reactive carbonyl compound having the structure $R^{**}-(C=O)-R^*$ is selected from the group consisting of phosgene, diphosgene, triphosgene, chloroformates and carbonic acid esters, more preferably from the group consisting of p-nitrophenylchloroformate, pentafluorophenylchloroformate, N,N'-disuccinimidyl carbonate, sulfo-N,N'-disuccinimidyl carbonate, dibenzotriazol-1-yl carbonate and
- 10 carbonyldiimidazol.

- Preferably upon reaction of at least one hydroxyl group with the reactive carbonyl compound $R^{**}-(C=O)-R^*$ prior to the coupling step according to step (a2)(ii) an activated hydroxyalkyl starch derivative is formed, which comprises at least one structural unit,
- 15 according to the following formula (Ib)



- 20 wherein R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS", $-[O-CH_2-CH_2]_s-OH$ and $-[O-CH_2-CH_2]_t-O-C(=O)-R^*$ wherein t is in the range of from 0 to 4, and wherein s is in the range of from 0 to 4, and wherein at least one of R^a , R^b and R^c comprises the group $-[O-CH_2-CH_2]_t-O-C(=O)-R^*$, and wherein R^* is a leaving group, preferably a group selected from the group consisting of p-nitrophenoxy,
- 25 2,4-dichlorophenoxy, 2,4,6-trichlorophenoxy, trichloromethoxy, imidazolyl, azides and halides, such as chloride or bromide.

- According to this embodiment, according to which the hydroxyalkyl starch is activated to give a hydroxyalkyl starch derivative comprising a reactive $-O-C(=O)-R^*$ group, Z^2 is
- 30 preferably a nucleophilic group, such as a group comprising an amino group. Possible groups are, for example, $-NHR^{Z^2}$, $-NH_2$, $-O-NH_2$, $-NH-O-alkyl$, $-(C=G)-NH-NH_2$, $-G-(C=G)-NH-NH_2$, $-NH-(C=G)-NH-NH_2$, and $-SO_2-NH-NH_2$ wherein G is O or S, and if present twice in one structural unit, may be the same or may be different, and wherein R^{Z^2} is an alkyl group, preferably methyl. More preferably Z^2 is $-NH_2$ or $-NHR^{Z^2}$, most
- 35 preferably $-NH_2$.

As described above, besides the functional group Z^2 , the linker comprises either the functional group Z^1 or a precursor thereof.

5 Preferably, the linker further comprises the functional group W, this functional group being a group capable of being transformed in at least one further step to give the functional group Z^1 . Preferably W is an epoxide or a functional group which is transformed in a further step to give an epoxide or W has the structure Z^1 -PG, with PG being a suitable protecting group.

10 The epoxide modified hydroxyalkyl starch derivative

According to a first preferred embodiment, in step (a2)(i), a first linker is used comprising the functional group W, wherein W is an epoxide or a functional group which is transformed in a further step to give an epoxide.

15 Thus, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate, as described above, and a hydroxyalkyl starch conjugate obtained or obtainable by said method, wherein step (a2)(i) comprises step (I)

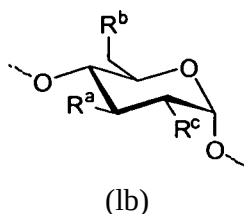
20 (I) coupling the hydroxyalkyl starch (HAS) via at least one hydroxyl group comprised in HAS to a first linker comprising a functional group Z^2 capable of being reacted with at least one hydroxyl group of the hydroxyalkyl starch, thereby forming a covalent linkage between the first linker and the hydroxyalkyl starch, the first linker further comprising a functional group W, wherein the functional group W is an epoxide or a
25 group which is transformed in a further step to give an epoxide.

Preferably, the first linker has the structure Z^2 -L^W-W, wherein Z^2 is a functional group capable of being reacted with at least one hydroxyl group of hydroxyalkyl starch, as described above, and wherein L^W is a linking moiety.

30 Thus, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate, as described above, and a hydroxyalkyl starch conjugate obtained or obtainable by said method, wherein step (a2)(i) comprises step (I)

35 (1) coupling the hydroxyalkyl starch via at least one hydroxyl group comprised in HAS to a first linker having a structure according to the following formula Z^2 -L^W-W, wherein Z^2 is a functional group capable of being reacted with at least one hydroxyl group of hydroxyalkyl starch, as described above, and wherein L^W is a linking moiety, and wherein, upon reaction of the hydroxyalkyl starch, a hydroxyalkyl starch

derivative is formed comprising at least one structural unit according to the following formula (lb)



5

wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS$ ", $-[O-(CR^wR^x)-(CR^yR^z)]_x-OH$ and $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^w-W$ wherein R^w , R^x , R^y and R^z are independently of each other selected from the group consisting of hydrogen and alkyl, y is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4, x is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4 and wherein at least one of R^a , R^b and R^c comprises the group $-[O-(CR^wR^xHCR^yR^z)]_y-[T^1]_p-L^w-W$, and wherein $[F^1]_p$ is the functional group being formed upon reaction of Z^2 with at least one hydroxyl group of the hydroxyalkyl starch,

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15

more preferably, wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS$ ", $-[O-CH_2-CH_2]_s-OH$ and $-[O-CH_2-CH_2]_t-[F^1]_p-L^w-W$, and wherein t is in the range of from 0 to 4 and wherein s is in the range of from 0 to 4, and p is 1, and wherein at least one of R^a , R^b and R^c comprises the group $-[O-CH_2-CH_2]_t-[F^1]_p-L^w-W$.

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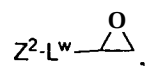
According to one embodiment of the present invention, the functionalization of at least one hydroxyl group of hydroxyalkyl starch to give the epoxide comprising hydroxyalkyl starch, is carried out in a one-step procedure, wherein at least one hydroxyl group is reacted with a first linker, as described above, wherein the first linker comprises the functional group W , and wherein W is an epoxide.

25

Therefore, the present invention also describes a method for preparing a hydroxyalkyl starch conjugate, as described above, as well as to a hydroxyalkyl starch conjugate obtained or obtainable by said method, wherein in step (a2)(i)(I) the hydroxyalkyl starch is reacted with a linker comprising a functional group Z^2 capable of being reacted with a hydroxyl group of the hydroxyalkyl starch, thereby forming a covalent linkage, the linker further comprising a functional group W , wherein the functional group W is an epoxide.

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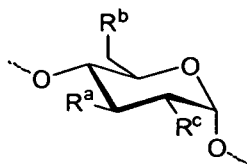
This linker has in this case a structure according to the following formula



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such as, for example, epichlorohydrine.

Upon reaction of this linker with at least one hydroxyl group of hydroxyalkyl starch, a hydroxyalkyl starch derivative is formed comprising at least one structural unit according to the following formula (Ib)



(Ib)

wherein R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS", $-[O-(CR^wR^xMCR^yR^z)]_x-OH$ and $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^w$ , and

wherein at least one of R^a , R^b and R^c comprises the group $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^w$ , preferably wherein R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS", $-[O-CH_2-CH_2]_s-OH$ and

$-[O-CH_2-CH_2]_t-[F^1]_p-L^w$ , and wherein t is in the range of from 0 to 4 and wherein s is in the range of from 0 to 4 (i.e. p is 1), and wherein at least one of R^a , R^b and R^c comprises

the group $-[O-CH_2-CH_2]_t-[F^1]_p-L^w$ .

According to a preferred embodiment of the invention, the epoxide is generated in a two step procedure, comprising the steps (I) and (II)

(I) coupling at least one hydroxyl group of the hydroxyalkyl starch, preferably of hydroxyethyl starch, to a first linker, comprising a functional group Z^2 capable of being reacted with a hydroxyl group of the hydroxyalkyl starch, thereby forming a covalent linkage between the first linker and the hydroxyalkyl starch, the linker further comprising a functional group W, wherein the functional group W is a functional group which is capable of being transformed in a further step to give an epoxide, such as an alkenyl group,

(II) transforming the functional group W to give an epoxide.

It was surprisingly found that this two step procedure is superior to the one step procedure in that higher loadings of the hydroxyalkyl starch with epoxide groups can be achieved

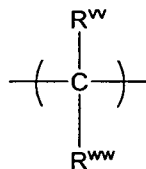
and/or undesired side reactions such as inter- and intra-molecular crosslinking can be substantially avoided.

Preferably the functional group W is an alkenyl group. In this case, step (II) preferably comprises the oxidation of the alkenyl group to give an epoxide and transforming the epoxide to give the functional group Z¹.

According to a preferred embodiment, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate, as described above, wherein the hydroxyalkyl starch, preferably the hydroxyethyl starch, is coupled in step (a2)(i) via at least one hydroxyl group to at least one suitable linker, the linker having the structure Z²-L^W-W, wherein upon reaction of a hydroxyl group of the hydroxyalkyl starch with the linker, the leaving group Z² departs, thereby forming a covalent linkage between the hydroxyalkyl starch and the linking moiety L^W, and wherein the functional group F¹ which links the hydroxyalkyl starch and the linking moiety L^W, is an -O- bond. Likewise, the present invention also relates to the respective hydroxyalkyl starch conjugates obtained or obtainable by said method.

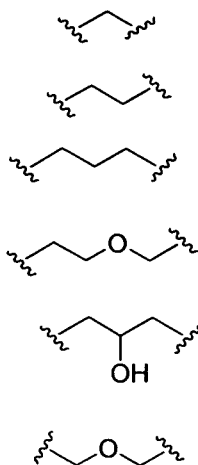
According to the present invention, the term "linking moiety L^W" as used in the context of the present invention relates to any suitable chemical moiety bridging the functional group Z² and the functional group W.

In general, there are no particular restrictions as to the chemical nature of the linking moiety L^W with the proviso that L^W has particular chemical properties enabling carrying out the inventive method for the preparation of the novel derivatives comprising the functional group Z¹, i.e. in particular, in case W is a functional group to be transformed to an epoxide, the linking moiety L^W has suitable chemical properties enabling the transformation of the chemical moiety W to the functional group Z¹. According to a preferred embodiment of the present invention, L^W bridging W and HAS' comprises at least one structural unit according to the following formula



wherein R^W and R^{WW} are independently of each other H or an organic residue selected from the group consisting of alkyl, alkenyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl and heteroarylalkyl groups.

Preferably, L^W is an optionally substituted, non-branched alkyl residue such as a group selected from the following groups:



- 5 According to a first preferred embodiment of the present invention, the functional group W is an optionally substituted, non-branched alkyl residue such as a group selected from the following groups, wherein the first linker Z^2-L^W-W has a structure according to the following formula



preferably with Z^2 being a leaving group or an epoxide.

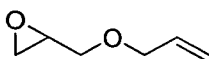
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Thus preferred structures of the first linker are by way of example, the following structures:

Hal-CH₂-CH=CH₂, such as Cl-CH₂-CH=CH₂ or Br-CH₂-CH=CH₂ or I-CH₂-CH=CH₂

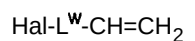
sulfonic esters such as TsO-CH₂-CH=CH₂ or MsO-CH₂-CH=CH₂

epoxides such as



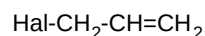
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More preferably Z^2 in the first linker Z^2-L^W-W is a leaving group, most preferably the first linker Z^2-L^W-W has a structure according to the following formula



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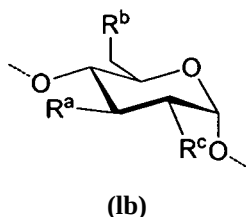
According to an especially preferred embodiment of the present invention, the linker Z^2-L^W-W has a structure according to the following formula



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with Hal being a halogen, preferably the halogen being I, Cl, or Br, more preferably Br.

Thus, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate, as described above, wherein in step (a2)(ii) the hydroxyalkyl starch, preferably the hydroxyethyl starch, is coupled via at least one hydroxyl group to at least one suitable linker having the structure $\text{Hal-CH}_2\text{-CH=CH}_2$, wherein upon reaction of the hydroxyalkyl starch with the linker, a hydroxyalkyl starch derivative is formed comprising at least one structural unit according to the following formula (Ib)



wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-\text{O-HAS''}$, $-\text{[O-(CR}^w\text{R}^x\text{HCR}^y\text{R}^z)]_x\text{-OH}$ and $-\text{[O-(CR}^w\text{R}^x\text{)-(CR}^y\text{R}^z)]_y\text{-O-CH}_2\text{-CH=CH}_2$, and wherein at least one of R^a , R^b and R^c comprises the group $-\text{[O-(CR}^w\text{R}^x\text{)-(CR}^y\text{R}^z)]_y\text{-O-CH}_2\text{-CH=CH}_2$, preferably wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-\text{OH}$, $-\text{O-HAS''}$, $-\text{[O-CH}_2\text{-CH}_2]_s\text{-OH}$ and $-\text{[O-CH}_2\text{-CH}_2]_t\text{-O-CH}_2\text{-CH=CH}_2$, wherein t is in the range of from 0 to 4, wherein s is in the range of from 0 to 4, and wherein at least one of R^a , R^b and R^c comprises the group $-\text{[O-CH}_2\text{-CH}_2]_t\text{-O-CH}_2\text{-CH=CH}_2$, and wherein the functional group $-\text{O-}$ linking the $-\text{CH}_2\text{-CH=CH}_2$ group to the hydroxyalkyl starch is formed upon reaction of the linker $\text{Hal-CH}_2\text{-CH=CH}_2$ with a hydroxyl group of the hydroxyalkyl starch. Likewise, the present invention also relates to a hydroxyalkyl starch conjugate obtained or obtainable by the above-mentioned method.

As regards, the reaction conditions used in this step (I), wherein the hydroxyalkyl starch is reacted with the first linker, in particular wherein the first linker comprises the functional group W with W being an alkenyl, in principle any reaction conditions known to those skilled in the art can be used. Preferably, the reaction is carried out in an organic solvent, such as *N*-methyl pyrrolidone, dimethyl acetamide (DMA), dimethyl formamide (DMF), formamide, dimethyl sulfoxide (DMSO) or mixtures of two or more thereof. More preferably, the reaction is carried out in anhydrous solvents or solvent mixtures.

Preferably, the hydroxyalkyl starch is dried prior to use, by means of heating to constant weight at a temperature range from 50 to 80 °C in a drying oven or with related techniques.

The temperature of the reaction is preferably in the range of from 5 to 55 °C, more preferably in the range of from 10 to 30 °C, and especially preferably in the range of from

15 to 25 °C. During the course of the reaction, the temperature may be varied, preferably in the above given ranges, or held essentially constant.

5 The reaction time for the reaction of HAS with the linker Z^2-L^W-W may be adapted to the specific needs and is generally in the range of from 1 h to 7 days, preferably 2 hours to 24 hours, more preferably 3 hours to 18 hours.

10 More preferably, the reaction is carried out in the presence of a base. The base may be added together with the linker Z^2-L^W-W , or may be added prior to the addition of the linker, to pre-activate the hydroxyl groups of the hydroxyalkyl starch. Preferably, a base, such as alkali metal hydrides, alkali metal hydroxides, alkali metal carbonates, amine bases such as diisopropylethyl amine (DIEA) and the like, amidine bases such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), amide bases such as lithium diisopropylamide (LDA) or alkali metal hexamethyldisilazyl bases (e.g. LiHMDS) may be used. Most
15 preferably the hydroxyalkyl starch is pre-activated with sodium hydride prior to the addition of the first linker Z^2-L^W-W .

20 The derivative comprising the functional group W, preferably the alkenyl group, may be isolated prior to transforming this group in at least one further step to give an epoxide comprising hydroxyalkyl starch derivative. Isolation of this polymer derivative comprising the functional group W may be carried out by a suitable process which may comprise one or more steps. According to a preferred embodiment of the present invention, the polymer derivative is first separated from the reaction mixture by a suitable method such as precipitation and subsequent centrifugation or filtration. In a second step, the separated
25 polymer derivative may be subjected to a further treatment such as an after-treatment like ultrafiltration, dialysis, centrifugal filtration or pressure filtration, ion exchange chromatography, reversed phase chromatography, HPLC, MPLC, gel filtration and/or lyophilization. According to an even more preferred embodiment, the separated polymer derivative is first precipitated, subjected to centrifugation, re-dissolved and finally
30 subjected to ultrafiltration.

Preferably, the precipitation is carried out with an organic solvent such as ethanol, isopropanol, acetone or tetrahydrofurane (THF). The precipitated derivative is subsequently subjected to centrifugation and subsequent ultrafiltration using water or an
35 aqueous buffer solution having a concentration preferably from 1 to 1000 mmol/l, more preferably from 1 to 100 mmol/l, and more preferably from 10 to 50 mmol/l such as about 20 mmol/l, a pH value in the range of preferably from 3 to 10, more preferably of from 4 to 8, such as about 7. The number of exchange cycles preferably is in the range of from 5 to 50, more preferably of from 10 to 30, and even more preferably of from 15 to 25, such as

about 20. Most preferably the obtained derivative comprising the functional group W is further lyophilized until the solvent content of the reaction product is sufficiently low according to the desired specifications of the product.

5 In case W is an alkenyl, the method preferably further comprises step (II), that is the oxidation of the alkenyl group to give an epoxide group. As to the reaction conditions used in the epoxidation (oxidation) step (II), in principle, any known method to those skilled in the art can be applied to oxidize an alkenyl group to yield an epoxide.

10 The following oxidizing reagents are mentioned, by way of example, metal peroxysulfates such as potassium peroxymonosulfate (Oxone®) or ammonium peroxydisulfate, peroxides such as hydrogen peroxide, tert.-butyl peroxide, acetone peroxide (dimethyldioxirane), sodium percarbonate, sodium perborate, peroxy acids such as peroxyacetic acid, meta-chloroperbenzoic acid (MCPBA) or salts like sodium hypochlorite or hypobromite.

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According to a particularly preferred embodiment of the present invention, the epoxidation is carried out with potassium peroxymonosulfate (Oxone®) as oxidizing agent.

20 Thus, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate, as described above, wherein step (a2)(i) comprises

- (I) coupling at least one hydroxyl group of the hydroxyalkyl starch, preferably of hydroxyethyl starch, to a first linker, comprising a functional group Z^2 capable of being reacted with a hydroxyl group of the hydroxyalkyl starch, thereby forming a covalent linkage between the first linker and the hydroxyalkyl starch, the linker further comprising a functional group W, wherein the functional group W is an alkenyl group,
- 25
- (II) oxidizing the alkenyl group to give an epoxide, wherein as oxidizing agent, preferably potassium peroxymonosulfate (Oxone®) is employed.

30 Further, the present invention also relates to a hydroxyalkyl starch conjugate obtained or obtainable by said method.

According to an even more preferred embodiment of the present invention, the reaction with potassium peroxymonosulfate (Oxone®) is carried out in the presence of a suitable catalyst. Catalysts may consist of transition metals and their complexes, such as manganese (Mn-salene complexes are known as Jacobsen catalysts), vanadium, molybdenum, titanium (Ti-dialkyltartrate complexes are known as Sharpless catalysts), rare earth metals and the like. Additionally, metal free systems can be used as catalysts. Acids such as acetic acid may form peracids in situ and epoxidize alkenes. The same accounts for ketones such

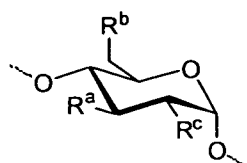
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as acetone or tetrahydrothiopyran-4-one, which react with peroxide donors under formation of dioxiranes, which are powerful epoxidation agents. In case of non-metal catalysts, traces of transition metals from solvents may lead to unwanted side reactions, which can be excluded by metal chelation with EDTA.

5

Preferably, said suitable catalyst is tetrahydrothiopyran-4-one.

Upon epoxidation, in step (II) a hydroxyalkyl starch derivative is formed comprising at least one structural unit according to the following formula (Ib)



10

(Ib)

wherein R^a , R^b and R^c are independently of each other selected from the group consisting

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of -O-HAS", $-[O-(CR^wR^xHCR^yR^z)]_x-OH$ and $\{O-(CR^wR^x)-(CR^yR^z)\}_y-[F^1]_p-L^w$, and wherein at least one of R^a , R^b and R^c comprises the group

$\{O-(CR^wR^x)-(CR^yR^z)\}_y-[F^1]_p-L^w$, preferably wherein R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS", $-[O-CH_2-CH_2]_s-OH$ and

$-[O-CH_2-CH_2]_s-F^1-L^w$, and wherein s is in the range of from 0 to 4 (i.e. p is 1) and wherein at least one of R^a , R^b and R^c comprises the

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group $-[O-CH_2-CH_2]_s-F^1-L^w$.

According to a preferred embodiment, the epoxidation of the alkenyl-modified hydroxyalkyl starch derivatives is carried out in aqueous medium, preferably at a temperature in the range of from 0 to 80 °C, more preferably in the range of from 0 to 50 °C and especially preferably in the range of from 10 to 30 °C.

25

During the course of the epoxidation reaction, the temperature may be varied, preferably in the above-given ranges, or held essentially constant. The term "aqueous medium" as used in the context of the present invention refers to a solvent or a mixture of solvents comprising water in an amount of at least 10 % per weight, preferably at least 20 % per weight, more preferably at least 30 % per weight, more preferably at least 40 % per weight, more preferably at least 50 % per weight, more preferably at least 60 % per weight, more

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preferably at least 70 % per weight, more preferably at least 80 % per weight, even more preferably at least 90 % per weight or up to 100 % per weight, based on the weight of the solvents involved. The aqueous medium may comprise additional solvents like formamide, dimethylformamide (DMF), dimethylsulfoxide (DMSO), alcohols such as methanol, ethanol or isopropanol, acetonitrile, tetrahydrofuran or dioxane. Preferably, the aqueous solution contains a transition metal chelator (disodium ethylenediaminetetraacetate, EDTA, or the like) in the concentration ranging from 0.01 to 100 mM, preferably from 0.01 to 1 mM, most preferably from 0.1 to 0.5 mM, such as about 0.4 mM.

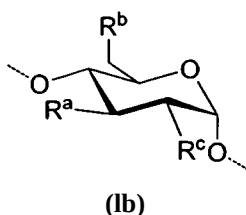
- 10 The pH value for the reaction of the HAS derivative with potassium peroxymonosulfate (Oxone®) may be adapted to the specific needs of the reactants. Preferably, the reaction is carried out in buffered solution, at a pH value in the range of from 3 to 10, more preferably of from 5 to 9, and even more preferably of from 7 to 8. Among the preferred buffers, carbonate, phosphate, borate and acetate buffers as well as
- 15 tris(hydroxymethyl)aminomethane (TRIS) may be mentioned. Among the preferred bases, alkali metal bicarbonates may be mentioned.

According to the invention, the epoxide-modified HAS derivative may be purified or isolated in a further step prior to the transformation of the epoxide group to the functional group Z¹.

The separated derivative is optionally lyophilized.

- After the purification step, the HAS derivative is preferably obtained as a solid. According to a further conceivable embodiment of the present invention, the HAS derivative solutions or frozen HAS derivative solutions may be mentioned.

The epoxide comprising HAS derivative is preferably reacted in a subsequent step (III) with at least one suitable reagent to yield the HAS derivative comprising the functional group Z¹. Preferably, the epoxide is reacted with a nucleophile comprising the functional group Z¹ or a precursor thereof. Preferably, the nucleophile reacts with the epoxide in a ring opening reaction and yields a HAS derivative comprising at least one structural unit according to the following formula (Ib)



wherein at least one of R^a , R^b and R^c is $-[O-(CR^wR^xHCR^yR^z)]_y-[F^1]_p-L^w-CHOH-CH_2-Nuc$, preferably wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-[F^1]_p-L^w-CHOH-CH_2-Nuc$, wherein the residue Nuc is the remaining part of the nucleophile covalently linked to the hydroxyalkyl starch after being reacted with the epoxide.

5

Any nucleophile capable of reacting with the epoxide thereby forming a covalent linkage and comprising the functional group Z^1 or a precursor thereof may be used. As nucleophile, for example, linker compounds comprising at least one nucleophilic functional group capable of reacting with the epoxide and at least one functional group W capable of being transformed to the functional Z^1 can be used. Alternatively, a linker such as an at least bifunctional linker comprising a nucleophilic group such as a thiol group and further comprising the functional group Z^1 may be used.

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As described above, according to a particularly preferred embodiment of the present invention, Z^1 is a thiol group.

According to a further preferred embodiment of the present invention, the nucleophilic group reacting with the epoxide is a thiol group.

20

Thus, the present invention also relates to a method as described above, wherein step (a2)(i) comprises

(III) reacting the epoxide with a nucleophile comprising the functional group Z^1 or a precursor of the functional group Z^1 , the nucleophile additionally comprising a nucleophilic group, preferably wherein Z^1 and the nucleophilic group are both -SH groups.

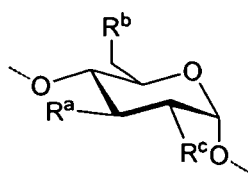
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According to an especially preferred embodiment of the present invention, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate, as well as to a hydroxyalkyl starch conjugate obtained or obtainable by said method, as described above, wherein the epoxide is reacted with a nucleophile comprising the functional group Z^1 , with Z^1 being a thiol group, and comprising a nucleophilic group, this group being a thiol. Thus, according to a preferred embodiment, the nucleophile is a dithiol.

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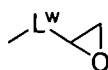
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The invention also relates to the respective derivative obtained or obtainable by said method, wherein said derivative is preferably transformed to the conjugate according of the invention, as described hereinunder and above, said derivative preferably comprising at least one structural unit according to the following formula (Ib)

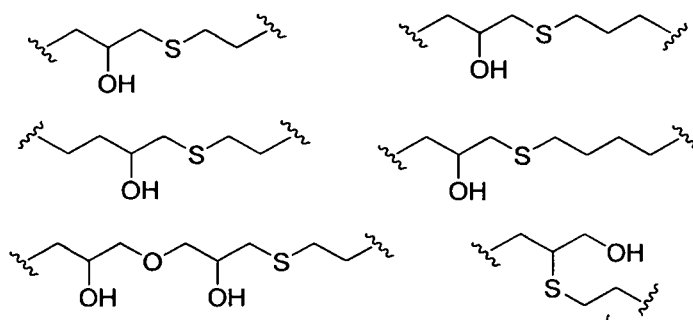


(1b)

- wherein at least one of R^a , R^b and R^c is $-\{O-(CR^wR^x)-(CR^yR^z)\}_y-[F^1]_p-L^1-SH$, preferably
 5 wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-SH$, wherein L^1 is a linking moiety which is obtained when reacting the structural unit

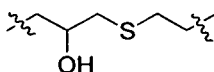


- 10 with the nucleophile and which links the functional group F^1 to the functional group Z^1 . According to the preferred embodiment, the linking moiety L^1 has a structure selected from the groups below:



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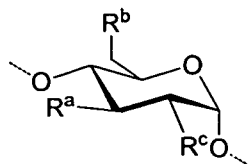
more preferably L^1 has a structure according to the following formula



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- According to an alternative embodiment of the present method, the epoxide is reacted with a nucleophile suitable for the introduction of thiol groups such as thiosulfate, alkyl or aryl thiosulfonates or thiourea, preferably sodium thiosulfate. Thus, the present invention also
 25 relates to a method as described above as well as to a hydroxyalkyl starch derivative obtained or obtainable by said method, wherein the epoxide-modified hydroxyalkyl starch is reacted with a nucleophile, said nucleophile being thiosulfate, alkyl or aryl thiosulfonates or thiourea, preferably sodium thiosulfate.

Upon reaction of the thiosulfate with the epoxide in a ring opening reaction, preferably a hydroxyalkyl starch derivative is formed comprising at least one structural unit according to the following formula (Ib)



(Ib)

wherein at least one of R^a , R^b and R^c is $-[0-(CR^wR^x)-(CR^yR^z)]_x-[F^1]_p-L^w-CHOH-CH_2-SSO_3Na$, preferably wherein at least one of R^a , R^b and R^c is $-[0-CH_2-CH_2r[F^1]_p-L^w-CHOH-CH_2-SSO_3Na$.

Preferably, this derivative is reduced in a subsequent step to yield the HAS derivative comprising the functional group Z^1 with Z^1 being $-SH$. Any suitable methods known to those skilled in the art can be used to reduce the respective intermediate shown above.

Preferably, the thiosulfonate is reduced with sodium borohydride in aqueous solution.

According to a preferred embodiment of the present invention, the hydroxyalkyl starch derivative comprising the functional Z^1 , obtained by the above-described method, is purified in a further step. Again, the purification of the HAS derivative from step (III) can be carried out by any suitable method such as ultrafiltration, dialysis or precipitation or a combined method using for example precipitation and afterwards ultrafiltration. Furthermore, the HAS derivative may be lyophilized, as described above, using conventional methods, prior to step (b).

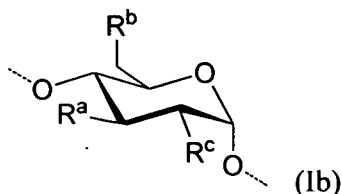
Carboxy activated hydroxyalkyl starch with a crosslinking compound (linker)

According to a second embodiment, in step (a2)(i), a linker is used, comprising the functional group Z^1 or the functional group W , wherein W has the structure $-Z'^*-PG$, with PG being a suitable protecting group. Preferably, in case this linker is used, the hydroxyalkyl starch is activated prior to the reaction using a reactive carbonate as described above.

Thus, the present invention also relates to a method, as described above, wherein step (a2)(i) comprises

- (aa) activating at least one hydroxyl group comprised in the hydroxyalkyl starch with a reactive carbonyl compound having the structure $R^{**}-(C=O)-R^*$, wherein R^* and

R^{**} may be the same or different, and wherein R^* and R^{**} are both leaving groups, wherein upon activation a hydroxyalkyl starch derivative comprising at least one structural unit according to the following formula (1b),



5 is formed, in which R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS", $-[O-CH_2-CH_2]_s-OH$, $-[O-CH_2-CH_2]_t-O-C(=O)-R^*$,

wherein s is in the range of from 0 to 4,

and wherein t is in the range of from 0 to 4,

10 and wherein at least one of R^a , R^b and R^c comprises the group $-[O-CH_2-CH_2]_t-O-C(=O)-R^*$, and

(bb) reacting the activated hydroxyalkyl starch derivative according to step (aa) with the suitable linker comprising the functional group Z^1 or a precursor of the functional group Z^1 .

15 The invention further relates to a conjugate obtained or obtainable by said method.

In particular, in step (a2)(i) the hydroxyalkyl starch is reacted with a linker comprising the functional group Z^1 or a precursor thereof and a functional group Z^2 , the linker having the structure $Z^2-L^1-Z^1$ or $Z^2-L^1-Z^{1*}-PG$, with Z^2 being a functional group capable of being
 20 reacted with the hydroxyalkyl starch or an activated hydroxyalkyl starch, preferably with an activated hydroxyalkyl starch, the method further comprising activating the hydroxyalkyl starch prior to the reaction with the linker using a reactive carbonate, and with Z^{1*} being the protected form of the functional group Z^1 .

25 As described above, the linker preferably comprises a functional group Z^2 , which in this case, is preferably a nucleophile, such as a group comprising an amino group, more preferably a group selected from the group consisting of $-NHR^{Z^2}$, $-NH_2$, $-O-NH_2$, $-NH-O$ -alkyl, $-(C=G)-NH-NH_2$, $-G-(C=G)-NH-NH_2$, $-NH-(C=G)-NH-NH_2$, and $-SO_2-NH-NH_2$ wherein G is O or S, and if present twice in one structural unit, may be the same or may be
 30 different, and wherein R^{Z^2} is an alkyl group, preferably methyl. More preferably Z^2 is $-NH_2$ or $-NHR^{Z^2}$, most preferably $-NH_2$.

In this case, the linker has preferably a structure $Z^2-L^1-Z^{1*}-PG$, wherein Z^{1*} is in particular -S- (and the respective unprotected functional group Z^1 a thiol group). According to this
 35 embodiment, the linking moiety L^1 is preferably an optionally substituted alkyl group. More preferably, the linking moiety L^1 is a spacer comprising at least one structural unit

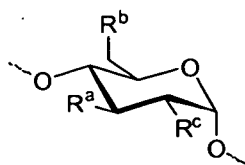
according to the formula $-\{[CR^{dR^f}]_h-[F^4]_u-[CR^{dd}R^{ff}]_z\}_{\alpha}$, as described above, wherein integer α is in the range of from 1 to 10, and wherein F^4 is preferably selected from the group consisting of -S-, -O- and -NH-, more preferably wherein F^4 , if present, is -O- or -S-, more preferably wherein F^4 is -S-. As described above, in the context of the preferred conjugates, residues R^d , R^f , R^{dd} and R^{ff} are, independently of each other, preferably selected from the group consisting of halogens, alkyl groups, H or hydroxyl groups. More preferably, these residues are independently from each other H, alkyl or hydroxyl groups. Preferably, integer u and integer z of the formula $-\{[CR^{dR^f}]_h-[F^4]_u-[CR^{dd}R^{ff}]_z\}_{\alpha}$ are 0, and α is 1, the linking moiety L^1 thus corresponds to the structural unit $-[CR^{dR^f}]_h$. The integer h is preferably in the range of from 1 to 20, more preferably of from 1 to 10, such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10, more preferably of from 1 to 5, most preferably of from 1 to 3. More preferably R^d and R^f are both H. Thus, by way of example, the following preferred linking moieties L^1 are mentioned: $-\text{CH}_2-$, $-\text{CH}_2-\text{CH}_2-$, $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$, $-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-$, $-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-$, more preferably $-\text{CH}_2-\text{CH}_2-$, in the context of this second embodiment.

In case Z^1 is a thiol group, and Z^{1*} is -S-, the group PG is preferably a thiol protecting group, more preferably a protecting group forming together with Z^{1*} a thioether (e.g. trityl, benzyl, allyl), a disulfide (e.g. S-sulfonates, S-tert.-butyl, S-(2-aminoethyl)), or a thioester (e.g. thioacetyl). In case the linker comprises a protecting group, the method further comprises a deprotection step.

In case the group Z^{1*} -PG is a disulfide, and Z^{1*} is -S-, the linker Z^2 - L^1 -S-PG is preferably a symmetrical disulfide, with PG having the structure $-\text{S}-L^1-Z^2$. As preferred linker compound, thus cystamine and the like, may be mentioned

In the context of this embodiment, the following linker compounds having the structure Z^2 - L^1 -Z¹-PG are mentioned by way of example: $\text{H}_2\text{N}-\text{CH}_2-\text{S}-\text{Trt}$, $\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{S}-\text{Trt}$, $\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{S}-\text{Trt}$, $\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{S}-\text{Trt}$, $\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{S}-\text{Trt}$, $\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{S}-\text{S}-\text{CH}_2-\text{CH}_2-\text{NH}_2$, $\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{S}-\text{S}-\text{tBu}$, wherein Trt is a trityl group.

Subsequent to the activation, the hydroxyalkyl starch is preferably reacted with the linker Z^2 - L^1 -Z¹-PG, thereby most preferably forming a derivative, comprising the functional group $-\text{Z}^{1*}$ -PG, more preferably this derivative comprises at least one structural unit according to the following formula (Ib)



(lb)

wherein at least one of R^a , R^b and R^c is $-[O-(CR^wR^x)-(CR^yR^z)]_x-F^1-L^1-Z^{1*}-PG$, more preferably wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS''$, $-[O-CH_2-CH_2]_s-OH$, and $-[O-CH_2-CH_2]_tF^1-L^1-Z^{1*}-PG$, wherein t is in the range of from 0 to 4, and wherein s is in the range of from 0 to 4, and wherein at least one of R^a , R^b and R^c comprises the group $-[O-CH_2-CH_2]_t-F^1-L^1-Z^{1*}-PG$, and wherein F^1 is the functional group being formed upon reaction of the group $-O-C(=O)-R^*$ with the functional group Z^2 . According to a preferred embodiment, the functional group Z^2 is $-NH_2$, thus F^1 preferably has the structure $-O-C(=O)-NH-$.

The coupling reaction between the activated hydroxyalkyl starch and the linker, comprising the functional Z^1 or the functional group W , wherein W has preferably the structure $-Z^{1*}-PG$, with PG being a suitable protecting group, in principle any reaction conditions known to those skilled in the art can be used. Preferably, the reaction is carried out in an organic solvent, such as N-methyl pyrrolidone, dimethyl acetamide (DMA), dimethyl formamide (DMF), formamide, dimethyl sulfoxide (DMSO), or mixtures of two or more thereof, preferably at a temperature in the range of from 5 to 80 °C, more preferably of from 5 to 50 °C and especially preferably of from 15 to 30 °C. The temperature may be held essentially constant or may be varied during the reaction procedure.

The pH value for this reaction may be adapted to the specific needs of the reactants. Preferably, the reaction is carried out in the presence of a base. Among the preferred bases pyridine, substituted pyridines, such as 4-(dimethylamino)-pyridine, 2,6-lutidine or collidine, tertiary amine bases such as triethyl amine, diisopropyl ethyl amine (DIEA), N-methyl morpholine, amidine bases such as 1,8-diazabicyclo[5.4.0]undec-7-ene or inorganic bases such as alkali metal carbonates may be mentioned.

The reaction time for the reaction of activated hydroxyalkyl starch with the linker $Z^2-L^*-Z^{1*}-PG$ or $Z^2-L^1-Z^1$ may be adapted to the specific needs and is generally in the range of from 1 h to 7 days, preferably of from 2 hours to 48 hours, more preferably of from 4 hours to 24 hours.

The derivative comprising the functional group $Z^{1*}-PG$ or Z^1 , may be subjected to at least one further isolation and/or purification step. According to a preferred embodiment of the present invention, the polymer derivative is first separated from the reaction mixture by a suitable method such as precipitation and subsequent centrifugation or filtration. In a second step, the separated polymer derivative may be subjected to a further treatment such

as an after-treatment like ultrafiltration, dialysis, centrifugal filtration or pressure filtration, ion exchange chromatography, reversed phase chromatography, HPLC, MPLC, gel filtration and/or lyophilization. According to an even more preferred embodiment, the separated polymer derivative is first precipitated, subjected to centrifugation, re-dissolved
5 and finally subjected to ultrafiltration.

Preferably, the precipitation is carried out with an organic solvent such as ethanol, isopropanol, acetone or tetrahydrofurane (THF). The precipitated conjugate is subsequently subjected to centrifugation and subsequent ultrafiltration using water or an
10 aqueous buffer solution having a concentration preferably from 1 to 1000 mmol/l, more preferably from 1 to 100 mmol/l, and more preferably from 10 to 50 mmol/l such as about 20 mmol/l, a pH value in the range of preferably from 3 to 10, more preferably of from 4 to 8, such as about 7. The number of exchange cycles preferably is in the range of from 5 to 50, more preferably of from 10 to 30, and even more preferably from 15 to 25, such as
15 about 20.

Most preferably the obtained derivative is further lyophilized until the solvent content of the reaction product is sufficiently low according to the desired specifications of the product.

In case the linker comprises a protecting group (PG), the method preferably further comprises a deprotection step. The reaction conditions used are adapted to the respective protecting group used. According to a preferred embodiment of the invention, Z^1 is a thiol group, and the group Z'^1 -PG is a disulfide, as described above. In this case, the
25 deprotection step comprises the reduction of this disulfide bond to give the respective thiol group. This deprotection step is carried out using specific reducing agents. As possible reducing agents, complex hydrides such as borohydrides, especially sodium borohydride, and thiols, especially dithiothreitol (DTT) and dithioerythritol (DTE) or phosphines such as tris-(2-carboxyethyl)phosphine (TCEP) are mentioned. The reduction is preferably carried
30 out using DTT.

The deprotection step is preferably carried out at a temperature in the range of from 0 to 80 °C, more preferably of from 10 to 50 °C and especially preferably of from 20 to 40 °C. During the course of the reaction, the temperature may be varied, preferably in the above-
35 given ranges, or held essentially constant.

Preferably, the reaction is carried out in aqueous medium. The term "aqueous medium" as used in the context of the present invention refers to a solvent or a mixture of solvents comprising water in an amount of at least 10 % per weight, preferably at least 20 % per

weight, more preferably at least 30 % per weight, more preferably at least 40 % per weight, more preferably at least 50 % per weight, more preferably at least 60 % per weight, more preferably at least 70 % per weight, more preferably at least 80 % per weight, even more preferably at least 90 % per weight or up to 100 % per weight, based on the weight of the solvents involved. The aqueous medium may comprise additional solvents like formamide, dimethylformamide (DMF), dimethylsulfoxide (DMSO), alcohols such as methanol, ethanol or isopropanol, acetonitrile, tetrahydrofuran or dioxane. Preferably, the aqueous solution contains a transition metal chelator (disodium ethylenediaminetetraacetate, EDTA, or the like) in a concentration ranging from 0.01 to 100 mM, preferably from 0.01 to 1 mM, most preferably from 0.1 to 0.5 mM, such as about 0.4 mM.

The pH value in the deprotection step may be adapted to the specific needs of the reactants. Preferably, the reaction is carried out in buffered solution, at a pH value in the range of from 3 to 14, more preferably of from 5 to 11, and even more preferably of from 7.5 to 8.5. Among the preferred buffers, carbonate, phosphate, borate and acetate buffers as well as tris(hydroxymethyl)aminomethane (TRIS) may be mentioned.

Again, at least one of the isolation steps/and or purification steps, as described above, may be carried out subsequent to the deprotection step. Most preferably, the obtained derivative is further lyophilized prior to step (b) until the solvent content of the reaction product is sufficiently low according to the desired specifications of the derivative.

Step (a2)(ii)

As regards step (a2)(ii) of the method according to the present invention, in this step, the functional group Z^1 is introduced by displacing a hydroxyl group present in the hydroxyalkyl starch in a substitution reaction with a precursor of the functional group Z^1 or with a functional linker comprising the functional group Z^1 or a precursor thereof.

Preferably, prior to the replacement of the hydroxyl group with the functional group Z^1 , the at least one hydroxyl group of the hydroxyalkyl starch is activated to generate a suitable leaving group. Preferably, a group R^L is added to the at least one hydroxyl group thereby generating a group $-O-R^L$, wherein the structural unit $-O-R^L$ is the leaving group.

Thus, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate, as described above, as well as to a hydroxyalkyl starch conjugate, obtained or obtainable by said method wherein in step (a2)(ii), prior to the substitution (displacement) of the hydroxyl group with the group comprising the functional group Z^1 or a precursor

thereof, a group R^L is added to at least one hydroxyl group thereby generating a group $-O-R^L$, wherein $-O-R^L$ is the leaving group.

5 The term "leaving group" as used in this context of the present invention is denoted to mean that the molecular fragment $-O-R^L$ departs when reacting the hydroxyalkyl starch derivative with a reagent, such as a crosslinking compound, comprising the functional group Z^1 or a precursor thereof.

10 As regards, preferred leaving groups used in this context of the present invention, according to a preferred embodiment, the hydroxyl group is transformed to a sulfonic ester, such as a mesylic ester ($-OMs$), tosylic ester ($-OTs$), imsylic ester (imidazylsulfonyl ester) or a carboxylic ester such as trifluoroacetic ester.

15 Preferably, the at least one leaving group is generated by reacting at least one hydroxyl group of hydroxyalkyl starch, preferably in the presence of a base, with the respective sulfonyl chloride to give the sulfonic ester, preferably the mesylic ester.

20 Thus, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate as described above, as well as to a hydroxyalkyl starch conjugate obtained or obtainable by said method, wherein in step (a2)(ii), prior to the substitution (displacement) of the hydroxyl group with the group comprising the functional group Z^1 or a precursor thereof, a group R^L is added to at least one hydroxyl group, thereby generating a group $-O-R^L$, wherein $-O-R^L$ is $-OMs$ or $-OTs$, and wherein the $-OMs$ group is preferably introduced by reacting at least one hydroxyl group of hydroxyalkyl starch with
25 methanesulfonyl chloride, and $-OTs$ is introduced by reacting at least one hydroxyl group with toluenesulfonylchloride.

30 The addition of the group R^L to at least one hydroxyl group of hydroxyalkyl starch, whereupon a group $-O-R^L$ is formed, is preferably carried out in an organic solvent, such as N-methyl pyrrolidone, dimethyl acetamide (DMA), dimethyl formamide (DMF), formamide, dimethylsulfoxide (DMSO) and mixtures of two or more thereof, preferably at a temperature in the range of from -60 to 80 °C, more preferably in the range of from -30 to 50 °C and especially preferably in the range of from -30 to 30 °C. The temperature may be held essentially constant or may be varied during the reaction procedure. The pH value
35 for this reaction may be adapted to the specific needs of the reactants. Preferably, the reaction is carried out in the presence of a base. Among the preferred bases pyridine, substituted pyridines such as collidine or 2,6-lutidine, tertiary amine bases such as triethylamine, diisopropyl ethyl amine (DIEA), N-methylmorpholine, N-methylimidazole or amidine bases such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and inorganic bases

such as metal hydrides and carbonates may be mentioned. Especially preferred are substituted pyridines (collidine) and tertiary amine bases (DIEA, N-methylmorpholine). The reaction time for this reaction step may be adapted to the specific needs and is generally in the range of from 5 min to 24 hours, preferably of from 15 min to 10 hours,
5 more preferably of from 30 min to 5 hours.

The derivative comprising the group $-O-R^L$, may be subjected to at least one further isolation and/or purification step such as precipitation and/or centrifugation and/or filtration prior to the substitution reaction according to step (a2)(ii). Likewise, instead or
10 additionally, the derivative comprising the $-O-R^L$ group may be subjected to an after-treatment like ultrafiltration, dialysis, centrifugal filtration or pressure filtration, ion exchange chromatography, reversed phase chromatography, HPLC, MPLC, gel filtration and/or lyophilization. According to a preferred embodiment, the derivative comprising the $-O-R^L$ is in situ reacted with the precursor of the functional group Z^1 or with the
15 bifunctional linker, comprising the functional group Z^1 or a precursor thereof.

As described above, the at least one hydroxyl group, preferably the at least one $-O-R^L$ group, more preferably the $-OMs$ or $-OTs$ group, is displaced, in a substitution reaction, with the precursor of the functional group Z^1 or with an at least bifunctional linker
20 comprising the functional group Z^1 or a precursor thereof.

According to a preferred embodiment of the present invention, the activated hydroxyl group, preferably the $-O-R^L$ group, more preferably the $-OMs$ or $-OTs$ group, is reacted with the precursor of the functional group Z^1 . The term "a precursor" as used in this
25 context of the present invention is denoted to mean a reagent which is capable of displacing the group, thereby forming a functional group Z^1 or a group, which can be modified in at least one further step to give the functional group Z^1 .

Thus, the present invention also relates to a method for preparing a hydroxyalkyl starch conjugate, as described above, as well as to a hydroxyalkyl starch conjugate, obtained or obtainable by said method wherein in step (a2)(ii), prior to the substitution (displacement) of the hydroxyl group with the group comprising the functional group Z^1 or a precursor thereof, a group R^L is added to at least one hydroxyl group, thereby generating a group $-O-R^L$, wherein $-O-R^L$ is a leaving group, and subsequently $-O-R^L$ is replaced by a
30 precursor of the functional group Z^1 , the method further comprising converting the precursor after the substitution reaction to the functional group Z^1 , and wherein Z^1 is preferably a thiol group.

In case Z^1 is an amine, reagents such as ammonia, hydrazine, acyl hydrazides, such as carbohydrazide, potassium phthalimide, azides, such as sodium azide, and the like, can be employed to introduce the functional group Z^1 .

- 5 In case Z^1 is a thiol group, reagents such as thioacetic acid, alkyl or aryl thiosulfonates such as sodium benzenethiosulfonate, thiourea, thiosulfate or hydrogen sulfide can be employed as precursor to introduce the functional group Z^1 .

- 10 According to an especially preferred embodiment of the present invention, the hydroxyl group present in the hydroxyalkyl starch is first activated and then reacted with thioacetate, thereby replacing the hydroxyl group with the structure $-S-C(=O)-CH_3$. A particularly preferred reagent is potassium thioacetate. Thus, the present invention also relates to a method, as described above, wherein in step (a2)(ii) the hydroxyl group present in the hydroxyalkyl starch is reacted with thioacetate giving a functional group having the
15 structure $-S-C(=O)-CH_3$.

- In this substitution step, in principle any reaction conditions known to those skilled in the art can be used. Preferably, the reaction is carried out in an organic solvent, such as N-methyl pyrrolidone, dimethyl acetamide (DMA), dimethyl formamide (DMF), formamide,
20 dimethyl sulfoxide (DMSO) and mixtures of two or more thereof. Preferably this step is carried out at a temperature in the range of from 0 to 80 °C, more preferably of from 20 to 70 °C and especially preferably of from 40 to 60 °C. The temperature may be held essentially constant or may be varied during the reaction procedure.

- 25 The pH value for this reaction may be adapted to the specific needs of the reactants. Optionally, the reaction is carried out in the presence of a scavenger, which reacts with the leaving group $-OR^L$, such as mercaptoethanol or the like.

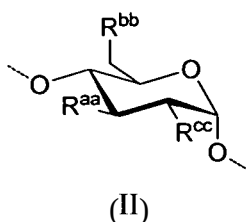
- The reaction time for the substitution step is generally in the range of from 1 hour to
30 7 days, preferably of from 3 to 48 hours, more preferably of from 4 to 18 hours.

The derivative obtained may be subjected to at least one further isolation and/or purification step, as described above.

- 35 Preferably, the derivative is subjected to at least one further step. In particular, in case the hydroxyl group present in the hydroxyalkyl starch is reacted with thioacetate, thereby replacing the hydroxyl group with the structure $-S-C(=O)-CH_3$, the derivative is preferably saponified in a subsequent step to give the functional group Z^1 with Z^1 being an $-SH$ group.

Thus, the present invention also relates to a method as described above as well as to a conjugate obtained or obtainable by said method, wherein in step (a2)(ii), the hydroxyl group present in the hydroxyalkyl starch is reacted with thioacetate giving a functional group having the structure $-S-C(=O)-CH_3$, wherein the method further comprises saponification of the group $-S-C(=O)-CH_3$ to give the functional group Z^1 .

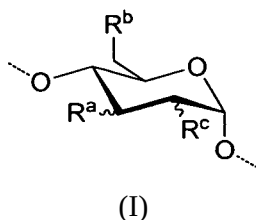
It has to be understood, that in case at least one hydroxyl group present in hydroxyalkyl starch, comprising the structural unit according to the following formula (II)



with R^{33} , R^{bb} and R^c being independently of each other selected from the group consisting of $-[O-(CR^{wR^x})-(CR^{yR^z})]_x-OH$ and $-O-HAS''$, is displaced in a substitution reaction, the stereochemistry of the carbon atom which bears the respective hydroxyl function, which is displaced may be inverted.

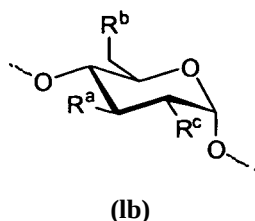
Thus, in case at least one of R^{33} and R^{bb} in the above shown structural unit is $-OH$, and in case, this at least one group is displaced by a precursor of the functional group Z^1 , thereby yielding a hydroxyalkyl starch derivative comprising the functional group Z^1 in this structural unit, the stereochemistry of the carbon atoms bearing this functional group Z^1 may be inverted.

Since, it cannot be excluded that such a substitution of secondary hydroxyl groups occur, in the method of the invention according to step (a2)(ii), the stereochemistry of the carbon atoms bearing the functional group R^a and R^c is not further defined, as shown in the structural unit according to the following formula (I)



However, without wanting to be bound to any theory, it is believed that mainly primary hydroxyl groups will be displaced in the substitution reaction according to step (a2)(ii). Thus, according to this theory, the stereochemistry of most carbon atoms bearing the

residues R^a or R^c will not be inverted but the respective structural unit of the hydroxyalkyl starch will comprise the stereochemistry as shown in the formula (Ib)



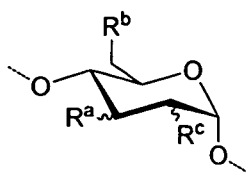
The thioacetate is preferably saponified in at least one further step to give the thiol comprising hydroxyalkyl starch derivatives. As regards the saponification of the functional group $-S-C(=O)-CH_3$, all methods known to those skilled in the art are encompassed by the present invention. This includes the use of at least one base (such as metal hydroxides) and strong nucleophiles (such as ammonia, amines, thiols or hydroxides) in order to saponify the present thioesters to give thiols. Preferred reagents are sodium hydroxide and ammonia.

Since thiols are well known to oxidize via the formation of disulfides, especially under basic conditions present in most saponification protocols, the molecular weight of the hydroxyalkyl starch derivative obtained may vary due to unspecific crosslinking. To prevent the formation of disulfides, preferably a reducing agent is added prior, during or after the saponification step. According to a preferred embodiment of the invention, a reducing agent is directly added to the saponification mixture in order to keep the forming thiol groups in their low oxidation state. Regarding the reduction of the thiol groups, all reduction methods known to those skilled in the art such as borohydrides, especially sodium borohydride, and thiols, especially dithiothreitol (DTT) and dithioerythritol (DTE) or phosphines such as tris-(2-carboxyethyl)phosphine (TCEP) are encompassed by the present invention. According to preferred embodiments of the present invention, dithiothreitol (DTT), dithioerythritol (DTE) or sodium borohydride are employed.

In an alternative embodiment of the reaction, aqueous sodium hydroxide is used as saponification agent together with sodium borohydride as reducing agent.

Optionally, mercaptoethanol can be used as an additive in this reaction.

Thus, the present invention also relates to a method, as described above, wherein in step (a2)(ii) the activated hydroxyl group present in the hydroxyalkyl starch is reacted with thioacetate giving a functional group having the structure $-S-C(=O)-CH_3$, wherein the method further comprises saponifying the group $-S-C(=O)-CH_3$ to give the functional group Z^1 , wherein the hydroxyalkyl starch derivative comprises at least one structural unit according to the following formula (I)



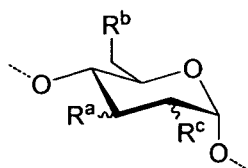
(I)

wherein R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS", $-[0-\text{CH}_2-\text{CH}_2]_s-\text{OH}$, $-[0-\text{CH}_2-\text{CH}_2]_t-\text{SH}$ and wherein at least one R^a , R^b and R^c is $-[0-\text{CH}_2-\text{CH}_2]_t-\text{SH}$ and wherein t is in the range of from 0 to 4, and wherein s is in the range of from 0 to 4.

Again, the hydroxyalkyl starch derivative, comprising the functional group SH, obtained by the above-described preferred embodiment, may be isolated/and or purified prior to step (b) in a further step. Again, the purification/isolation of the HAS derivative from step (a2)(ii) can be carried out by any suitable method such as ultrafiltration, dialysis or precipitation or a combined method using for example precipitation and afterwards ultrafiltration.

Furthermore, the hydroxyalkyl starch derivative may be lyophilized, as described above, using conventional methods.

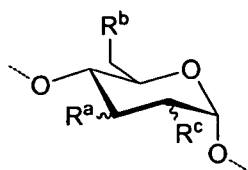
According to an especially preferred embodiment, the hydroxyalkyl starch derivative, obtained in step (a2)(ii), comprises at least one structural unit according to the following formula (1)



(I)

wherein R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS", $-[0-\text{CH}_2-\text{CH}_2]_s-\text{OH}$, $-[0-\text{CH}_2-\text{CH}_2]_t-\text{Z}^1$, wherein t is in the range of from 0 to 4, and wherein s is in the range of from 0 to 4, and wherein at least one of R^a , R^b and R^c is $-[0-\text{CH}_2-\text{CH}_2]_t-\text{Z}^1$, with Z^1 being -SH. This derivative is preferably reacted in step (b) with a crosslinking compound L having a structure according to the following formula $\text{K}^2-[\text{L}^2]_g-[\text{E}]_e-[\text{CR}^m\text{R}^n]_f-\text{K}^1$ with g and e being 0, and wherein K^2 is a halogene.

According to an especially preferred embodiment the hydroxyalkyl starch derivative obtained in step (a2)(ii) comprises at least one structural unit according to the following formula (I)



(I)

wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS^n$, $-[O-CH_2-CH_2]_s-OH$, and $-[O-CH_2-CH_2]_t-Z^1$, wherein t is in the range of from 0 to 4, and wherein s is in the range of from 0 to 4, and wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-Z^1$, with Z^1 being $-SH$. This derivative is preferably reacted in step (b) with a crosslinking compound L having a structure according to the formula $K^2-[L^2]_g-[E]_e-[CR^mR^n]_f-K^1$, wherein K^2 is maleimide, and wherein upon reaction of Z^1 with K^2 , a functional group $-X-F^2-$ is formed.

Step (b)

As already described above, the hydroxyalkyl starch derivative obtained according to step (a) is, optionally after at least one purification and/or isolation step, further reacted in step (b).

In step (b) the HAS derivative is coupled via the functional group Z^1 to at least one cytotoxic agent via the at least bifunctional crosslinking compound L , wherein L comprises the functional groups K^1 and K^2 , wherein L is coupled to Z^1 via a functional group K^2 comprised in L , and wherein each cytotoxic agent is coupled via the primary hydroxyl group to the HAS derivative via the functional group K^1 , comprised in L .

Thus, step (b) preferably comprises the steps (b1) and (b2)

(b1) coupling the cytotoxic agent to the crosslinking compound L , thereby forming a derivative of the cytotoxic agent having the structure $-L-M$, wherein M is the residue of the cytotoxic agent;

(b2) coupling the derivative of the cytotoxic agent having the structure $-L-M$ to the hydroxyalkyl starch derivative according to step (a), thereby forming the hydroxyalkyl starch conjugate.

As to the preferred reaction conditions used in step (b1), reference is made to the details given above.

As regards to the reaction conditions used in step (b2), in principle any reaction conditions
5 known to those skilled in the art can be used. Preferably, the reaction is carried out in an aqueous reaction medium, preferably in a mixture comprising water and at least one organic solvent, preferably at least one water miscible solvent, in particular a solvent selected from the group such as N-methyl pyrrolidone, dimethyl acetamide (DMA), dimethyl formamide (DMF), formamide, dimethyl sulfoxide (DMSO), acetonitrile,
10 tetrahydrofuran (THF), dioxane, alcohols such as methanol, ethanol, isopropanol and mixtures of two or more thereof. More preferably, the reaction is carried out in DMF.

The temperature of the reaction is preferably in the range of from 5 to 55 °C, more preferably of from 10 to 30 °C, and especially preferably of from 15 to 25 °C. During the
15 course of the reaction, the temperature may be varied, preferably in the above given ranges, or held essentially constant.

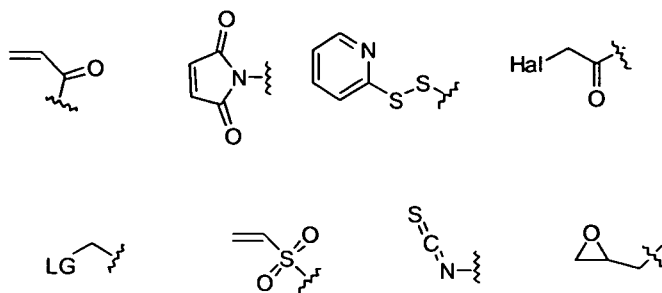
The reaction time for the reaction of step (b2) may be adapted to the specific needs and is generally in the range of from 30 min to 2 days, preferably of from 1 hour to 18 hours,
20 more preferably of from 2 hours to 6 hours.

The pH value for the reaction of step (b) may be adapted to the specific needs of the reactants. Preferably, the reaction is carried out in a buffered solution, at a pH value in the range of from 3 to 10, more preferably of from 5 to 9, and even more preferably of from 6
25 to 8. Among the preferred buffers, citrate buffers (pH 6.4), phosphate buffers (pH 7.5) and bicarbonate buffers (pH 8) may be mentioned.

As described above, the hydroxyalkyl starch may comprise more than one functional group Z^1 , such as multiple thiol groups. Preferably, all groups Z^1 present in the hydroxyalkyl
30 starch derivative participate in the coupling reaction in step (b2). However, it is also possible that in step (b2) not all of the functional groups Z^1 are coupled to the at least bifunctional crosslinking compound L, or preferably with the derivative of the cytotoxic agent having the structure -L-M. Thus, in this case, the hydroxyalkyl starch conjugate according to step (b2) may comprise at least one unreacted functional group Z^1 .

35 To avoid side effects due to the presence of such unreacted functional groups Z^1 , the hydroxyalkyl starch conjugate may be further reacted, as described above, in a subsequent step to step (c) with a suitable capping reagent D^* . In case Z^1 is a thiol group, possible free thiol groups present in the conjugate, which may lead to unwanted side effects such as

- oxidative disulfide formation and consequently crosslinking, may be reacted, for example, with small molecules comprising a thiol reactive group. Examples of thiol reactive groups are given above. Preferred capping reagents D* thus in particular comprise a group selected from the group consisting of pyridyl disulfides, maleimide group, haloacetyl groups, haloacetamides, vinyl sulfones and vinyl pyridines. Preferably, the capping reagent D* comprises a thiol reactive group selected from the group consisting of the following structures:



- wherein Hal is a halogen, such as Cl, Br, or I, and LG is a leaving group (or nucleofuge).

In particular D* is iodoacetic acid and/or ethylbromoacetate.

- Optionally, a reducing agent such as tris-(2-carboxyethyl)phosphine (TCEP) may be added prior to the capping step in order to break existing disulfides and to keep thiols in their low oxidation state.

- Thus, the present invention also describes a method, as described above, the method further comprises
(c) reacting the hydroxyalkyl starch conjugate with a capping reagent D*.

- Likewise, in case the crosslinking compound L is either reacted with the hydroxyalkyl starch derivative prior to the coupling to the cytotoxic agent, or only in a subsequent step with the cytotoxic agent, the hydroxyalkyl starch conjugate may comprise at least one unreacted functional group Z¹ and/or at least one unreacted group K¹.

- In this case, the present invention may comprise a further capping step
(cl) reacting the hydroxyalkyl starch conjugate with a further capping reagent D**, wherein D** may be the same or may differ from D*, depending on the nature of functional group to be capped.

Most preferably the hydroxyalkyl starch conjugate according to step (b) comprises no unreacted functional groups Z¹ and/or no unreacted group K¹.

5 Preferably, the hydroxyalkyl starch conjugate obtained according to step (b), optionally according to step (c) and/or (cl), is subjected to at least one isolation and/or purification step. Isolation of the conjugate may be carried out by a suitable process which may comprise one or more steps.

10 According to a preferred embodiment of the present invention, the conjugate is first separated from the reaction mixture by a suitable method such as precipitation and subsequent centrifugation or filtration. In a second step, the separated conjugate may be subjected to a further treatment such as an after-treatment like ultrafiltration, dialysis, centrifugal filtration or pressure filtration, ion exchange chromatography, reversed phase chromatography, HPLC, MPLC, gel filtration and/or lyophilization. According to an even
15 more preferred embodiment, the separated polymer derivative is first precipitated, subjected to centrifugation, re-dissolved and finally subjected to ultrafiltration.

20 Preferably, the precipitation is carried out with an organic solvent such as ethanol or isopropanol. The precipitated conjugate is subsequently subjected to centrifugation and subsequent ultrafiltration using water or an aqueous buffer solution having a concentration preferably from 1 to 1000 mmol/l, more preferably from 1 to 100 mmol/l, and more preferably from 10 to 50 mmol/l such as about 20 mmol/l, a pH value in the range of preferably from 3 to 10, more preferably of from 4 to 8, such as about 5. The number of exchange cycles preferably is in the range of from 5 to 50, more preferably of from 10 to
25 30, and even more preferably of from 15 to 25, such as about 20.

Most preferably, the obtained conjugate is further lyophilized until the solvent content of the reaction product is sufficiently low according to the desired specifications of the product.

30

Pharmaceutical Composition

Furthermore, the present invention relates to a pharmaceutical composition comprising in a therapeutically effective amount a HAS conjugate, as described above, or a HAS conjugate
35 obtained or obtainable by the above described method.

As far as the pharmaceutical compositions according to the present invention comprising the hydroxyalkyl starch conjugate, as described above, are concerned, the hydroxyalkyl starch conjugate may be used in combination with a pharmaceutical excipient. Generally,

the hydroxyalkyl starch conjugate will be in a solid form which can be combined with a suitable pharmaceutical excipient that can be in either solid or liquid form. As excipients, carbohydrates, inorganic salts, antimicrobial agents, antioxidants, surfactants, buffers, acids, bases, and combinations thereof may be mentioned. A carbohydrate such as a sugar, a derivatized sugar such as an alditol, aldonic acid, an esterified sugar, and/or a sugar polymer may be present as an excipient. Specific carbohydrate excipients include, for example: monosaccharides, such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol, sorbitol (glucitol), pyranosyl sorbitol, myoinositol, and the like. The excipient may also include an inorganic salt or buffer such as citric acid, sodium chloride, potassium chloride, sodium sulfate, potassium nitrate, sodium phosphate monobasic, sodium phosphate .dibasic, and combinations thereof. The pharmaceutical composition according to the present invention may also comprise an antimicrobial agent for preventing or determining microbial growth, such as, e.g., benzalkonium chloride, benzethonium chloride, benzyl alcohol, cetylpyridinium chloride, chlorobutanol, phenol, phenylethyl alcohol, phenylmercuric nitrate, thimersol, and combinations thereof.

The pharmaceutical composition according to the present invention may also comprise an antioxidant, such as, e.g., ascorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, hypophosphorous acid, monothioglycerol, propyl gallate, sodium bisulfite, sodium formaldehyde sulfoxylate, sodium metabisulfite, and combinations thereof.

The pharmaceutical composition according to the present invention may also comprise a surfactant, such as, e.g., polysorbates, or pluronics sorbitan esters; lipids, such as phospholipids and lecithin and other phosphatidylcholines, phosphatidylethanolamines, acids and fatty esters; steroids, such as cholesterol; and chelating agents, such as EDTA or zinc.

The pharmaceutical composition according to the present invention may also comprise acids or bases such as, e.g., hydrochloric acid, acetic acid, phosphoric acid, citric acid, malic acid, lactic acid, formic acid, trichloroacetic acid, nitric acid, perchloric acid, phosphoric acid, sulfuric acid, fumaric acid, and combinations thereof, and/or sodium hydroxide, sodium acetate, ammonium hydroxide, potassium hydroxide, ammonium acetate, potassium acetate, sodium phosphate, potassium phosphate, sodium citrate, sodium formate, sodium sulfate, potassium sulfate, potassium fumarate, and combinations thereof.

Generally, the excipient will be present in a pharmaceutical composition according to the present invention in an amount of 0.001 to 99.999 wt.-%, preferably from 0.01 to 99.99 wt.-%, more preferably from 0.1 to 99.9 wt.-%, in each case based on the total weight of the pharmaceutical composition.

5

The present invention also relates to a method of treating cancer, comprising administering to a patient suffering from cancer a therapeutically effective amount of the hydroxyalkyl starch conjugate as defined herein, or the hydroxyalkyl starch conjugate obtained or obtainable by the method according to the present invention, or the pharmaceutical composition according to the present invention.

10

The term "patient", as used herein, relates to animals and, preferably, to mammals. More preferably, the patient is a rodent such as a mouse or a rat. Even more preferably, the patient is a primate. Most preferably, the patient is a human. It is, however, envisaged by the method of the present invention that the patient shall suffer from cancer.

15

The term "cancer", as used herein, preferably refers to a proliferative disorder or disease caused or characterized by the proliferation of cells which have lost susceptibility to normal growth control. Preferably, the term encompasses tumors and any other proliferative disorders. Thus, the term is meant to include all pathological conditions involving malignant cells, irrespective of stage or of invasiveness. The term, preferably, includes solid tumors arising in solid tissues or organs as well as hematopoietic tumors (e.g. leukemias and lymphomas).

20

The cancer may be localized to a specific tissue or organ (e.g. in the breast, the prostate or the lung), and, thus, may not have spread beyond the tissue of origin. Furthermore the cancer may be invasive, and, thus may have spread beyond the layer of tissue in which it originated into the normal surrounding tissues (frequently also referred to as locally advanced cancer). Invasive cancers may or may not be metastatic. Thus, the cancer may be also metastatic. A cancer is metastatic, if it has spread from its original location to distant parts of the body. E.g., it is well known in the art that breast cancer cells may spread to another organ or body part, such as the lymph nodes.

25

30

Preferred cancers are biliary cancer, bladder cancer, breast cancer, cervical cancer, colorectal cancer, gastrointestinal cancer, head and neck cancer, leukaemia, lymphoma, malignant melanoma, mesothelioma, non-small cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, sarcoma and small cell lung cancer.

35

Moreover, it is also envisaged that the cancer is selected from cancer is selected from the group consisting of Acute Lymphoblastic Leukemia (adult), Acute Lymphoblastic Leukemia (childhood), Acute Myeloid Leukemia (adult), Acute Myeloid Leukemia (childhood), Adrenocortical Carcinoma, Adrenocortical Carcinoma (childhood), AIDS-Related Cancers, AIDS-Related Lymphoma, Anal Cancer, Appendix Cancer, Astrocytomas (childhood), Atypical Teratoid/Rhabdoid Tumor (childhood), Central Nervous System Cancer, Basal Cell Carcinoma, Bile Duct Cancer (Extrahepatic), Bladder Cancer, Bladder Cancer (childhood), Bone Cancer, Osteosarcoma and Malignant Fibrous Histiocytoma, Brain Stem Glioma (childhood), Brain Tumor (adult), Brain Tumor (childhood), Brain Stem Glioma (childhood), Central Nervous System Brain Tumor, Atypical Teratoid/Rhabdoid Tumor (childhood), Brain Tumor, Central Nervous System Embryonal Tumors (childhood), Astrocytomas (childhood) Brain Tumor, Craniopharyngioma Brain Tumor (childhood), Ependymoblastoma Brain Tumor (childhood), Ependymoma Brain Tumor (childhood), Medulloblastoma Brain Tumor (childhood), Medulloepitheliom Brain Tumor (childhood), Pineal Parenchymal Tumors of Intermediate Differentiation Brain Tumor (childhood), Supratentorial Primitive Neuroectodermal Tumors and Pineoblastoma Brain Tumor, (childhood), Brain and Spinal Cord Tumors (childhood), Breast Cancer , Breast Cancer (childhood), Breast Cancer (Male), Bronchial Tumors (childhood), Burkitt Lymphoma, Carcinoid Tumor (childhood), Carcinoid Tumor, Gastrointestinal, Carcinoma of Unknown Primary, Central Nervous System Atypical Teratoid/Rhabdoid Tumor (childhood), Central Nervous System Embryonal Tumors (childhood), Central Nervous System (CNS) Lymphoma, Primary Cervical Cancer, Cervical Cancer (childhood), Childhood Cancers, Chordoma (childhood), Chronic Lymphocytic Leukemia, Chronic Myelogenous Leukemia, Chronic Myeloproliferative Disorders, Colon Cancer, Colorectal Cancer (childhood), Craniopharyngioma (childhood), Cutaneous T-Cell Lymphoma, Embryonal Tumors, Central Nervous System (childhood), Endometrial Cancer, Ependymoblastoma (childhood), Ependymoma (childhood), Esophageal Cancer, Esophageal Cancer (childhood), Esthesioneuroblastoma (childhood), Ewing Sarcoma Family of Tumors, Extracranial Germ Cell Tumor (childhood), Extragonadal Germ Cell Tumor, Extrahepatic Bile Duct Cancer, Eye Cancer, Intraocular Melanoma, Eye Cancer, Retinoblastoma, Gallbladder Cancer, Gastric (Stomach) Cancer, Gastric (Stomach) Cancer (childhood), Gastrointestinal Carcinoid Tumor, Gastrointestinal Stromal Tumor (GIST), Gastrointestinal Stromal Cell Tumor (childhood), Germ Cell Tumor, Extracranial (childhood), Germ Cell Tumor, Extragonadal, Germ Cell Tumor, Ovarian, Gestational Trophoblastic Tumor, Glioma (adult), Glioma (childhood) Brain Stem, Hairy Cell Leukemia, Head and Neck Cancer, Heart Cancer (childhood), Hepatocellular (Liver) Cancer (adult) (Primary), Hepatocellular (Liver) Cancer (childhood) (Primary), Histiocytosis, Langerhans Cell, Hodgkin Lymphoma (adult), Hodgkin Lymphoma (childhood), Hypopharyngeal Cancer, Intraocular

Melanoma, Islet Cell Tumors (Endocrine Pancreas), Kaposi Sarcoma, Kidney (Renal Cell) Cancer, Kidney Cancer (childhood), Langerhans Cell Histiocytosis, Laryngeal Cancer, Laryngeal Cancer (childhood), Leukemia, Acute Lymphoblastic (adult), Leukemia, Acute Lymphoblastic (childhood), Leukemia, Acute Myeloid (adult), Leukemia, Acute Myeloid (childhood), Leukemia, Chronic Lymphocytic, Leukemia, Chronic Myelogenous, Leukemia, Hairy Cell, Lip and Oral Cavity Cancer, Liver Cancer (adult) (Primary), Liver Cancer (childhood) (Primary), Non-Small Cell Lung Cancer, Small Cell Lung Cancer, Non-Hodgkin Lymphoma, (adult), Non-Hodgkin Lymphoma, (childhood), Primary Central Nervous System (CNS) Lymphoma, Waldenstrom, Macroglobulinemia, Malignant Fibrous Histiocytoma of Bone and Osteosarcoma, Medulloblastoma (childhood), Medulloepithelioma (childhood), Melanoma, Intraocular (Eye) Melanoma, Merkel Cell Carcinoma, Mesothelioma (adult) Malignant, Mesothelioma (childhood), Metastatic Squamous Neck Cancer with Occult Primary, Mouth Cancer, Multiple Endocrine Neoplasia Syndromes (childhood), Multiple Myeloma/Plasma Cell Neoplasm, Mycosis Fungoides, Myelodysplastic Syndromes, Myelodysplastic/Myeloproliferative Neoplasms, Myelogenous Leukemia, Chronic, Myeloid Leukemia (adult) Acute, Myeloid Leukemia (childhood) Acute, Myeloma, Multiple, Nasal Cavity and Paranasal Sinus Cancer, Nasopharyngeal Cancer, Nasopharyngeal Cancer (childhood), Neuroblastoma, Oral Cancer (childhood), Lip and Oral Cavity Cancer, Oropharyngeal Cancer, Osteosarcoma and Malignant Fibrous, Histiocytoma of Bone, Ovarian Cancer (childhood), Ovarian Epithelial Cancer, Ovarian Germ Cell Tumor, Ovarian Low Malignant Potential Tumor, Pancreatic Cancer, Pancreatic Cancer (childhood), Pancreatic Cancer, Islet Cell Tumors, Papillomatosis (childhood), Paranasal Sinus and Nasal Cavity Cancer, Parathyroid Cancer, Penile Cancer, Pharyngeal Cancer, Pineal Parenchymal Tumors of Intermediate Differentiation (childhood), Pineoblastoma and Supratentorial Primitive Neuroectodermal Tumors (childhood), Pituitary Tumor, Plasma Cell Neoplasm/Multiple Myeloma, Pleuropulmonary Blastoma, Pregnancy and Breast Cancer, Primary Central Nervous System (CNS) Lymphoma, Prostate Cancer, Rectal Cancer, Renal Cell (Kidney) Cancer, Renal Pelvis and Ureter Transitional Cell Cancer, Respiratory Tract Cancer with Chromosome 15 Changes, Retinoblastoma, Rhabdomyosarcoma (childhood), Salivary Gland Cancer, Salivary Gland Cancer (childhood), Sarcoma, Ewing Sarcoma Family of Tumors, Kaposi Sarcoma, Soft Tissue (adult) Sarcoma, Soft Tissue (childhood) Sarcoma, Uterine Sarcoma, Sezary Syndrome, Skin Cancer (Nonmelanoma), Skin Cancer (childhood), Skin Cancer (Melanoma), Merkel Cell Skin Carcinoma, Small Cell Lung Cancer, Small Intestine Cancer, Soft Tissue Sarcoma (adult), Soft Tissue Sarcoma (childhood), Squamous Cell Carcinoma, see Skin Cancer (Nonmelanoma), Stomach (Gastric) Cancer, Stomach (Gastric) Cancer (childhood), Supratentorial Primitive Neuroectodermal Tumors (childhood), Cutaneous T-Cell Lymphoma, Testicular Cancer, Testicular Cancer (childhood), Throat Cancer, Thymoma and Thymic Carcinoma,

Thymoma and Thymic Carcinoma (childhood), Thyroid Cancer, Thyroid Cancer (childhood), Transitional Cell Cancer of the Renal Pelvis and Ureter, T Gestational rophoblastic Tumor, Unknown Primary Site, Carcinoma of adult, Unknown Primary Site, Cancer of (childhood), Unusual Cancers of childhood, Ureter and Renal Pelvis, 5 Transitional Cell Cancer, Urethral Cancer, Uterine Cancer, Endometrial, Uterine Sarcoma, Vaginal Cancer, Vaginal Cancer (childhood), Vulvar Cancer, Waldenstrom Macroglobulinemia and Wilms Tumor.

10 The terms "treating cancer" and "treatment of cancer", preferably, refer to therapeutic measures, wherein the object is to prevent or to slow down (lessen) an undesired physiological change or disorder, such as the growth, development or spread of a hyperproliferative condition, such as cancer. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, 15 diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. It is to be understood that a treatment can also mean prolonging survival as compared to expected survival if not receiving treatment.

20 The term "administering" as used herein, preferably, refers to the introduction of the hydroxyalkyl starch conjugate as defined herein, the hydroxyalkyl starch conjugate obtained or obtainable by the method according to the present invention, or the pharmaceutical composition according to the present invention into cancer patients. Methods for administering a particular compound are well known in the art and include 25 parenteral, intravascular, paracancer, transmucosal, transdermal, intramuscular (i.m.), intravenous (i.v.), intradermal, subcutaneous (s.c), sublingual, intraperitoneal (i.p.), intraventricular, intracranial, intravaginal, intratumoral, and oral administration. It is to be understood that the route of administration may depend on the cancer to be treated. Preferably, the hydroxyalkyl starch conjugate as defined herein, the hydroxyalkyl starch 30 conjugate obtained or obtainable by the method according to the present invention, or the pharmaceutical composition according to the present invention are administered parenterally. More preferably, it is administered intravenously. Preferably, the administration of a single dose of a therapeutically effective amount of the aforementioned compounds is over a period of 5 min to 5 h.

35 Preferably, the conjugates are administered together with a suitable carrier, and/or a suitable diluent, such as preferably a sterile solution for i.v., i.m., i.p. or s.c. application.

The term "therapeutically effective amount", as used herein, preferably refers to an amount of the hydroxyalkyl starch conjugate as defined herein, the hydroxyalkyl starch conjugate obtained or obtainable by the method according to the present invention, or the pharmaceutical composition according to the present invention that (a) treats the cancer, (b) attenuates, ameliorates, or eliminates the cancer. More preferably, the term refers to the amount of the cytotoxic agent present in the hydroxyalkyl starch conjugate as defined herein, the hydroxyalkyl starch conjugate obtained or obtainable by the method according to the present invention, or the pharmaceutical composition according to the present invention that (a) treats the cancer, (b) attenuates, ameliorates, or eliminates the cancer.

How to calculate the amount of a cytotoxic agent present in the aforementioned conjugates or pharmaceutical composition is described elsewhere herein. It is particularly envisaged that the therapeutically effective amount of the aforementioned compounds shall reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, at least to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. Whether a particular amount of the aforementioned compounds exerts these effects (and, thus is pharmaceutically effective) can be determined by well known measures. Particularly, it can be determined by assessing cancer therapy efficacy. Cancer therapy efficacy, e. g., can be assessed by determining the time to disease progression and/or by determining the response rate. Thus, the required dosage will depend on the severity of the condition being treated, the patient's individual response, the method of administration used, and the like. The skilled person is able to establish a correct dosage based on his general knowledge.

Advantageously, it has been shown in the studies carried out in the context of the present invention that

- i) the cytotoxic agent is less toxic when present in the conjugates described herein as compared to an agent not being present in a conjugate and/or
- ii) that the use of said conjugate, or of the pharmaceutical composition comprising said conjugate allows for a more efficient treatment of cancer in a subject (see Example 2).

Moreover, the present invention relates to the hydroxyalkyl starch conjugate as defined above, or the hydroxyalkyl starch conjugate obtained or obtainable by the method according to the present invention, or the pharmaceutical composition according to the present invention for use as a medicament.

Moreover, the present invention relates to the hydroxyalkyl starch conjugate as defined above, or the hydroxyalkyl starch conjugate obtained or obtainable by the method

according to the present invention, or the pharmaceutical composition according to the present invention for the treatment of cancer.

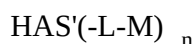
Also envisaged by the present invention is the hydroxyalkyl starch conjugate as defined above, or the hydroxyalkyl starch conjugate obtained or obtainable by the method according to the present invention, or the pharmaceutical composition according to the present invention for the treatment of cancer selected from the group consisting of biliary cancer, bladder cancer, breast cancer, cervical cancer, colorectal cancer, gastrointestinal cancer, head and neck cancer, leukaemia, lymphoma, malignant melanoma, mesothelioma, non-small cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, sarcoma and small cell lung cancer.

Finally, the present invention pertains to the use of the hydroxyalkyl starch conjugate as defined above, or the hydroxyalkyl starch conjugate obtained or obtainable by the method according to the present invention, or the pharmaceutical composition according to the present invention for the manufacture of a medicament for the treatment of cancer. Preferably, the cancer is selected from the group consisting of biliary cancer, bladder cancer, breast cancer, cervical cancer, colorectal cancer, gastrointestinal cancer, head and neck cancer, leukaemia, lymphoma, malignant melanoma, mesothelioma, non-small cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, sarcoma and small cell lung cancer.

How to administer the conjugates, compositions or medicaments has been explained elsewhere herein.

The following especially preferred embodiments are described:

1. A hydroxyalkyl starch (HAS) conjugate comprising a hydroxyalkyl starch derivative and a cytotoxic agent, said conjugate having a structure according the following formula



wherein

M is a residue of a cytotoxic agent, wherein the cytotoxic agent comprises a primary hydroxyl group,

L is a linking moiety,

HAS' is a residue of the hydroxyalkyl starch derivative,

n is greater than or equal to 1,

wherein the hydroxyalkyl starch derivative has a mean molecular weight MW above the renal threshold, preferably an MW greater than or equal to 60 kDa,

and a molar substitution MS in the range of from 0.6 to 1.5, .
and wherein the linking moiety L is linked to a primary hydroxyl group of the cytotoxic agent.

5

2. The conjugate according to embodiment 1, wherein the hydroxyalkyl starch conjugate is a hydroxyethyl starch (HES) conjugate comprising a hydroxyethyl starch derivative.

10

3. The conjugate according to embodiment 1 or 2, wherein the hydroxyalkyl starch derivative has a mean molecular weight MW in the range of from 80 to 1200 kDa, preferably in the range of from 90 to 800 kDa.

15

4. The conjugate according to any of embodiments 1 to 3, wherein the hydroxyalkyl starch derivative has a molar substitution MS in the range of from 0.70 to 1.45, preferably in the range of from 0.80 to 1.40, more preferably in the range of from 0.90 to 1.35.

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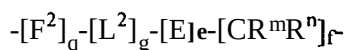
5. The conjugate according to any of embodiments 1 to 4, wherein the linking moiety L has a structure $-L'-F^3-$, wherein F^3 is a functional group linking L' to M via the group $-O-$ derived from the primary hydroxyl group of the cytotoxic agent, thereby forming a group $-F^3-O-$, preferably wherein F^3 is a $-C(=Y)-$ group, with Y being O, NH or S, preferably with Y being O or S, and wherein L' is a linking moiety.

25

6. The conjugate according to embodiment 5, wherein the bond between the functional group $-F^3-$ and the functional group $-O-$ of the residue of the cytotoxic agent M is a cleavable linkage, which is capable of being cleaved *in vivo* so as to release the cytotoxic agent, wherein the functional group $-O-$ is derived from the primary hydroxyl group of the cytotoxic agent.

30

7. The conjugate according to embodiment 5 or 6, wherein L' has a structure according to the following formula

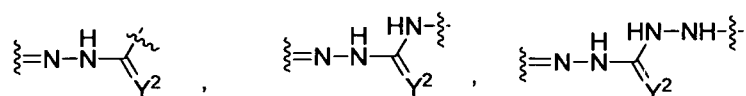
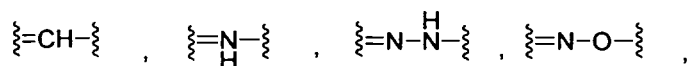


wherein E is an electron-withdrawing group, preferably selected from the group consisting of -O-, -S-, -SO-, -SO₂-, -NR^e-, succinimide, -C(=Y^e)-, -NR^e-C(=Y^e)-, -C(=Y^e)-NR^e-, -NO₂ comprising groups, -CN comprising groups, aryl moieties or an at least partially fluorinated alkyl moiety, wherein Y^e is either O, S or NR^e, and R^e is

5 hydrogen or alkyl,

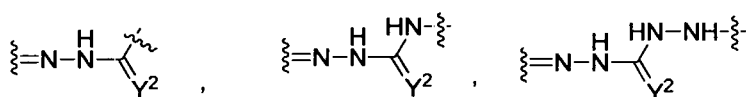
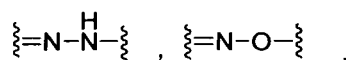
preferably wherein E is selected from the group consisting of -NH-C(=O)-, -C(=O)-NH-, -NH-, -O-, -S-, -SO-, -SO₂- and -succinimide-,

F² is a group consisting of -Y¹-, -C(=Y²)-, -C(=Y²)-NR^{F2}-,



10 and -CH₂-CH₂-C(=Y²)-NR^{F2}-,

more preferably, F² is a group consisting of -Y¹-, -C(=Y²)-, -C(=Y²)-NR^{F2}-,



15 and -CH₂-CH₂-C(=Y²)-NR^{F2}-,

wherein Y¹ is selected from the group consisting of -S-, -O-, -NH-, -NH-NH-, -CH₂-CH₂-SO₂-NR^{F2}-, -CH₂-CHOH-, and cyclic imides, such as succinimide, and wherein Y² is selected from the group consisting of NH, S and O, and wherein R^{F2} is

20 selected from the group consisting of

hydrogen, alkyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl group

L² is a linking moiety, preferably an alkyl, alkenyl, alkylaryl, arylalkyl, aryl,

25 heteroaryl, alkylheteroaryl or heteroarylalkyl groupf is in the range of from 1 to 20,

g is 0 or 1,

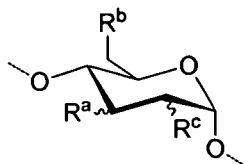
q is 0 or 1,

e is 0 or 1,

and wherein R^m and Rⁿ are, independently of each other, H, alkyl, aryl or a side chain of a natural or unnatural amino acid, preferably H or alkyl, more preferably H or methyl.

30

8. The conjugate according to any of embodiments 1 to 7, wherein the hydroxyalkyl starch derivative comprises at least one structural unit according to the following formula (I)



(I)

wherein R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS", $-[0-(CR^wR^xMCR^yR^z)]_x-OH$, $-[0-(CR^wR^x)-(CR^yR^z)]_y-X$, $-[0-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^1-X$,

wherein R^w , R^x , R^y and R^z are independently of each other selected from the group consisting of hydrogen and alkyl,

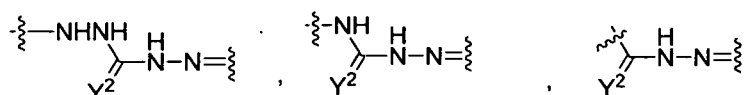
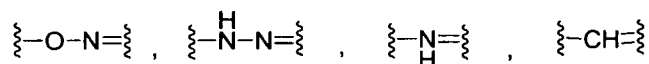
y is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4, and

x is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4,

and wherein at least one of R^a , R^b and R^c is $[0-(CR^wR^x)-(CR^yR^z)]_y-X$ or

$-[0-(CR^wR^xHCR^yR^z)]_y-[F^1]_p-L^1-X$,

wherein X is selected from the group consisting of $-Y^{xx}$, $-C(=Y^x)-$, $-C(=Y^x)-NR^{xx}-$,



and $-CH_2-CH_2-C(=Y^x)-NR^{xx}-$,

wherein Y^{TM} is selected from the group consisting of -S-, -O-, -NH-, -NH-NH-, $-CH_2-CH_2-SO_2-NR^{xx}-$, and cyclic imids, and wherein Y^x is selected from the group consisting of NH, S and O, and wherein R^a is selected from the group consisting of hydrogen, alkyl, alkenyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl group,

X preferably being -S—,

F^1 is a functional group, preferably selected from the group consisting of $-Y^7$, $-Y^7-C(=Y^6)-$, $-C(=Y^6)-$, $-Y^7-C(=Y^6)-Y^8-$,

with Y^7 and Y^8 being, independently of each other, selected from the group consisting of -NH-, -O- and -S-, and wherein Y^6 is O, NH or S,

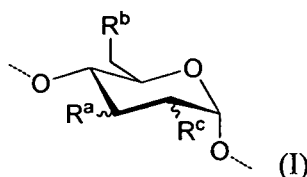
and wherein p is 0 or 1,

L^1 is a linking moiety, preferably an, alkyl, alkenyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl group,

and wherein HAS'' is a remainder of HAS.

5

9. The conjugate according to any of embodiments 1 to 8, wherein the hydroxyalkyl starch derivative comprises at least one structural unit according to the following formula (I)



- 10 wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS''$, $-[O-CH_2-CH_2]_s-OH$, $-[O-CH_2-CH_2]_t-X-$ and $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X-$, and wherein

and wherein s is in the range of from 0 to 4,

and wherein t is in the range of from 0 to 4,

- 15 p is 0 or 1,

wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-X-$ or $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X-$,

and wherein HAS'' is a remainder of HAS.

- 20 10. The conjugate according to embodiment 8 or 9, wherein the linking moiety L is covalently linked to X .

11. The conjugate according to embodiment 9 or 10, wherein at least one of R^a , R^b and R^c is

25

(i) $-[O-CH_2-CH_2]_t-X-$, or

(ii) $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X-$, and wherein p is 1 and $-F^1-$ is $-O-$, or

(iii) $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X-$, and wherein p is 1 and $-F^1-$ is $-O-C(=O)-NH-$.

and wherein X is S,

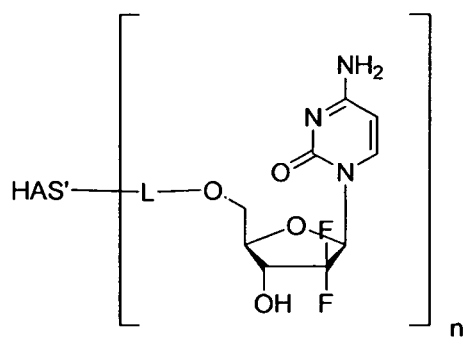
and wherein s is in the range of from 0 to 4,

and wherein t is in the range of from 0 to 4.

5 12. The conjugates according to any of embodiments 1 to 11, wherein the cytotoxic agent is an antimetabolite, more preferably a nucleoside analogue, most preferably a cytidine analogue.

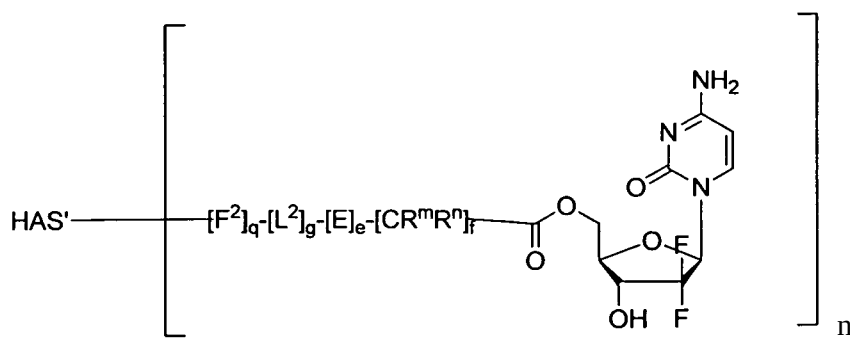
10 13. The conjugates according to any of embodiments 1 to 12, wherein the cytotoxic agent is selected from the group consisting of clofarabine, nelarabine, cytarabine, cladribine, decitabine, azacitidine, floxuridine, pentostatin and gemcitabine,
or wherein the cytotoxic agent is a kinase inhibitor including rapamycin and rapamycin analogues, preferably the cytotoxic agent is a rapamycin analogue, in
15 particular, temsirolimus or everolimus.

14. The conjugate according to embodiments 1 to 13, wherein the conjugate has a structure according to the following formula:



20

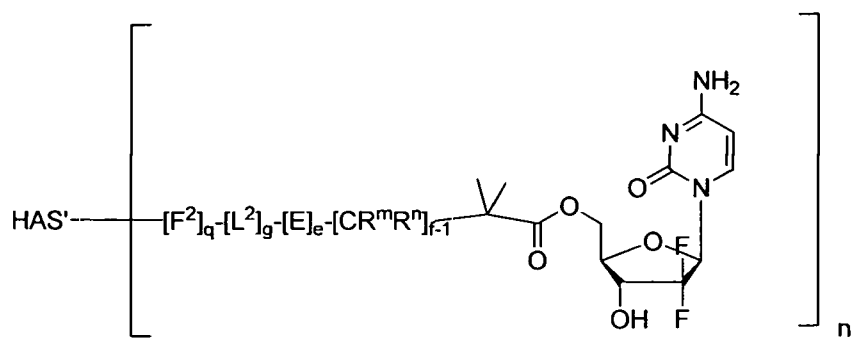
15. The conjugate according embodiment 7, said conjugate having a structure according to the following formula



16. The conjugate according to embodiment 15, wherein at least one of R^m or R^n of at least one repeating unit of the structural unit $-[CR^mR^n]_f-$ is an alkyl group.

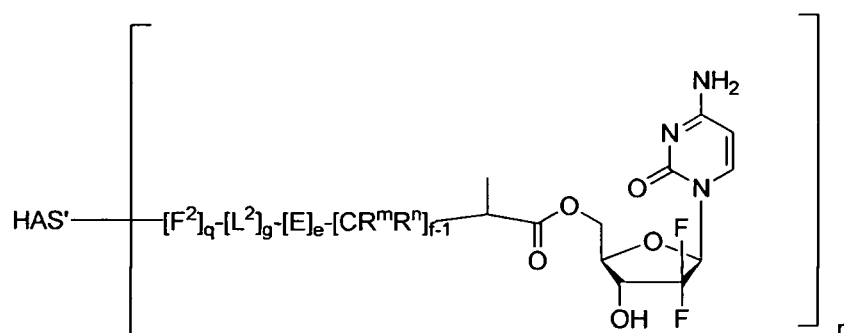
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17. The conjugate according to embodiment 16, the conjugate having a structure according to the following formula



10

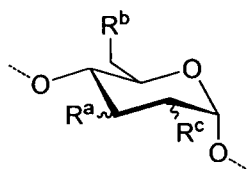
or of the following formula:



wherein R^m and R^n are, independently of each other, H or alkyl.

15

18. The conjugate according to any of embodiments 14 to 17, wherein HAS' comprises at least one structural unit according to the following formula (1)



(I)

20

wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS''$, $-[O-CH_2-CH_2]_s-OH$ and $-[O-CH_2-CH_2]_t-X-$,

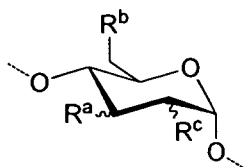
and wherein s is in the range of from 0 to 4,

and wherein t is in the range of from 0 to 4

and wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-X-$, wherein X is -S-

and wherein HAS'' is a remainder of HAS.

- 5 19. The conjugate according to any of embodiments 14 to 18, wherein HAS' comprises at least one structural unit according to the following formula (I)



(1)

- 10 wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS''$, $-[O-CH_2-CH_2]_s-OH$, and $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X-$, and wherein

s is in the range of from 0 to 4,

t is in the range of from 0 to 4,

p is 0 or 1,

- 15 and wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X-$, wherein F^1 is $-O-$,

wherein L^1 is a linking moiety having a structure according to the following formula $-[CR^{df}]_h-[F^4]_u-[CR^{dd}R^{ff}]_z-$ wherein F^4 is a functional group, preferably selected from the group consisting of $-S-$, $-O-$ and $-NH-$, in particular $-S-$, wherein

- 20 z is in the range of from 0 to 20, more preferably of from 0 to 10, more preferably of from 0 to 3, and most preferably of from 0 to 2,

or in the range of from 1 to 5, preferably in the range of from 1 to 3, more preferably 2,

h is in the range of from 1 to 5, preferably in the range of from 1 to 3, more preferably

- 25 3,

u is 0 or 1,

a is in the range of from 1 to 10,

and R^d , R^f , R^{dd} and R^{ff} are, independently of each other, selected from the group

- 30 consisting of H, alkyl, hydroxyl, and halogene, preferably selected from the group consisting of H, methyl and hydroxyl, and wherein each repeating unit of $-[CR^{df}]_h-$

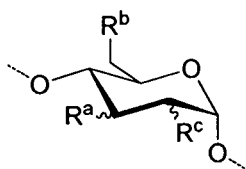
may be the same or may be different, and wherein each repeating unit of $-[CR^{dd}R^{ff}]_z-$

may be the same or may be different and wherein each repeating unit of F^4 may be the same or may be different,

- wherein, more preferably, L^1 has a structure selected from the group consisting of
- CH₂-CHOH-CH₂-, -CH₂-CHOH-CH₂-S-CH₂-CH₂-,
 - CH₂-CHOH-CH₂-S-CH₂-CH₂-CH₂-, -CH₂-CHOH-CH₂-NH-CH₂-CH₂-,
 - CH₂-CHOH-CH₂-NH-CH₂-CH₂-CH₂-, -CH₂-, -CH₂-CH₂-, -CH₂-CH₂-CH₂-,
 - CH₂-CH₂-CH₂-CH₂-CH₂-, -CH₂-CH₂-CH₂-CH₂-,
 - CH₂-CH(CH₂OH)-, -CH₂-CH(CH₂OH)-S-CH₂-CH₂-,
 - CH₂-CHOH-CH₂-O-CH₂-CHOH-CH₂-,
 - CH₂-CHOH-CH₂-O-CH₂-CHOH-CH₂-S-CH₂-CH₂-,
 - CH₂-CH₂-CH₂-S-CH₂-CH₂-, -CH₂-CH₂-S-CH₂-CH₂- and
 - CH₂-CH₂-O-CH₂-CH₂-, more preferably from the group consisting of
 - CH₂-CHOH-CH₂-, -CH₂-CHOH-CH₂-S-CH₂-CH₂-,
 - CH₂-CHOH-CH₂-S-CH₂-CH₂-CH₂-, -CH₂-CHOH-CH₂-NH-CH₂-CH₂- and
 - CH₂-CHOH-CH₂-NH-CH₂-CH₂-CH₂-, more preferably from the group consisting
 - of -CH₂-CHOH-CH₂-, -CH₂-CHOH-CH₂-S-CH₂-CH₂- and
 - CH₂-CHOH-CH₂-S-CH₂-CH₂-CH₂-,
- wherein X is S,

and wherein HAS'' is a remainder of HAS.

20. The conjugate according to any of embodiments 14 to 18, wherein HAS' comprises at least one structural unit according to the following formula (I)



(I)

- wherein R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS'', $-[O-CH_2-CH_2]_s-OH$, and $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X$, and
- wherein

s is in the range of from 0 to 4,

t is in the range of from 0 to 4,

p is 0 or 1,

and wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X$,

- wherein F^1 is -O-(C=O)-NH-,

wherein L^1 is an, optionally substituted, alkyl group,

wherein X is -S-,

and wherein HAS'' is a remainder of HAS.

21. A method for preparing a hydroxyalkyl starch (HAS) conjugate comprising a hydroxyalkyl starch derivative and a cytotoxic agent, said conjugate having a structure according to the following formula



wherein

M is a residue of a cytotoxic agent, wherein the cytotoxic agent comprises a primary hydroxyl group,

L is a linking moiety,

HAS' is a residue of the hydroxyalkyl starch derivative,

and n is equal or greater than 1,

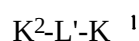
said method comprising

- (a) providing a hydroxyalkyl starch (HAS) derivative having a mean molecular weight MW above the renal threshold, preferably a mean molecular weight MW greater than or equal to 60 kDa, and a molar substitution MS in the range of from 0.6 to 1.5, said HAS derivative comprising a functional group Z^1 ; and providing a cytotoxic agent comprising a primary hydroxyl group;
- (b) coupling the HAS derivative to the cytotoxic agent via an at least bifunctional crosslinking compound L comprising a functional group K^1 and a functional group K^2 , wherein K^2 is capable of being reacted with Z^1 comprised in the HAS derivative and wherein K^1 is capable of being reacted with the primary hydroxyl group comprised in the cytotoxic agent.

22. The method according to embodiment 21, wherein the functional group K^1 comprises the group $-\text{C}(=\text{Y})-$, with Y being O, NH or S, wherein K^1 is preferably a carboxylic acid group or a reactive carboxy group.

23. The method according to embodiment 21 or 22, wherein the cytotoxic agent is reacted with the crosslinking compound L prior to the reaction with the HAS derivative.

24. The method according to any of embodiments 21 to 23, wherein the crosslinking compound L has a structure according to the following formula



wherein K^1 comprises the group $-C(=Y)-$, with Y being O, NH or S,

and L' is a linking moiety.

5

25. The method according to any of embodiments 21 to 24, wherein K^2 is reacted with the functional group Z^1 , which is selected from the group consisting of an aldehyde group, a keto group, a hemiacetal group, an acetal group, an alkynyl group, an azide, a carboxy group, an alkenyl group, a thiol reactive group, -SH, $-NH_2$, $-O-NH_2$, $-NH-$ O-alkyl, $-(C=G)-NH-NH_2$, $-G-(C=G)-NH-NH_2$, $-NH-(C=G)-NH-NH_2$, and $-SO_2-$ $NH-NH_2$, where G is O or S and, if G is present twice, it is independently O or S.

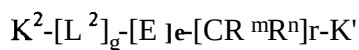
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26. The method according to embodiment 25, wherein upon reaction of the primary hydroxyl group comprised in the cytotoxic agent with K^1 , a functional group $-F^3-O-$ is formed, wherein F^3 comprises the functional group $-C(=Y)-$, with Y being O, NH or S, in particular wherein F^3 is the functional group $-C(=Y)-$, with Y being O.

15

27. The method according to any of embodiments 21 to 26, wherein the at least one crosslinking compound L has a structure according to the following formula:

20



wherein E is an electron-withdrawing group, preferably selected from the group consisting of $-C(=O)-NH-$, $-NH-C(=O)-$, $-NH-$, $-O-$, $-S-$, $-SO-$, $-SO_2-$ and -succinimide-,

25

L^2 is a linking moiety, preferably an alkyl, alkenyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl groupf is in the range of from 1 to 20, g is 0 or 1,

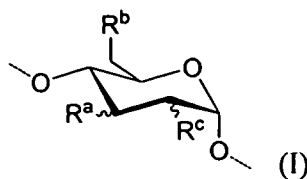
30

e is 0 or 1,

and wherein R^m and R^n are, independently of each other, H, alkyl, aryl or a residue of a natural or unnatural amino acid, preferably H or alkyl, more preferably H or methyl, in particular H.

35

28. The method according to any of embodiments 21 to 27, wherein the hydroxyalkyl starch derivative provided in step (a) comprises at least one structural unit according to the following formula (I)



wherein at least one of R^a , R^b or R^c comprises the functional group Z^1 , preferably consisting of -O-HAS", $-[O-(CR^wR^xHCR^yR^z)]_x-OH$, $-[O-(CR^wR^x)-(CR^yR^z)]_y-X-$, $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^1-X-$,

5 wherein R^w , R^x , R^y and R^z are independently of each other selected from the group consisting of hydrogen and alkyl,

y is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4, and

x is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4,

F^1 is a functional group,

10 p is 0 or 1,

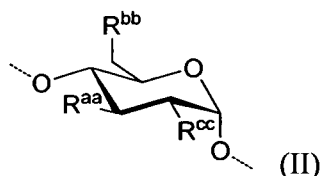
L^1 is a linking moiety,

wherein HAS" is a remainder of HAS,

and wherein step (a) comprises

(a1) providing a hydroxyalkyl starch having a mean molecular weight MW greater

15 than or equal to 60 kDa and a molar substitution MS in the range of from 0.6 to 1.5 comprising the structural unit according to the following formula (II)



wherein R^{aa} , R^{bb} and R^{cc} are, independently of each other, selected from the group consisting of -O-HAS" and $-[O-(CR^wR^xMCR^yR^z)]_x-OH$,

20 wherein R^w , R^x , R^y and R^z are independently of each other selected from the group consisting of hydrogen and alkyl,

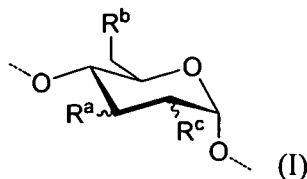
and x is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4;

25 (a2) introducing at least one functional group Z^1 into HAS by

(i) coupling the hydroxyalkyl starch via at least one hydroxyl group comprised in HAS to at least one suitable linker comprising the functional group Z^1 or a precursor of the functional group Z^1 , or

30 (ii) displacing at least one hydroxyl group comprised in HAS in a substitution reaction with a precursor of the functional group Z^1 or with a suitable linker comprising the functional group Z^1 or a precursor thereof.

29. The method according to embodiment 28, wherein the HAS derivative formed in step (a2) comprises at least one structural unit according to the following formula (I)



- 5 wherein R^A , R^b and R^C are, independently of each other, selected from the group consisting of -O-HAS", $-[O-CH_2-CH_2]_s-OH$, $-[O-CH_2-CH_2]_t-X$ and $-[O-CH_2-CH_2]_t-[F^1]_p-L'-X$, and wherein

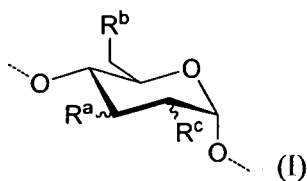
and wherein s is in the range of from 0 to 4,

and wherein t is in the range of from 0 to 4,

p is 0 or 1,

- 10 wherein at least one of R^A , R^b and R^C is $-[O-CH_2-CH_2]_t-X$ or $-[O-CH_2-CH_2]_t-[F^1]_p-L'-X$,
and wherein HAS" is a remainder of HAS.

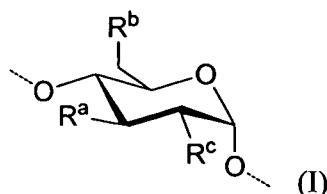
- 15 30. The method according to embodiment 28 or 29, wherein in (a2)(i), the hydroxyalkyl starch is reacted with a suitable linker comprising the functional group Z^1 or a precursor of the functional group Z^1 , and comprising a functional group Z^2 , the linker preferably having the structure $Z^2-L^1-Z^1$ or $Z^2-L^1-Z^1-PG$, with Z^2 being a functional group capable of being reacted with the hydroxyalkyl starch, thereby forming a hydroxyalkyl starch derivative comprising at least one structural unit according to the
20 following formula (I)



- 25 wherein at least one of R^A , R^b and R^c is $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-Z^1$ or $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-Z^{1*}-PG$ with PG being a suitable protecting group and Z^{1*} being the protected form of the functional group Z^1 ,
wherein Z^1 is preferably -SH, Z^{1*} is preferably -S- and PG is preferably a suitable thiol protecting group, more preferably a protecting group forming together with Z^{1*} a group selected from the group consisting of thioethers, thioesters and disulfides,
30 and wherein in case the linker comprises the protecting group PG, the method further comprises deprotection of Z^{1*} to give Z^1 .

31. The method according to embodiment 30, wherein step (a2)(i) comprises

- (aa) activating at least one hydroxyl group comprised in the hydroxyalkyl starch with a reactive carbonyl compound having the structure $R^{**}-(C=O)-R^*$, wherein R^* and R^{**} may be the same or different, and wherein R^* and R^{**} are both leaving groups, wherein upon activation a hydroxyalkyl starch derivative comprising at least one structural unit according to the following formula (1)



is formed, in which R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS''$, $-[O-CH_2-CH_2]_s-OH$, $-[O-CH_2-CH_2]_t-O-C(=O)-R^*$,

wherein s is in the range of from 0 to 4,

and wherein t is in the range of from 0 to 4,

and wherein at least one of R^a , R^b and R^c comprises the group $-[O-CH_2-CH_2]_t-O-C(=O)-R^*$, and

- (bb) reacting the activated hydroxyalkyl starch according to step (aa) with the suitable linker comprising the functional group Z^1 or a precursor of the functional group Z^1 .

32. The method according to embodiment 31, wherein the reactive carbonyl compound having the structure $R^{**}-(C=O)-R^*$ is selected from the group consisting of phosgene, diphosgene, triphosgene, chloroformates and carbonic acid esters, preferably wherein the reactive carbonyl compound is selected from the group consisting of p-nitrophenylchloroformate, pentafluorophenylchloroformate, N,N'-disuccinimidyl carbonate, sulfo-N,N'-disuccinimidyl carbonate, dibenzotriazol-1-yl carbonate and carbonyldiimidazol.

33. The method according to embodiment 31 or 32, wherein in step (bb), the activated hydroxyalkyl starch derivative is reacted with a linker comprising the functional group Z^2 and the functional group Z^1 or a precursor of the functional group Z^1 , the linker preferably having the structure $Z^1-L^1-Z^1$ or $Z^1-L^1-Z^1-PG$, wherein Z^{1*} is the protected form of Z^1 and PG is a protecting group, preferably wherein Z^1 is $-SH$ and Z^{1*} is $-S-$

Z^2 is a functional group capable of being reacted with the

$-[O-CH_2-CH_2]_t-O-C(=O)-R'$ group,

L^1 is an alkyl group,

wherein upon reaction of the $-O-C(=O)-R'$ group with the functional group Z^2 , the

functional group F^1 is formed, and

Z^2 is preferably $-NH_2$.

34. The method according to embodiment 33, wherein the linker has the structure Z^2-L^1-

$Z^{1*}-PG$, wherein Z^{1*} is $-S-$ and PG is a thiol protecting group, more preferably a

protecting group forming together with Z^{1*} a group selected from the group consisting

of thioethers, thioesters and disulfides, and wherein the method further comprises

deprotection of Z^1 .

35. The method according to embodiment 28 or 29, wherein step (a2)(i) comprises

(I) coupling the hydroxyalkyl starch via at least one hydroxyl group comprised in the hydroxyalkyl starch to a first linker comprising a functional group Z^2 , Z^2 being capable of being reacted with a hydroxyl group of the hydroxyalkyl starch, thereby forming a covalent linkage, the first linker further comprising a functional group W , wherein the functional group W is an epoxide or a group which is transformed in a further step to give an epoxide.

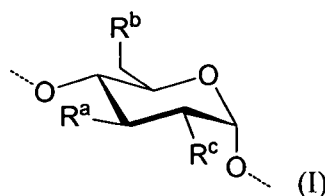
36. The method according to embodiment 35, wherein the first linker has a structure

according to the formula Z^2-L^W-W , wherein

Z^2 is a functional group capable of being reacted with a hydroxyl group of the hydroxyalkyl starch,

L^W is a linking moiety,

wherein upon reaction of the hydroxyalkyl starch with the first linker, a hydroxyalkyl starch derivative is formed comprising at least one structural unit according to the following formula (I)



wherein R^a , R^b and R^c are, independently of each other, selected from the group consisting of $-O-HAS$, $-[O-CH_2-CH_2]_s-OH$, and $-[O-CH_2-CH_2]_t-[F']_p-L^W-W$,

wherein s is in the range of from 0 to 4,

and wherein t is in the range of from 0 to 4,

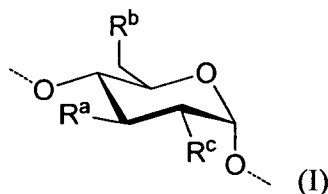
and wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-[F^1]_p-L^w-W$,
 and wherein F^1 is the functional group being formed upon reaction of Z^2 with a
 hydroxy1 group of the hydroxyalkyl starch, wherein F^1 is preferably $-O-$ or $-CH_2-$
 $CHOH-$, preferably $-O-$,
 5 and wherein HAS'' is a remainder of HAS.

37. The method according to embodiment 35 or 36, wherein W is an alkenyl group and the
 method further comprises

10 (II) oxidizing the alkenyl group W to give the epoxide, wherein as oxidizing agent,
 potassium peroxymonosulfate (Oxone®) is preferably employed.

38. The method according to any of embodiments 35 to 37, wherein Z^2 is a halogene (Hal)
 or an epoxide, and wherein the linker preferably has the structure $Hal-CH_2-CH=CH_2$.

15 39. The method according to embodiment 37, the method comprising
 (HI) reacting the epoxide with a nucleophile comprising the functional group Z^1 or a
 precursor of the functional group Z^1 , wherein the nucleophile is preferably a
 dithiol or a thiosulfate, thereby forming a hydroxyalkyl starch derivative
 comprising at least one structural unit, preferably 3 to 100 structural units,
 20 according to the following formula (I)



wherein R^a , R^b and R^c are independently of each other selected from the group
 consisting of $-O-HAS''$, $-[O-CH_2-CH_2]_s-OH$, and $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-Z^1$,
 wherein

25 and wherein s is in the range of from 0 to 4,

and wherein t is in the range of from 0 to 4,

P is 1,

at least one of R^a , R^b and R^c comprises the group $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-Z^1$,
 and wherein Z^1 is $-SH$.

30

40. The method according to embodiment 41, wherein the nucleophile is ethanedithiol or
 sodium thiosulfate.

41. The method according to embodiment 28, wherein in step (a2)(ii), prior to the displacement of the hydroxyl group, a group R^L is added to at least one hydroxyl group thereby generating a group $-O-R^L$, wherein $-O-R^L$ is a leaving group, in particular an $-O$ -Mesyl ($-OMs$) or $-O$ -Tosyl (OTs) group.

5

42. The method according to embodiment 28 or embodiment 41, wherein Z^1 is $-SH$, and wherein in step (a2)(ii) the at least one hydroxyl group comprised in the hydroxyalkyl starch is displaced by a suitable precursor of the functional group Z^1 , the method further comprising converting the precursor after the substitution reaction to the functional group Z^1 .

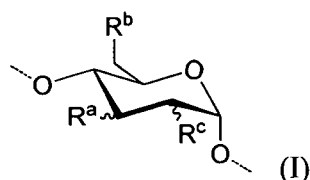
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43. The method according to embodiment 42, wherein in step (a2)(ii) the at least one hydroxyl group comprised in the hydroxyalkyl starch is displaced with thioacetate giving a precursor of the functional group Z^1 having the structure $-S-C(=O)-CH_3$, wherein the method further comprises the conversion of the group $-S-C(=O)-CH_3$ to give the functional group Z^1 , preferably wherein the conversion is carried out using sodium hydroxide and sodium borohydride.

15

44. The method according to any of embodiments 41 to 43, wherein the hydroxyalkyl starch derivative obtained according to step (a2)(ii) comprises at least one structural unit according to the following formula (I)

20



wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS''$, $-[O-CH_2-CH_2]_s-OH$, and $-[O-CH_2-CH_2]_t-Z^1$, wherein

25

and wherein s is in the range of from 0 to 4,

and wherein t is in the range of from 0 to 4,

and wherein at least one of R^a , R^b and R^c comprises the group $-[O-CH_2-CH_2]_t-Z^1$,

wherein Z^1 is $-SH$,

and wherein HAS'' is a remainder of HAS .

30

45. The method according to any of embodiments 28 to 44, wherein in step (b) the hydroxyalkyl starch derivative obtained according to step (a) is coupled to a crosslinking compound L having a structure according to the formula $K^2-[L^2]_g-[E]_e-[CR^mR^n]_f-K^1$ wherein E is an electron-withdrawing group, L^2 is a linking moiety, and wherein
- 5 — g and e are 0,
f is in the range of from 1 to 20,
 R^m and R^n are, independently of each other, H or alkyl, preferably H or methyl, and K^2 is a halogene,
- 10 and wherein upon reaction of Z^1 with K^2 the covalent linkage $-X-[CR^mR^n]_f-$ is formed;
- or
- g and e are 0,
f is in the range of from 1 to 20,
- 15 R^m and R^n are, independently of each other, H or alkyl, preferably H or methyl, in particular H,
and K^2 is maleimide,
and wherein upon reaction of Z^1 with K^2 the covalent linkage $-X$ -succinimide- is formed.
- 20
46. The method according to embodiment 45, wherein Z^1 is $-SH$, and X is $-S-$.
47. The method according to any embodiments 21 to 46, wherein the cytotoxic agent is an antimetabolite, more preferably a nucleoside analog, in particular wherein the
- 25 cytotoxic agent is selected from the group consisting of clofarabine, nelarabine, cytarabine, cladribine, decitabine, azacitidine, fludarabine, floxuridine, doxifluridine, pentostatin and gemcitabine or
- wherein the cytotoxic agent is a kinase inhibitor including rapamycin and rapamycin analogues, preferably the cytotoxic agent is a rapamycin analogue, in particular,
- 30 temsirolimus or everolimus.
48. A hydroxyalkyl starch conjugate obtained or obtainable by a method according to any of embodiments 21 to 47.
- 35 49. A pharmaceutical composition comprising a conjugate according to any of embodiments 1 to 20 or according to embodiment 48.

50. A hydroxyalkyl starch conjugate according to any of embodiments 1 to 20 or according to embodiment 48, or a pharmaceutical composition according to embodiment 49 for use as medicament.
- 5 51. A hydroxyalkyl starch conjugate according to any of embodiments 1 to 20 or according to embodiment 48, or a pharmaceutical composition according to embodiment 49 for the treatment of cancer.
- 10 52. A hydroxyalkyl starch conjugate according to any of embodiments 1 to 20 or according to embodiment 48, or a pharmaceutical composition according to embodiment 49 for the treatment of cancer selected from the group consisting of biliary cancer, bladder cancer, breast cancer, cervical cancer, colorectal cancer, gastrointestinal cancer, head and neck cancer, leukaemia, lymphoma, malignant melanoma, mesothelioma, non-small cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, sarcoma and small cell lung cancer.
- 15 53. Use of a hydroxyalkyl starch conjugate according to any of embodiments 1 to 20 or according to embodiment 48, or a pharmaceutical composition according to embodiment 49 for the manufacture of a medicament for the treatment of cancer.
- 20 54. Use of a hydroxyalkyl starch conjugate according to claim 53, wherein the cancer is selected from the group consisting of biliary cancer, bladder cancer, breast cancer, cervical cancer, colorectal cancer, gastrointestinal cancer, head and neck cancer, leukaemia, lymphoma, malignant melanoma, mesothelioma, non-small cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, sarcoma and small cell lung cancer.
- 25 55. A method of treating a patient suffering from cancer comprising administering a therapeutically effective amount of a hydroxyalkyl starch conjugate according to any of embodiments 1 to 20 or according to embodiment 48, or a pharmaceutical composition according to embodiment 49.
- 30 56. The method of embodiment 55 wherein the patient suffers from a cancer being selected from the group consisting of biliary cancer, bladder cancer, breast cancer, cervical cancer, colorectal cancer, gastrointestinal cancer, head and neck cancer, leukaemia, lymphoma, malignant melanoma, mesothelioma, non-small cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, sarcoma and small cell lung cancer.
- 35

Description of the Figures:

Figure 1: *Time course of the median RTV values after administering conjugate CGtl (dosage 7.5 mg/kg body weight; human pancreas carcinoma model ASPC-1)*

5

Figure 1 shows the time course of the relative tumor volume of *human pancreas carcinoma ASPC-1* growing in nude mice treated with conjugate CGtl vs. mice in the control group (untreated mice (saline)) as well as vs. mice treated with gemcitabine.

10 The following symbols are used:

■ = saline, * = gemcitabine (Gemzar®), ◇ = CGtl.

The X-axis shows the time after start [d], the Y-axis shows the mean relative tumor volume, mean RTV [%].

15

Each measurement was carried out with a group of 8 mice. The conjugate CGtl was administered at a dosage of 7.5 mg/kg body weight on days 9, 16, 23, 30, 24 and 40. Gemcitabine was administered at a dosage of 60 mg/kg body weight at days 9, 13, 16, 20, 23, 27, 30, 34 and 40. Median values are given. Further details are given in Table 11.

20

Figure 2: *Time course of the body weight change after administering conjugate CGtl (dosage 7.5 mg/kg body weight; human pancreas carcinoma model ASPC-1)*

25 Figure 2 shows the time course of the body weight change in nude mice *human pancreas carcinoma ASPC-1 xenografts* treated with conjugate CGtl vs. mice in the control group (untreated mice (saline)) as well as vs. mice treated with gemcitabine.

The following symbols are used:

■ = saline, * = gemcitabine (Gemzar®), ◇ = CGtl.

30

The X-axis shows the time after start [d], the Y-axis shows the mean BWC [%]

Each measurement was carried out with a group of 8 mice. The conjugate CGtl was administered at a dosage of 7.5 mg/kg body weight on days 9, 16, 23, 30, 24 and 40. 35 Gemcitabine was administered at a dosage of 60 mg/kg body weight at days 9, 13, 16, 20, 23, 27, 30, 34 and 40. Median values are given. Further details are given in Table 11.

Figure 3: *Time course of the median RTV values after administering conjugates CGtl, CGL6, CGt3, CGt5 (dosage 7.5 mg/kg body weight; human pancreas carcinoma model ASPC-1)*

40

Figure 3 shows the time course of the relative tumor volume of **human pancreas carcinoma ASPC-1** growing in nude mice treated with conjugates CGt2, CGt6, CGt3, CGt5 vs. mice in the control group (untreated mice (saline)) as well as vs. mice treated with gemcitabine.

The following symbols are used: ■ = Saline, * = gemcitabine (Gemzar®), o = CGt2, Δ = CGt6, V = CGt3, ◇ = CGt5.

The X-axis shows the time after tumor implantation [d], the Y-axis shows the relative tumor volume (RTV) [%].

Each measurement was carried out with a group of 8 mice. The conjugates were administered 5 to 9 times, each time at a dosage of 7.5 mg/kg body weight. Gemcitabine was administered 8 times at a dosage of 60 mg/kg body weight each. Median values are given. Further details are given in Table 12.

Figure 4: Time course of the body weight change after administering conjugates CGt2, CGt6, CGt3, CGt5 (dosage 7.5 mg/kg body weight; human pancreas carcinoma model ASPC-1)

Figure 4 shows the time course of the body weight change in nude mice **human pancreas carcinoma ASPC-1 xenografts** treated with conjugates CGt2, CGt6, CGt3, CGt5 vs. mice in the control group (untreated mice (saline)) as well as vs. mice treated with gemcitabine.

The following symbols are used: ■ = Saline, ★ = gemcitabine (Gemzar®), o = CGt2, Δ = CGt6, V = CGt3, ◇ = CGt5.

The X-axis shows the time after tumor transplantation [d], the Y-axis shows the BWC [%].

Each measurement was carried out with a group of 8 mice. The conjugates were administered 5 to 9 times, each time at a dosage of 7.5 mg/kg body weight. Gemcitabine was administered 8 times at a dosage of 60 mg/kg body weight each. Median values are given. Further details are given in Table 12.

Figure 5: Cleavage Kinetics of Everolimus conjugates

Figure 5 shows the cleavage kinetics of Everolimus conjugates in 5 mg/ml PBS buffer pH 7.4 / DMF 1:1 at a temperature of 37°C, determined by RP-HPLC.

X-axis: time [h], Y-axis: conjugate [%].

5

The following symbols are used: ■ = CEv2, ♦ = CEvl.

Figure 6: Cleavage Kinetics of Temsirolimus conjugates

10 Figure 6 shows the cleavage kinetics of Temsirolimus conjugates in 5 mg/ml PBS buffer pH 7.4 / ACN 1:1, measured at 37°C, determined by RP-HPLC.

X-axis: time [h], Y-axis: conjugate [%].

15 The following symbols are used: ♦ = CTml, ■ = CTm3, A = CTm2, X = CTm5.

Figure 7: Cleavage Kinetics of Gemcitabine conjugates

20 Figure 7 shows the cleavage kinetics of Gemcitabine conjugates in 5 mg/ml PBS buffer pH 7.4, measured at 37°C, determined by RP-HPLC).

X-axis: time [h], Y-axis: conjugate [%].

The following symbols are used: ♦ = CGt9, ■ = CGt3, ▲ = CGt8.

25

Figure 8: Cleavage Kinetics of Cytarabine conjugates

Figure 8 shows the cleavage kinetics of Cytarabine conjugates, in 5 mg/ml PBS buffer pH 7.4, measured at 37°C, and determined by RP-HPLC).

30

X-axis: time [h], Y-axis: conjugate [%].

The following symbols are used: ♦ = CCtl, ■ = CCt2.

35 **Figure 9: Time course of the median RTV values after administering conjugates CEvl and CEv2 (dosage 10 to 15 mg/kg body weight; human large cell lung carcinoma LXFL-529**

Figure 9 shows the time course of the relative tumor volume of *human large cell lung carcinoma LXFL-529 xenografts* growing in nude mice treated with conjugates *CEvl and*

CEv2 vs. mice in the control group (untreated mice (saline)) as well as vs. mice treated with everolimus.

The following symbols are used: ■ = Saline, x = Everolimus, ♦ = CEv1, · = CEv2,

5

The X-axis shows the days after treatment [d], the Y-axis shows the median relative tumor volume (RTV) [%].

Each measurement was carried out with a group of 5 mice. The conjugates were administered 5 times, each time at a dosage of 10 or 15 mg/kg body weight. Everolimus was administered 5 times, each time at a dosage of 10 or 15 mg/kg body weight each. Median values are given. Further details are given in Table 14.

10

Figure 10: Time course of the body weight change after administering conjugates CEv1 and CEv2 (dosage 10 to 15 mg/kg body weight; human large cell lung carcinoma LXFL-529

15

Figure 10 shows the time course of the body weight change in nude mice *human large cell lung carcinoma LXFL-529 xenografts* growing in nude mice treated with conjugates CEv1 and CEv2 vs. mice in the control group (untreated mice (saline)) as well as vs. mice treated with everolimus.

20

The following symbols are used: The following symbols are used: ■ = Saline, x = Everolimus, ♦ = CEv1, · = CEv2,

25

The X-axis shows the time after start, the Y-axis shows the median BWC [%].

Each measurement was carried out with a group of 5 mice. The conjugates were administered 5 times, each time at a dosage of 10 or 15 mg/kg body weight. Everolimus was administered 5 times, each time at a dosage of 10 or 15 mg/kg body weight each. Median values are given. Further details are given in Table 14.

30

Figure 11: Time course of the median RTV values after administering conjugates CTm1, CTm2 and CTm3 (dosage 20 mg/kg body weight; human large cell lung carcinoma LXFL-529

35

Figure 11 shows the time course of the relative tumor volume of human large cell lung carcinoma LXFL-529 xenografts growing in nude mice treated with conjugates CTm1, CTm2 and CTm3 vs. mice in the control group (untreated mice (saline)) as well as vs. mice treated with temsirolimus.

40

The following symbols are used: ■ = saline, x = temsirolimus, ♦ = CTm1, A = CTm2, • = CTm3.

- 5 The X-axis shows the days after treatment [d], the Y-axis shows the relative tumor volume (RTV) [%].

Each measurement was carried out with a group of 5 mice. The conjugates were administered 4 times, each time at a dosage of 20 mg/kg body weight. Temsirolimus was administered 4 times, each time at a dosage of 20 mg/kg body weight. Median values are given. Further details are given in Table 14.

Figure 12: Time course of the body weight change after administering conjugates CTm1, CTm2 and CTm3 (dosage 20 mg/kg body weight; human large cell lung carcinoma LXFL-529)

Figure 12 shows the time course of the body weight change in nude mice human large cell lung carcinoma LXFL-529 xenografts growing in nude mice treated with conjugates CTm1, CTm2 and CTm3 vs. mice in the control group (untreated mice (saline)) as well as vs. mice treated with temsirolimus.

The following symbols are used: ■ = saline, x = temsirolimus, ♦ = CTm1, A = CTm2, • = CTm3.

- 25 The X-axis shows the time after treatment [d], the Y-axis shows the mean body weight [% of start value].

Each measurement was carried out with a group of 5 mice. The conjugates were administered 4 times, each time at a dosage of 20 mg/kg body weight. Temsirolimus was administered 4 times, each time at a dosage of 20 mg/kg body weight. Median values are given. Further details are given in Table 14.

Figure 13: Time course of the relative tumor volume after administering conjugates CGt10, CGt9 and CGt7 (dosage between 3 and 10 mg/kg body weight; human pancreatic carcinoma ASPC-1)

Figure 13 shows the time course of the relative tumor volume of human pancreatic carcinoma ASPC-1 xenografts growing in nude mice treated with conjugates CGt10, CGt9

and CGt7 vs. mice in the control group (untreated mice (saline)) as well as vs. mice treated with gemcitabine (Gemzar®).

5 The following symbols are used: ■ = saline, * = gemcitabine (Gemzar®), V = CGt10, Δ = CGt9, ◇ = CGt7.

The X-axis shows the time after treatment [d], the Y-axis shows the median relative tumor volume.

10 Each measurement was carried out with a group of 9 mice. Conjugates were administered 3 times, each time at a dosage of 3 mg/kg for CGt10 and CGt7 and 7.5 mg/kg for CGt9. gemcitabine (Gemzar®) was administered 3 times, each time at a dosage of 60 mg/kg body weight each. Median values are given. Further details are given in Table 13.

15 **Figure 14: Time course of the body weight change after administering conjugates CGt10, CGt9 and CGt7 (dosage between 3 and 10 mg/kg body weight; human pancreatic carcinoma ASPC-1**

20 Figure 12 shows the time course of the body weight change in nude mice human pancreatic carcinoma xenografts growing in nude mice treated with conjugates CGt10, CGt9 and CGt7 vs. mice in the control group (untreated mice (saline)) as well as vs. mice treated with gemcitabine (Gemzar®).

25 The following symbols are used: ■ = saline, * = gemcitabine (Gemzar®), V = CGt10, Δ = CGt9, ◇ = CGt7.

The X-axis shows the time after treatment [d], the Y-axis shows the mean body weight [% of start value.

30 Each measurement was carried out with a group of 9 mice. Conjugates were administered 3 times, each time at a dosage of 3 mg/kg for CGt10 and CGt7 and 7.5 mg/kg for CGt9. gemcitabine (Gemzar®) was administered 3 times, each time at a dosage of 60 mg/kg body weight each. Median values are given. Further details are given in Table 13.

35

Examples:**A. Materials and Methods**

5 **Centrifugation** was performed using a Sorval! Evolution RC centrifuge (Thermo Scientific) equipped with a SLA-3000 rotor (6x 400 mL vessels) at 9000 g and 4°C for 5-10 min.

Ultrafiltration was performed using a Sartoflow Slice 200 Benchtop (Sartorius AG) equipped with two Hydrosart Membrane cassettes (10 kDa Cutoff, Sartorius). Pressure settings: p_i = 2 bar, p₂ = 0.5 bar.

10 **Filtration:** Solutions were filtered prior to size exclusion chromatography and HPLC using syringe filters (0.45 µm, GHP-Acrodisc, 13 mm) or Steriflip (0.45 µm, MilliQ).

Analytical HPLC spectra were measured on a Ultimate 3000 (Dionex) using a LPG-3000 pump, a DAD-3000a diode array detector and a C18 reverse phase column (Dr. Maisch, Reprosil Gold 300A, C18, 5µm, 150x4.6 mm). Eluents were purified water (Millipore) +
15 0.1% TFA (Uvasol, MERCK) and acetonitrile (HPLC grade, MERCK) + 0.1% TFA. Standard gradient was: 2% ACN to 98% ACN in 30 min.

Size exclusion chromatography was performed using an Äkta Purifier (GE-Healthcare) system equipped with a P-900 pump, a P-960 sample pump using an UV-900 UV detector and a pH/IC-900 conductivity detector. A HiPrep 26/10 desalting column (53 mL, GE-
20 Healthcare) was used together with a HiTrap desalting column as pre-column (5 mL, GE-Healthcare). Fractions were collected using the Frac-902 fraction collector.

Freeze-drying: Samples were frozen in liquid nitrogen and lyophilized using a Christ alpha 1-2 LD plus (Martin Christ, Germany) at p = 0.2 mbar.

UV-vis absorbances were measured at a Cary 100 BIO (Varian) in either plastic cuvettes
25 (PMMA, d = 10 mm) or quartz cuvettes (d = 10 mm, Hellma, Suprasil, 100-QS) using the Cary Win UV simple reads software.

B. Reagents

30 **Table 3: Reagents used**

Entry	Name	Quality	Supplier	Lot#
1	Sodium hydride	60% w/w in paraffin	Merck	S4977752
2	Allyl bromide	reagent grade 97%	Aldrich	S77053-109
3	Potassium monopersulfate Triplesalt (Oxone®)	technical grade	Aldrich	82070
4	Sodium bicarbonate	puriss.	Merck	26533223
5	Tetrahydrothiopyran-4-one	99%	Aldrich	1370210

				42708159
6	Ethanedithiol		Fluka	1377608
7	5,5'-Dithiobis(2-nitrobenzoic acid), Ellman's reagent	>97.5%	Fluka	1334177
8	Isopropanol	puriss. ACS	Fluka	
9	Methyl tert. butyl ether	99%	Acros	
10	Dimethyl formamide	pept. syn. grade	Acros	A0256931
11	Dimethyl formamide	extra dry 99.8%	Acros	A00954967
12	Acetic acid	>99.8%	Fluka	9 1190

Table 4: Hydroxyalkyl starches used

Name	Lot	Mw	Mn	PDI	MS
HESI	073121	84.5	55.2	1.47	1.3
HES2	073421	89.1	78.1	1.14	0.4
HES3	08051 1	77.1	62.2	1.24	0.7
HES4	17093341	83.0	61.4	1.35	1.0
HES5	17091931	273.8	214.5	1.28	0.5
HES6	17091071	275.8	200.2	1.38	0.7
HES7	080741	258.9	196.6	1.32	1.0
HES8	06321 1	78.2	65.9	1.19	1.0
HES9	171 101 1	92.4	66.4	1.39	1.0

5 A Synthesis

I Synthesis of HES derivatives

1.1 Example 1.1: Synthesis of HES derivative DI

10

(a) Synthesis of allyl-HES

Hydroxyethyl starch (Lot. 073121, Fresenius Kabi, Linz) (HESI) was thoughtfully dried prior to use on an infra-red heated balance at 80°C until the mass remained constant. In a 500 ml round bottom flask equipped with a magnetic stirring bar and a rubber septum, 20 g of hydroxyethyl starch was dissolved in 200 ml of dry DMF under an inert atmosphere. After the HES had dissolved, 0.63 g of sodium hydride (60% w/w in paraffin) were added

15

in one portion and the resulting cloudy solution was allowed to stir for 1 h at room temperature followed by the addition of 0.94 ml allyl bromide. The reaction mixture was allowed to stir over night, resulting in a yellow, clear solution. The solution was then slowly poured into 1400 ml of isopropanol and the precipitate collected by centrifugation.

- 5 The precipitated polymer was re-dissolved in water and subjected to ultrafiltration (20 volume exchanges with water). Freeze-drying of the retentate yielded 18.9 g (94%) of a colourless solid.

10 (b) Synthesis of thiol-HES DI

- In a glass beaker, 10.0 g of allyl-HES were dissolved in 200 ml of a 4×10^{-4} M EDTA solution. 35 mg of tetrahydrothiopyran-4-one were added and the solution was allowed to stir on a magnetic stirring plate. 5 g of potassium persulfate (Oxone®) and 2.12 g sodium hydrogen carbonate were mixed in dry state and the mixture was added in small portions to the HES-solution resulting in the formation of thick foam. The mixture was stirred at ambient temperature for 2 h. The mixture was directly subjected to ultrafiltration (20 volume exchanges with water). The retentate was split into two aliquots, each of them used for independent preparations.

- 20 One aliquot (~ 5 g HES) was poured into 180 ml of a 1:1 mixture of isopropanol and MTBE resulting in precipitation of the polymer, which was collected by centrifugation. The precipitate was re-dissolved in 50 ml of DMF, transferred into a 100 ml screw-cap bottle. 23 ml of ethanedithiol were added and the resulting mixture degassed by purging with inert gas for several minutes. 7 ml of a 0.1 M sodium bicarbonate solution were added, the bottle was tightly closed and stirred for 2 days at ambient temperature. The resulting clear solution was poured into 500 ml of isopropanol and the precipitate collected by ultrafiltration. The polymer was re-dissolved in 100 ml of water and subjected to ultrafiltration (20 volume exchanges with water). The resulting retentate (100 ml) was transferred into a 250 ml round bottom flask equipped with a magnetic stirring bar. The solution was degassed by purging with argon for several minutes. 0.5 g of sodium borohydride were added and the resulting mixture was allowed to stir over night at ambient temperature. The reaction was quenched by addition of 1 ml of acetic acid. The solution was subjected to ultrafiltration (15 volume exchanges against 20 mM acetic acid + 2 mM EDTA followed by 5 volume exchanges against 20 mM acetic acid). The retentate was freeze dried to give 5.13 g of a colorless solid.

Molecular weight: Mw = 88.7 kD; Mn = 60.9 kD; PDI = 1.46.

1.2 General procedure for the synthesis of SH-HES-Derivatives D1 to D12 (GP 1)**(a) General procedure for the synthesis of thioacetyl-HES (GP 1.1)**

5

Hydroxyethyl starch as used in the preparation was thoughtfully dried prior to use either on an infra-red heated balance at 80°C until the mass remained constant or by leaving in a drying oven over night at 80°C. In a round bottom flask equipped with a magnetic stirring bar and a rubber septum under inert gas, HES was dissolved in formamide to give a 20% solution. After the addition of collidine, the clear solution was cooled in an ice-water bath. Then, mesyl chloride was added dropwise and the reaction mixture kept in the ice bath for ~1 h. The cooling bath was removed and the solution allowed to warm up to room temperature. After additional 1 h of stirring, potassium thioacetate was added as a solid and the resulting amber solution was allowed to stir over night at the given temperature. After cooling to room temperature, the reaction mixture was diluted 5:1 with water and subjected to ultrafiltration (concentration to a 10% w/w HES solution followed by 15-20 volume exchanges with water). The retentate was used immediately in the next step. Alternatively, the thioacetyl-HES can be lyophilized and stored without signs of degradation.

10

15

20

(b) General procedure for the synthesis of SH-HES derivatives by saponification of thioacetyl-HES using sodium hydroxide (GP 1.2)

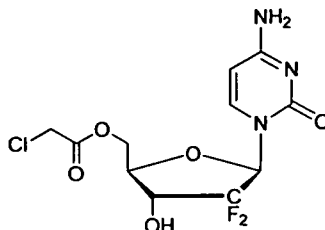
A 10% (w/v) solution of thioacetyl-HES derived from GP 1.1 in water was filled in a round bottom flask equipped with a magnetic stirring bar and a rubber septum under an inert gas atmosphere. The solution was degassed by passing a stream of inert gas through the mixture while continuous stirring for ~10 minutes. A 1 M sodium hydroxide solution was added (20% of total volume), followed by addition of solid sodium borohydride (10% w/w of HES). The resulting solution was allowed to stir under inert gas for 2 h. The reaction was quenched by addition of acetic acid (-0.5 ml/gram HES, pH = 5-7). The product was purified by ultrafiltration (15-20 volume exchanges with a 20 mM solution of acetic acid in water). Freeze-drying of the retentate afforded SH-HES as a colorless solid.

25

30

II. Synthesis of the derivatives of the cytotoxic agents

11:1 Example II.1: Synthesis of chloroacetyl gemcitabine GEM1



The cytostatic compound gemcitabine was used as compound M. Such cytostatic compound is commercially available and sold, for example, by company Sigma-Aldrich as Gemcitabine hydrochloride.

In a round bottom flask, 1.0 g of gemcitabine hydrochloride was dissolved in 5 ml of DMF. 0.5 ml of triethylamine were added and the resulting solution was stirred for 5 minutes at room temperature. The solution was cooled to 0-5°C followed by the dropwise addition of chloroacetyl chloride (0.55 ml) in 5 minutes time, stirred for 2 h at 0-5°C and allowed to warm up to room temperature. The solvents were removed under reduced pressure and the crude product purified via column chromatography on silica (DCM / methanol 9:1) to give 1.0 g (77.5%) of an off white solid.

IR (KBr; cm^{-1}): 1682.82, 1737.48, 3227.37

^1H NMR (400 MHz; DMSO-d_6): δ = 4.11 (m, 1H), 4.23 (m, 1H), 4.38-4.45 (m, 2H), 4.47 (s, 2H), 6.12 (t, $J=7.6$ Hz, 1H), 6.20 (d, $J=7.6$ Hz, 1H), 6.65 (bs, 1H, OH), 7.86 (d, $J=7.6$ Hz, 1H), 8.80 (s, 1H, NH), 9.89 (s, 1H, NH).

MS (ESI): m/z = 340.

II.2 Example II.2: Synthesis of 5'-bromoisopropyl gemcitabine (GEM-2)

A 100 ml 2-neck round bottom flask was charged with gemcitabine hydrochloride (2.0 g; 6.67 mmol), triethylamine (0.9 ml, 6.66 mmol) and DMF (10 ml) under nitrogen atmosphere. The reaction mixture was cooled to 0-5°C and a solution of 2-bromopropionyl bromide (1.05 ml, 9.99 mmol) in 5 ml of DMF was added dropwise. The reaction mixture was warmed to 20-25°C and stirred for 2 h. The reaction was quenched by adding water

(40 ml) and extracted with DCM (2x40 ml). The combined DCM layer was evaporated at 40°C under vacuum. The residue was purified by column chromatography on silica using 4% methanol in DCM as eluant to furnish the product as off-white solid (550 mg, 17 %; 1.14 mmol).

5

IR (KBr; cm⁻¹): 1680.48, 1735.64, 3393.97

¹H NMR (400 MHz; DMSO-d₆): δ = 1.61 & 1.74 (2><d, 3H, J=6.4 Hz), 4.15 (m, 1H), 4.26 (t, 1H), 4.47-4.50 (m, 2H), 4.81 (m, 1H), 6.11-6.17 (m, 2H), 6.58 (br s, 1H), 7.86 (d, 1H), 8.43 & 9.43 (br s, 2H)

10

MS (ESI): m/z = 398 (M+H)⁺ & 400 (M+2+H)⁺

15 **II.3 Example II.3: Synthesis of 5'-bromoisobutyryl gemcitabine (GEM-3)**

A 100 ml 2-neck round bottom flask was charged with gemcitabine hydrochloride (3.0 g; 10.0 mmol), triethylamine (1.39 ml, 10.0 mmol) and DMF (20 ml) under nitrogen atmosphere. The mixture thus obtained was cooled to 0-5°C and a solution of 2-bromo-2-methylpropionyl bromide (1.2 ml, 10.0 mmol) in DMF (15 ml) was added dropwise over 1 h. The reaction mixture was warmed to 20-25°C and stirred for 1 h followed by evaporation at 50°C. The residue thus obtained was purified by column chromatography on silica using 3 % methanol in DCM to 5 % methanol in DCM to yield gemcitabine 5'-bromoisobutyryl ester, 420 mg (8.5 %; 0.85 mmol).

20

IR (KBr; cm⁻¹): 1733.98, 3328.87

¹H NMR (400 MHz; DMSO-d₆): δ = 1.98 (s, 6H), 4.14 (m, 1H), 4.27 (br s, 1H), 4.44-4.54 (m, 2H), 5.85 (d, 1H), 6.23 (s, 1H), 6.53 (d, 1H), 7.52 (d, 2H), 7.59 (d, 1H)

25

MS (ESI): m/z = 412 (M+H)⁺ & 414 (M+2+H)⁺

30 **II.4 Example II.4: Synthesis of chloroacetyl cytarabine (CYT-1)**

35

a) Preparation of cytarabine hydrochloride

A 50 ml Schlenk flask was charged with cytarabine (2.0 g; 8.22 mmol) and 20 ml of dry methanol. The suspension was stirred and hydrochloric acid (7.0 ml, 1.25 M in methanol;

8.75 mmol) was added under nitrogen. The mixture was stirred for 3 h at room temperature followed by evaporation. The residue was treated with dry diethyl ether in an ultrasonic bath. The ether was removed under vacuum to yield cytarabine hydrochloride (2.07 g; 7.39 mmol; 89.9 %) as a white powder.

5

b) Preparation of title compound

A Schlenk tube was charged with cytarabine hydrochloride (500 mg; 1.79 mmol) and 20 ml of dry DMPU under nitrogen. Chloroacetyl chloride (71 μ l; 893 μ mol) was added at room temperature. The reaction was monitored by HPLC. Portions of chloroacetyl chloride had to be added to complete the reaction (in this case: 12 portions of totally 140 μ l chloroacetyl chloride (**2**) (1.76 mmol)). The reaction mixture was poured into a mixture of diethyl ether and pentane (2:1, totally 600 ml). The precipitate was dissolved in 5 ml of methanol. This solution was added dropwise into 300 ml of ether. The precipitate was dried under vacuum to yield 770 mg of chloroacetyl cytarabine hydrochloride containing residual DMPU as a pale pink solid, which was used in the conjugation step without further purification.

¹H-NMR (DMSO-*d*₆): δ = 9.80 (s, 1H), 8.75 (s, 1H), 7.83 (d, *J*=7.8 Hz, 1H), 6.16 (d, *J*=7.8 Hz, 1H), 6.05 (d, *J*=3.9 Hz, 1H), 4.43 (s, 2H), 4.44-4.34 (m, 2H), 4.09 (dd, *J*=3.9 Hz, *J*=2.6 Hz, 1H), 4.05 (m, 1H), 3.96 (t, *J*=2.8 Hz, 1H)

MS (ESI): *m/z* = 396.04 [*M*+H⁺, ³⁵C1]⁺, 398.03 [*M*+H⁺, ³⁷C1]⁺

25

II.5 Example II.5: Synthesis of maleimidopropyl cytarabine (CYT-2)

a) Preparation of maleimidopropyl chloride

A Schlenk tube was charged with maleimidopropionic acid (2.0 g; 11.82 mmol) and thionyl chloride (4.0 ml; 54.86 mmol). The mixture was heated at reflux for 20 minutes followed by evaporation. The residue was washed three times with each 10 ml of dry pentane. Then it was dried at 10⁻³ mbar to yield maleimidopropionyl chloride (2.17 g; 11.57 mmol; 98 %) as a yellow solid.

35

b) Preparation of title compound

A Schlenk tube was charged with cytarabine hydrochloride (prepared as described above, 509.7 mg; 1.82 mmol) and 17.5 ml of dry DMPU. Maleimidopropionyl chloride

(288.4 mg; 1.54 mmol) was added under nitrogen followed by 1.5 ml of dry DMPU. The resulting suspension was stirred at room temperature to yield a solution after 10 minutes. The reaction was monitored by HPLC. Portions of a maleimidopropionyl chloride solution (prepared from 600 mg of maleimidopropionyl chloride and 600 μ l of DMPU) were added until the HPLC indicated full conversion (625 μ l added). The solution was poured into diethyl ether / pentane (2:1; 600 ml) followed by centrifugation. The residue was dissolved in 10 ml of methanol. This solution was dropped into 300 ml of diethyl ether followed by centrifugation. The residue was dried under vacuum to yield 900 mg of maleimidopropionyl cytarabine hydrochloride containing residual DMPU. The material was used in the conjugation experiments without further purification.

¹H-NMR (DMSO-d₆): δ = 9.84 (s, 1H), 8.75 (s, 1H), 7.81, (d, J =7.8 Hz, 1H), 7.07 (s, 2H), 6.15 (d, J =7.9 Hz, 1H), 6.02 (d, J =3.6 Hz, 1H), 4.30 (dd, J =11.6 Hz, J =8.3 Hz, 1H), 4.18 (dd, J =11.7 Hz, J =4.0 Hz, 1H), 4.05-3.97 (m, 2H), 3.92 (m, 1H), 3.66 (t, J =6.9 Hz, 2H), 2.63 (t, J =7.0 Hz, 2H)

MS (ESI): m/z = 395.12 [M+FT]⁺

11.6 Example 11.6: Synthesis of temsirolimus bromoacetyl monoester (TEM-1)

A 100 ml round bottom flask was charged with temsirolimus (2.0 g; 1.94 mmol) and 15 ml of DCM. The clear solution was cooled to -15°C to -10°C and 4-pyrrolidino pyridine (0.32 g, 2.1 mmol) was added under nitrogen. A solution of bromoacetyl bromide (0.39 g, 1.9 mmol) in 2 ml of DCM was added dropwise (20 minutes) into the reaction. The mixture was stirred further for 40 minutes when TLC analysis indicated formation of three non-polar products. The reaction mixture was diluted with 10 ml of DCM followed by water (5 ml). The DCM layer was separated, dried over MgSO₄ and evaporated under vacuum to give white foam. The crude product was subjected to column chromatography on silica using a gradient of 10 % acetone in hexane to 20% acetone in hexane to furnish temsirolimus bromoacetyl monoester (682 mg, 30 %; 0.59 mmol) as white foam.

IR (KBr; cm⁻¹): 1643.7, 1731.0, 3456.7

MS (ESI): m/z = 1172 (M+Na)⁺ & 1174 (M+2+Na)⁺

11.7 Example 11.7: Preparation of temsirolimus (2-bromopropionyl) monoester (TEM-2)

A 100 ml round bottom flask was charged with temsirolimus (3.0 g; 2.90 mmol) and 15 ml of DCM. The clear solution was cooled to -20°C to -15°C and 4-pyrrolidino pyridine (0.43 g, 2.90 mmol) was added under nitrogen. A solution of 2-bromopropionyl bromide (0.63 g, 2.90 mmol) in 2 ml of DCM was added dropwise (20 minutes) into the reaction.

5 The mixture was stirred further for 40 minutes when TLC analysis indicated formation of three non-polar products. The reaction mixture was diluted with 10 ml of DCM followed by water (5 ml). The DCM layer was separated, dried over MgSO_4 and evaporated under vacuum to give white foam. The crude product was subjected to column chromatography on silica using a gradient of 10 % acetone in hexane to 20 % acetone in hexane to furnish
10 temsirolimus (2-bromopropionyl) monoester (850 mg, 25 %, 0.72 mmol) as white foam.

IR (KBr; cm^{-1}): 1640.56, 1731.4, 3438.7

MS (ESI): $m/z = 1181 (\text{M} + \text{NH}_4)^+ \text{ \& } 1183 (\text{M} + 2 + \text{N}^{3/4})^+$

15

11.8 Example II.8: Preparation of temsirolimus (2-bromoisobutyryl) monoester (TEM-3)

20 A 100 ml round bottom flask was charged with temsirolimus (2.0 g; 1.90 mmol) and 10 ml of DCM. The clear solution was cooled to -20°C to -15°C and 4-pyrrolidino pyridine (0.37 g, 2.40 mmol) was added under nitrogen. The solution of 2-bromo-2-methylpropionyl bromide (0.26 ml, 2.00 mmol) in 2 ml of DCM was added dropwise over a period of 20 minutes. The mixture was stirred further for 30 minutes when TLC analysis indicated
25 formation of three non-polar products. The reaction mixture was diluted with 10 ml of DCM followed by water (5 ml). The DCM layer was separated, dried over MgSO_4 and evaporated under vacuum to give white foam. The crude product was subjected to column chromatography on silica using a gradient of 5 % acetone in hexane to 20 % acetone in hexane to furnish temsirolimus (2-bromoisobutyryl) monoester (1.4 g, 61 %, 1.19 mmol)
30 as white foam.

IR (KBr; cm^{-1}): 1640.7, 1732.7, 3444.0

MS (ESI): $m/z = 1195 (\text{M} + \text{N}^{3/4})^+ \text{ \& } 1197 (\text{M} + 2 + \text{N}^{3/4})^+$

35

11.9 Example II.9: Preparation of temsirolimus 42-methacryloyl monoester (TEM-4)

A 100 ml round bottom flask was charged with temsirolimus (3.0 g; 2.9 mmol) and 15 ml of DCM. The clear solution was cooled to -20°C to -15°C and 4-pyrrolidino pyridine (0.6 g, 4.0 mmol) was added under nitrogen. The solution of methacryloyl chloride (0.32 g, 3.1 mmol) in 2 ml of DCM was added dropwise into the reaction. The mixture was stirred
5 further for 2 h when TLC analysis indicated formation of two non-polar products. The reaction mixture was diluted with 10 ml of DCM followed by water (5 ml). The DCM layer was separated, dried over MgSO₄ and evaporated under vacuum to give white foam. The crude product was subjected to column chromatography on silica using a gradient of 2 % acetone in DCM to 15 % acetone in DCM to furnish temsirolimus 42-methacryloyl
10 monoester (900 mg, 28 %; 0.82 mmol) as white foam.

IR (KBr; cm⁻¹): 1642.1, 1722.9, 3443.9

MS (ESI): m/z = 1115.6 (M+NH₄)⁺

11.10 Example 11.10: Preparation of temsirolimus maleimidopropionyl ester (TEM-5)

A 100 ml 2-neck round bottom flask was charged with temsirolimus (538 mg; 0.52 mmol) and 5 ml of DCM under nitrogen atmosphere. The solution was cooled to -15 to -10°C and a solution of maleimidopropionyl chloride (98 mg, 0.53 mmol) in DCM (5 ml) was added dropwise. The reaction mixture was stirred for 30 minutes and directly subjected to column chromatography on silica using 50 % ethyl acetate in hexane to obtain temsirolimus
25 maleimidopropionyl ester (59 mg, 9 %, 0.04 mmol).

IR (KBr; cm⁻¹): 1641.2, 1715.3, 3448.0

MS (ESI): m/z = 1198.7 (M+NH₄)⁺

11.11 Example 11.11: Synthesis of bromoacetyl-everolimus (EVE-I)

In a 100 ml 3-neck flask equipped with a magnetic stirring bar, a dropping funnel, and a
35 thermometer, 500 mg of everolimus, 94 mg of bromoacetic acid and 32 mg of DMAP were dissolved in 20 ml of dichloroethane. The mixture was cooled to 0°C. 132 mg of diisopropylcarbodiimide (DIC) were dissolved separately in 5 ml of dichloroethane and then added to the reaction mixture under control of the temperature (0°C to 2°C). The reaction was kept at 0°C and monitored by HPLC. After 45 minutes, the reaction mixture

was diluted with 100 ml of DCM and quenched with 100 ml of a NaHCO_3 solution (0.5 %). After the phases were separated, the organic phase was washed with 100 ml of 0.1N HCl solution and 50 ml of brine. The organic phase was dried with sodium sulfate. Afterwards the solvent was evaporated under reduced pressure. The crude product purified by column chromatography on silica (dichloromethane:methanol 60:1) to give 310 mg (0.287 mmol, 55 %) of the title compound as off-white solid.

TLC (DCM:MeOH // 10:1): R_f = 0.55

MS (ESI; MeOH): m/z = 1102.48 [M (^{81}Br) + Na^+]; 1100.95 [M (^{79}Br) + Na^+]

11.12 Example 11.12: Synthesis of maleimidopropyl-everolimus (EVE-2)

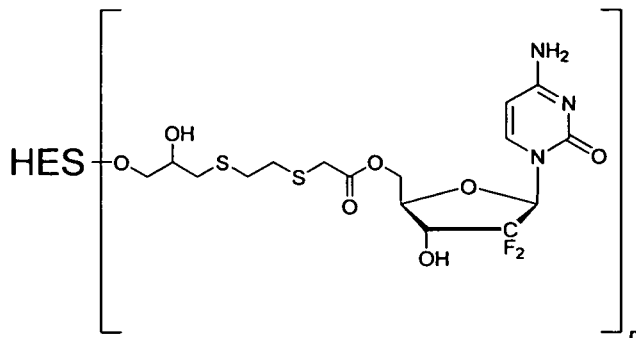
A 100 ml 3-neck flask was equipped with a magnetic stirring bar, a dropping funnel and an inside thermometer. The flask was loaded with 500 mg of everolimus, 115 mg of 3-maleimido-propionic acid and 32 mg of DMAP. The mixture was dissolved in 20 ml of dichloroethane and cooled to 0°C. 0.162 ml of diisopropylcarbodiimide was diluted with 20 ml of DCE and then added to the reaction mixture at 0-2°C. After 2 h at 0°C, the reaction was diluted with 100 ml of DCM and quenched with 100 ml of a 0.5% NaHCO_3 solution. After the phases were separated the organic phase was washed with 100 ml of 0.1 N HCl solution and 50 ml of brine. The organic phase was dried with sodium sulfate and evaporated under reduced pressure. The crude product was purified by column chromatography on silica (DCM:methanol // 60:1) to give the title compound (240 mg, 0.216 mmol, 43 %) as colorless solid.

TLC (DCM:MeOH // 10:1): R_f = 0.6

MS (ESI): m/z = 1131.60 [M + Na] $^+$

III: Synthesis of HES conjugates

111.1 Example III.1: Synthesis of HES-gemcitabine conjugate CGd



5

In a 100 ml round bottom flask equipped with a magnetic stirring bar, 2.5 g of thiol HES (2) were dissolved in 71 ml DMF. 284 mg of chloroacetyl-gemcitabine (3) was added and the resulting solution degassed by purging with inert gas for several minutes. 477 μ L of diisopropyl ethylamine (DIPEA) were added and the reaction mixture was stirred over night at ambient temperature. The reaction was quenched by addition of 1.24 g of iodoacetic acid, stirred for additional 30 minutes and finally poured into 500 ml of cooled isopropanol. The precipitate was collected by centrifugation. The crude conjugate was dissolved in 100 ml water, filtered (0.45 μ m bottle top filter) and purified via size exclusion chromatography. The fractions, containing polymer, were pooled and freeze-dried to yield 2.43 g (97%) of a colorless solid.

Molecular weight: M_w = 104.6 kD; M_n = 60.9 kD; PDI = 1.72.

20 III.2 General procedure for the preparation of HES-gemcitabine conjugates using DIPEA as base (GP 2.1)

In a round bottom flask equipped with a magnetic stirring bar, septum and inert gas inlet, the appropriate thiol-HES derivative was dissolved in DMF under an argon atmosphere. After addition of the drug derivative, argon was bubbled through the solution for several minutes under constant stirring. DIPEA was added and the resulting reaction mixture stirred at room temperature over night. Ethyl bromoacetate was added, stirring was continued for 30 minutes and the reaction mixture poured into isopropanol (~7 times the volume of the solution). The precipitated polymer was collected by centrifugation, dissolved in water, filtered and purified by size exclusion chromatography.

30

III.3 General procedure for the preparation of HES-gemcitabine conjugates using DBU as base (GP 2.2)

5 In a round bottom flask equipped with a magnetic stirring bar, septum and inert gas inlet, the appropriate thiol-HES derivative was dissolved in DMF under an argon atmosphere. After addition of the drug derivative, argon was bubbled through the solution for several minutes under constant stirring. DBU was added and the resulting reaction mixture stirred for 2 h at room temperature. Ethyl bromoacetate was added, stirring was continued for 30 minutes and the reaction mixture poured into isopropanol (~7 times the volume of the solution). The precipitated polymer was collected by centrifugation, dissolved in water, 10 filtered and purified by size exclusion chromatography.

111.4 General procedure for the preparation of HES-gemcitabine conjugates using buffer (GP 2.3)

20 In a round bottom flask equipped with a magnetic stirring bar, septum and inert gas inlet, the appropriate thiol-HES derivative was dissolved in a mixture of DMF and either 0.1 M phosphate buffer pH 7 or 0.04 M citrate buffer pH 6.4 (as indicated in table 5) under an argon atmosphere. Argon was bubbled through the solution for several minutes under constant stirring. The drug derivative was added and the resulting reaction mixture stirred for 2 h at room temperature. Ethyl bromoacetate or iodoacetic acid (as indicated in table 5) was added, stirring was continued for 30 minutes and the reaction mixture poured into isopropanol (~7 times the volume of the solution). The precipitated polymer was collected 25 by centrifugation, dissolved in water, filtered and purified by size exclusion chromatography.

IV. General procedure for the determination of thiol groups (GP3)

30 A stock solution of 4 mg/ml of 5,5'-dithio-bis(2-nitrobenzoic acid), Ellman's reagent, in 0.1 M sodium phosphate buffer + 1 mM EDTA (pH 8) buffer was freshly prepared.

A 0.2 mg/ml solution of sample in buffer was prepared and 1 ml of this solution filled into a 2 ml vial. An additional vial containing 1 ml of plain buffer was used as blank. The 35 samples were treated with 100 μ L of the reagent stock solution, placed into a mixer and mixed at 750 rpm, 21°C for 15 minutes. The sample solutions were transferred into plastic cuvettes (d = 10 mm) and measured for absorbance at 412 nm. The amount of thiols present in the vial was calculated according to the following formula (A = absorbance of sample, A⁰ = absorbance of blank):

$$c[\mu\text{mol/l cm}^3] = \frac{1.1 * (A_{412} - A_{412}^0)}{14.150 \frac{\mu\text{mol}}{\text{cm}^3} * \text{cm}}$$

considering the concentration of 0.2 mg/ml and $1 \text{ cm}^3 = 1 \text{ ml}$:

5

$$\text{Loading}[\text{nmol/mg}] = \frac{1000 * c}{0.2 \frac{\text{mg}}{\text{mL}}}$$

The final value was calculated as the average loading from the three samples.

10 The thiol content of the product of the thio-HES (2) was determined to be 223 nmol/mg.

V. General procedure for the determination of the drug content (GP4)

15 1 ml of a 0.5 mg/ml solution of a HES-drug conjugate in the appropriate solvent was measured at the absorbance maximum (see table 4a) (gemcitabine at 270 nm) in a plastic cuvette ($d = 1 \text{ cm}$) using pure water as blank. The absorption of the blank was subtracted from the conjugate and the drug content calculated as follows:

$$c_{\text{drug}} [\text{pmol/ml}] = \frac{(A_{270} - A_{270}^0)}{10.071 \frac{\text{cm}^2}{\mu\text{mol}} * \text{cm}}$$

20

The molar extinction coefficients were obtained from a calibration curve of the drugs in the specific solvents at the appropriate wavelength.

The loading is calculated as

25

$$\text{Loading} [\mu\text{mol/g}] = \frac{1000 * c_{\text{drug}} [\mu\text{mol/ml}]}{c_{\text{conjugate}} [\text{mg/ml}]}$$

with $c_{\text{conjugate}}$ being the concentration of the sample solution, e.g. 0.5 mg/ml.

30 With a known molecular weight for the drug (e.g. 263.2 g/mol for gemcitabine), the drug loading can also be expressed in mg drug / gram conjugate:

$$\text{Loading [mg/g]} = \text{Loading [\mu mol/g]} * M_w [\text{g/mol}] / 1000$$

The gemcitabine content of CGtl was, e.g., determined to be 163 $\mu\text{mol/g}$ or 42.9 mg/g.

5 Table 4: Extinction coefficients determined from calibration curves in TFE/H₂O and H₂O

#	Drug	Solvent	Wavelength [nm]	ϵ [$\text{cm}^2/\mu\text{mol l}$]	M_w [g/mol]
1	Sirolimus	TFE/H ₂ O 9:1	276	49.458	914.17
2	Gemcitabine	H ₂ O	270	10.022	263.20
3	Temsirolimus	TFE/H ₂ O 9:1	275	46.412	1030.28

10 VI. General procedure for the determination of the mean molecular weight MW (GP5)

The "mean molecular weight" as used in the context of the present invention relates to the weight as determined according to MALLS-GPC (Multiple Angle Laser Light Scattering).

15 For the determination, 2 Tosoh BioSep GMPWXL columns connected in line (13 μm particle size, diameter 7.8 mm, length 30 cm, Art.no. 08025) were used as stationary phase. The mobile phase was prepared as follows: In a volumetric flask 3.74 g Na-Acetate*3H₂O, 0.344 g NaN₃ are dissolved in 800 ml Milli-Q water and 6.9 ml acetic acid anhydride are added and the flask filled up to 1 l.

20 Approximately 10 mg of the hydroxyalkyl starch derivative were dissolved in 1 ml of the mobile phase and particle filtrated with a syringe filter (0.22 μm , mStarII, CoStar Cambridge, MA)

25 The measurement was carried out at a flow rate of 0.5 ml/min.

As detectors a multiple-angle laser light scattering detector and a refractometer maintained at a constant temperature, connected in series, were used.

30 Astra software (Vers. 5.3.4.14, Wyatt Technology Cooperation) was used to determine the mean M_w and the mean M_n of the sample using a dn/dc of 0.147. The value was determined at $\lambda=690$ nm (solvent NaOAc/H₂O/0.02%NaN₃, T=20°C) in accordance to literature (W.M. Kulicke, U. Kaiser, D. Schwengers, R. Lemmes, *Starch*, **Vol. 43**, Issue 10 (1991), 392-396).

35

Table 5a: Synthesis of HES derivatives according to General procedure GPI

Derivative	HES		V (Collidine) [μl]	V (MsCl) [μl]	m (KSAc) [g]	Yield [%]	Loading [nmol/mg]	Mw [kD]	Mn [kD]
	Type	m[g]							
D2	HES2	5.1	1101	324	2.38	96	350.7	106.3	82.1
D3	HES3	5.0	1024	301	2.21	93	331.6	81.1	64.7
D4	HES4	10.0	1915	563	4.13	96	338.5	90.8	63.4
D5	HES5	4.9	1302	382	2.78	92	262.4	344.3	242.4
D6	HES6	5.0	1242	364	2.66	81	245.3	322.0	222.2
D7	HES7*	5.0	1196	352	2.56	95	273.2	355.4	231.8
D8	HES4	10.0	1532	450	3.31	95	241.0	87.9	62.1
D9	HES8	10.0	1928	567	4.95	89	292.5	91.6	46.9
D10	HES8	10.0	1928	567	4.95	56	260.1	85.6	67.5
D11	HES8	27.0	3102	912	6.70	n.d.	169.6	83.3	67.0
D12	HES9	606	68700	20350	304	91	172.0	94.1	67.0

* prepared from a 10% solution of HES in formamide

FK 1011 IPC

Fresenius Kabi Deutschland GmbH

Table 5b: Synthesis of HES conjugates according to general procedures GP2.1-GP2.3

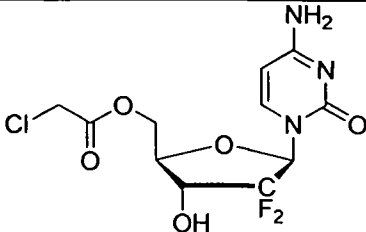
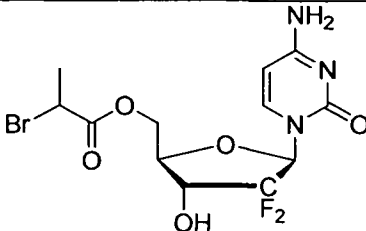
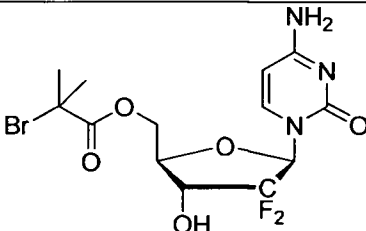
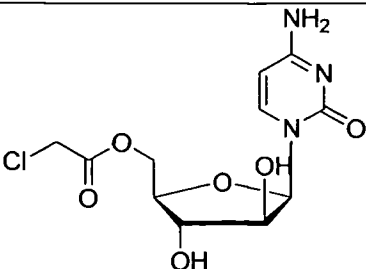
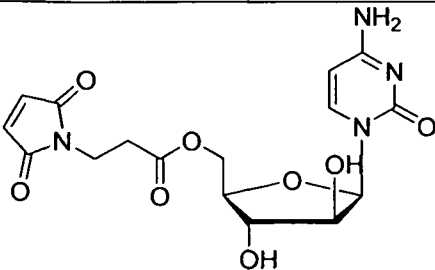
#	Derivative	GP	Cytotox. Derivative	DMF	DIPEA	DBU	Buffer	Reaction time	Capping *	Yield
			m[mg]	V[ml]	V[μl]	V[μl]	V[μl]	h	BrAAee V[μl]	[g]
CGt2	D2	2.1	GEM-1	14.3	150	-	-	o.n.	97.2	0.51
CGt3	D3	2.1	GEM-1	14.3	142	-	-	o.n.	91.9	0.50
CGt4	D4	2.1	GEM-1	14.3	145	-	-	o.n.	93.8	0.48
CGt5	D5	2.1	GEM-1	14.3	112	-	-	o.n.	72.7	0.46
CGt6	D6	2.1	GEM-1	14.3	105	-	-	o.n.	68.0	0.45
CGt7	D7	2.1	GEM-1	14.3	117	-	-	o.n.	75.7	0.46
CGt8	D8	2.2	GEM-3	4.0	-	8.6	-	2	13.4	0.09
CGt9	D9	2.2	GEM-2	20.0	-	131.3	-	2	162.5	1.05
CGt10	D8	2.1	GEM-1	34.3	248	-	-	o.n.	160.3	1.16
CCt1	D8	2.1	CYT-1	34.4	248	-	-	o.n.	160.3	1.09
CCt2	D10	2.3	CYT-2	21.6	-	-	240 ^a	2	173.0	1.20
									IAA [mg]	
CTm1	D11	2.2	TEM-1	50.5	-	-	4840 ^b	4	832.4	2.30
CTm2	D11	2.2	TEM-2	50.0	-	69.7	-	2	945.9	2.60
CTm3	D12	2.3	TEM-3	19.8	-	-	2200 ^a	2.5	319.8	0.89
CTm4	D12	2.0	TEM-4	40	-	77.2	-	2	767.6	2.00
CTm5	D11	0.1	TEM-5	2.0	-	2.8	-	2	37.8	0.07
CEv1	D12	2.3	EVE-2	18	-	-	2000 ^b	2	383.8	0.90
CEv2	D12	2.3	EVE-1	27.6	-	-	2640 ^a	4	460.6	1.13

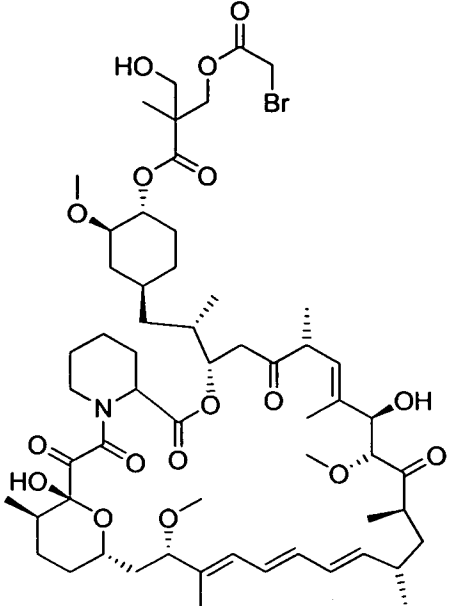
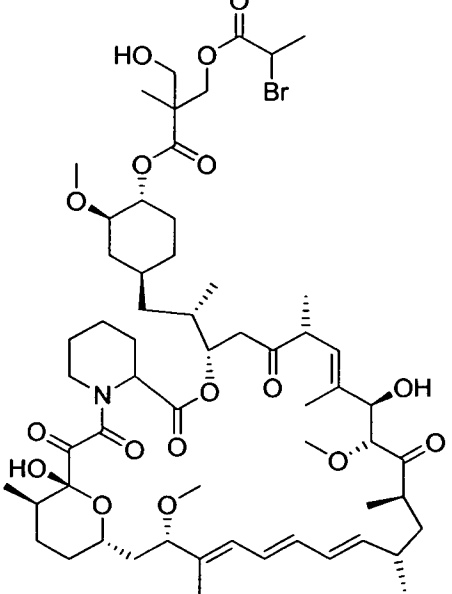
^a phosphate buffer pH 7 used; ^b citrate buffer pH 6.4 used. * BrAAee = ethyl bromoacetate, IAA = iodoacetic acid

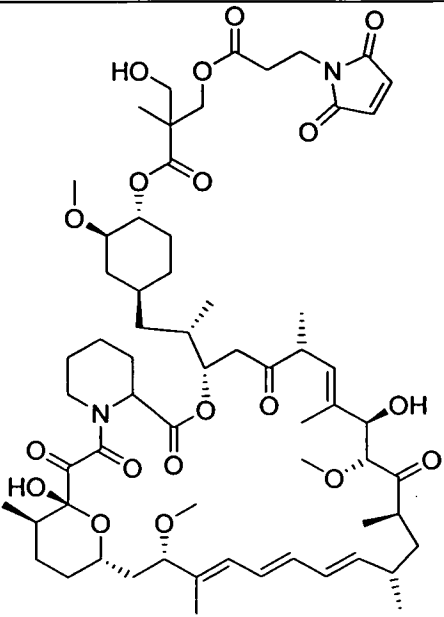
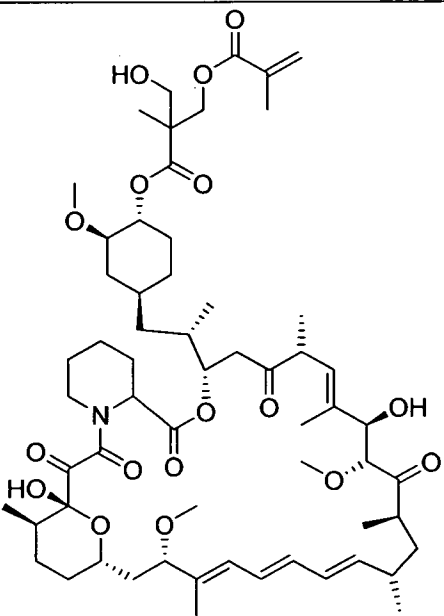
Table 5c: *Characterization of HEs conjugates*

#	Purity ^a [%]	Loading		Mw [kD]	Mn [kD]
		[mg drug/g]	[μ mol/g]		
CGt2	>99.9	60.0	228	121.7	88.4
CGt3	>99.9	57.0	217	98.5	70.4
CGt4	>99.9	56.0	213	112.6	70.8
CGt5	>99.9	45.6	173	339.6	246.4
CGt6	>99.9	44.0	167	331.3	226.7
CGt7	>99.9	46.1	175	353.9	248.3
CGt8	>99.9	30.7	117	99.0	65.2
CGt9	99.9	53.9	205	104.3	52.9
CGt10	>99.9	51.7	196	96.6	64.2
CCt1	99.7	42.7	176	101.7	65.7
CCt2	99.8	45.0	185	155.3	90.6
CTm1	96.8	123.4	120	223.5	204.9
CTm2	96.4	110.9	108	265.8	228.0
CTm3	98.0	65.3	63	3707	713.6
CTm4	97.4	61.3	60	336.3	112.9
CTm5	95.4	93.6	91	238.6	200.6
CEv1	99.2	93.9	98	276.7	150.9
CEv2	99.9	109.6	114	276.7	150.9

Table 6 Overview over synthesized drug- derivatives

Code	Name	Formula
GEM-1	5'-chloroacetyl-gemcitabine	
GEM-2	5'-(2-bromopropionyl)-gemcitabine	
GEM-3	5'-(2-isobutyryl)-gemcitabine	
CYT-1	5'-chloroacetyl-cytarabine	
CYT-2	5'-(3-maleimidopropionyl)-cytarabine	

TEM-1	mono-bromoacetyl- temsirolimus	 The chemical structure of mono-bromoacetyl-temsirolimus is a complex macrocyclic molecule. It features a central 14-membered ring with multiple stereocenters. A bromoacetyl group (-CH₂COCH₂Br) is attached to the macrocycle via an ester linkage. The structure includes several oxygen-containing functional groups, including hydroxyl groups and ether linkages, and a long unsaturated side chain.
TEM-2	mono-(2-bromopropionyl)- temsirolimus	 The chemical structure of mono-(2-bromopropionyl)-temsirolimus is a complex macrocyclic molecule, similar to the one in TEM-1. It features a central 14-membered ring with multiple stereocenters. A 2-bromopropionyl group (-CH₂CH(Br)CO-) is attached to the macrocycle via an ester linkage. The structure includes several oxygen-containing functional groups, including hydroxyl groups and ether linkages, and a long unsaturated side chain.

TEM-3	mono-(3-maleimidopropionyl)- temsirolimus	 The chemical structure of mono-(3-maleimidopropionyl)-temsirolimus is shown. It features a complex polycyclic core with multiple stereocenters, including a piperidine ring, a tetrahydropyran, and a long unsaturated side chain. The structure is modified with a 3-maleimidopropionyl group at the C-3 position, which is attached via an ester linkage to a hydroxyl group. The maleimide ring is a five-membered heterocycle with two carbonyl groups and a nitrogen atom.
TEM-4	mono-metacroyl- temsirolimus	 The chemical structure of mono-metacroyl-temsirolimus is shown. It features a complex polycyclic core with multiple stereocenters, including a piperidine ring, a tetrahydropyran, and a long unsaturated side chain. The structure is modified with a metacroyl group at the C-3 position, which is attached via an ester linkage to a hydroxyl group. The metacroyl group is a three-carbon chain with a terminal vinyl group and a carbonyl group.

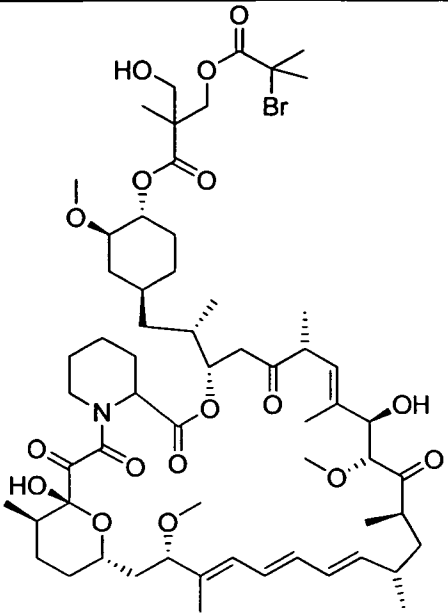
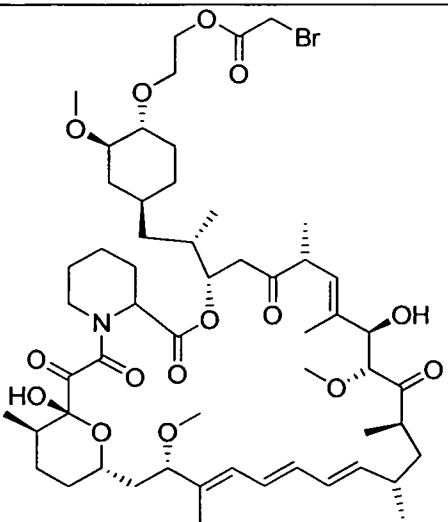
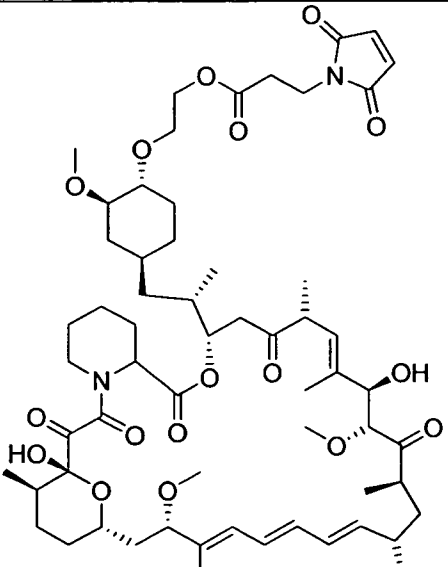
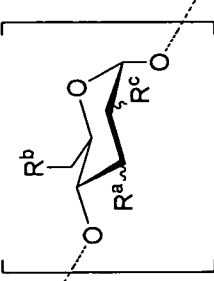
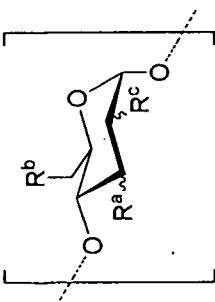
TEM-5	mono-(2-isobutyryl)- temsirolimus	 The chemical structure of mono-(2-isobutyryl)-temsirolimus features a complex macrocyclic core. This core includes a piperidine ring, a cyclohexane ring with a methoxy group, and a long chain with multiple double bonds and hydroxyl groups. Attached to the cyclohexane ring is a side chain containing a 2-isobutyryl group, which is a four-carbon chain with a branched isobutyryl moiety at the end.
EVE-1	bromoacetyl-everolimus	 The chemical structure of bromoacetyl-everolimus is similar to the previous one, with the same macrocyclic core. However, the side chain attached to the cyclohexane ring is a bromoacetyl group, consisting of a two-carbon chain ending in a bromine atom.
EVE-2	3-maleimidopropionyl- everolimus	 The chemical structure of 3-maleimidopropionyl-everolimus features the same macrocyclic core. The side chain attached to the cyclohexane ring is a 3-maleimidopropionyl group, which is a three-carbon chain ending in a maleimide ring (a five-membered ring with two carbonyl groups and a nitrogen atom).

Table 7: Overview of synthesized hydroxyethyl starch derivatives

Code	Structure		
	HES used	Structure of HES derivative	Cytotoxic agent M
		 with at least one of R^a, R^b or R^c of the shown structural unit being: ...[O-CH₂-CH₂]-[F¹]_p-[L¹]_{0,1}-Z¹ with t = 1-4 and with -[F¹]_p-[L¹]_{0,1}-Z¹ being: -O-CH₂CH(OH)-CH₂-S-(CH₂)₂-SH	
D1	HES 1	-O-CH ₂ CH(OH)-CH ₂ -S-(CH ₂) ₂ -SH	---
D2	HES 2	-SH	---
D3	HES 3	-SH	---
D4	HES 4	-SH	---
D5	HES 5	-SH	---
D6	HES 6	-SH	---
D7	HES 7	-SH	---
D8	HES 4	-SH	---
D9	HES 8	-SH	---
D10	HES 8	-SH	---
D11	HES 8	-SH	---
D12	HES 9	-SH	---

Code	Structure			Cytotoxic agent <i>M</i>
	HES used	Structure of HES derivative	Linking moiety <i>L</i>	
		 with at least one of R^a, R^b or R^c of the shown structural unit being: $\cdots[\text{O}-\text{CH}_2-\text{CH}_2]_t-[\text{F}^1]_p-[\text{L}^1]_{0,1}-\text{X}-$ with $t = 1 - 4$ and with $-\text{[F}^1\text{]}_p-[\text{L}^1]_{0,1}-\text{X}-$:		
CGt1	HES 1	-O-CH ₂ -CH(OH)-CH ₂ -S-(CH ₂) ₂ -S-	-CH ₂ -C(=O)-	5'-GEM
CGt2	HES 2	-S-	-CH ₂ -C(=O)-	5'-GEM
CGt3	HES 3	-S-	-CH ₂ -C(=O)-	5'-GEM
CGt4	HES 4	-S-	-CH ₂ -C(=O)-	5'-GEM
CGt5	HES 5	-S-	-CH ₂ -C(=O)-	5'-GEM
CGt6	HES 6	-S-	-CH ₂ -C(=O)-	5'-GEM
CGt7	HES 7	-S-	-CH ₂ -C(=O)-	5'-GEM
CGt8	HES 4	-S-	-CH(CH ₃)-C(=O)-	5'-GEM
CGt9	HES 8	-S-	-C(CH ₃) ₂ -C(=O)-	5'-GEM
CGt10	HES 4	-S-	-CH ₂ -C(=O)-	5'-GEM
CCt1	HES 4	-S-	-CH ₂ -C(=O)-	5'-CYT

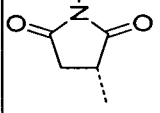
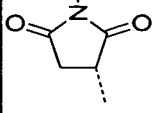
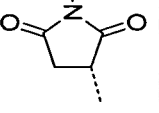
CCt2	HES 8	-S-	 N-CH ₂ -CH ₂ -C(=O)-	5'-CYT
CTm1	HES 8	-S-	-CH ₂ -C(=O)-	TEM
CTm2	HES 8	-S-	-CH(CH ₃)-C(=O)-	TEM
CTm3	HES 9	-S-	 N-CH ₂ -CH ₂ -C(=O)-	TEM
CTm4	HES 9	-S-	-CH ₂ -CH(CH ₃)-C(=O)-	TEM
CTm5	HES 8	-S-	-C(CH ₃) ₂ -C(=O)-	TEM
CEv1	HES 9	-S-	-CH ₂ -C(=O)-	EVE
CEv2	HES 9	-S-	 N-CH ₂ -CH ₂ -C(=O)-	EVE

Table 8: Overview of synthesized Hydroxyethyl starch - drug conjugates

B In vivo testing - Gemcitabine

1.1 Test animals

- 5 Adult female NMRJ:nu/nu mice (TACONIC Europe, Lille Skensved, Denmark) bred in the own (EPO) colony were used throughout the study. At the start of experiment they were 6-8 weeks of age and had a median body weight of 19.0 to 32.6 g.

- 10 All mice were maintained under strictly controlled and standardized barrier conditions. They were housed —maximum five mice/cage - in individually ventilated cages (Macrolon Typ-II, system Techniplast, Italy). The mice were held under standardized environmental conditions: $22 \pm 1^\circ\text{C}$ room temperature, $50 \pm 10\%$ relative humidity, 12 hour-light-dark-rhythm. They received autoclaved food and bedding (Ssniff, Soest, Germany) and acidified (pH 4.0) drinking water ad libitum.

- 15 Animals were randomly assigned to 12 experimental groups with 8 mice each. At treatment initiation the ears of the animals were marked and each cage was labeled with the cage number, study number and animal number per cage.

- 20 Table 9 provides an overview of the animal conditions.

Table 9 Summary of animal conditions

Subject	Conditions
Animals, gender and strain	female NMRI:nu/nu mice
Age	6-8 weeks
Body weight	19.0 to 32.6 g at the start of treatment
Supplier	EPO
Environmental Conditions	Strictly controlled and standardised barrier conditions, IVC System Techniplast DCC (TECNIPLAST DEUTSCHLAND GMBH, Hohenpeißenberg)
Caging	Macrolon Type-II wire-mesh bottom,
Feed type	Ssniff NM, Soest, Germany
Drinking water	autoclaved tap water in water bottles (acidified to pH 4 with HCl)
Feeding and	ad libitum 24 hours per day

drinking time	
Room temperature	22±1°C
Relative humidity	50±10%
Light period	artificial; 12-hours dark/12 hours light rhythm (light 06.00 to 18.00 hours)
Health control	The health of the mice was examined at the start of the experiment and twice per day during the experiment.
Identification	Ear mark and cage labels

1.2 Tumor model

5 Table 10

Name	tumor model	ATCC number	described in
ASPC-1	human pancreas carcinoma	CRL-1682	Tan, MH, et al. J. Natl. Cancer Inst. 67:563-569 (1981).

The human pancreas carcinoma ASPC-1 was used as s.c. xenotransplantation model in immunodeficient female NMRI:nu/nu mice.

10

The cells were obtained from ATCC and are cryo-preserved within the EPO tumor bank. They were thawed, expanded *in vitro* and transplanted as cell suspension subcutaneously (s.c.) in female NMRI:nu/nu mice. The tumor line ASPC-1 is used for testing new anticancer drugs or novel therapeutic strategies. It was therefore selected for this study.

15

ASPC-1 xenografts are growing relatively fast and uniform.

Experimental procedure

For experimental use 10^7 tumor cells/mouse from the *in vitro* passage were transplanted s.c. into the flank of each of 10 mice/group at day 0.

20

Treatment

At palpable tumor size (30-100 mm³) treatment started. The application volume was 0.2 ml/20g mouse body weight. The test compounds, the vehicle controls and the reference compounds were all given intravenously (i.v.).

25

1.3 Therapeutic evaluation

Tumor growth inhibition was used as therapeutic parameter. Additionally, body weight change was determined as signs for toxicity (particularly, potential hematological or gastrointestinal side effects).

Tumor measurement

Tumor diameters were measured twice weekly with a caliper. Tumor volumes were calculated according to $V = (\text{length} \times (\text{width})^2)/2$. For calculation of the relative tumor volume (RTV) the tumor volumes at each measurement day were related to the day of first treatment. At each measurement day the median and mean tumor volumes per group and also the treated to control (T/C) values in percent were calculated (Tables 11-13).

Body weight

Individual body weights of mice were determined twice weekly and mean body weight per group was related to the initial value in percent (body weight change, BWC).

End of experiment

On the day of necropsy mice were sacrificed by cervical dislocation and inspected for gross organ changes.

Statistics

Descriptive statistics were performed on the data of body weight and tumor volume. These data are reported in tables as median values, means and standard derivations, see Tables 11-13. Statistical evaluation was performed with the U-test of Mann and Whitney with a significance level of $p \leq 0.05$, using the Windows program STATISTICA 6.

1.4 Analysis of the effects of Gemcitabine conjugates on tumor growth and body weight

1.4.1 Tested substances

All Gemcitabine-conjugates were stored in a freeze-dried form at -20°C until use. Solutions were prepared immediately before injection by solving the conjugates in saline solution by vortexing in combination with centrifugation until a clear solution of the necessary concentration of the drug was obtained.

All solutions were prepared and injected under sterile conditions.

Gemcitabine (Gemzar ®, charge A4781790 200 mg) was obtained from Lilly Deutschland GmbH and was stored in the dark at -20°C until use. The final solution of Gemzar® was prepared immediately before injection by mixing the appropriate volume of the original stock solution (200 mg) with saline (0.9%, infusion solution, Ch.-Nr 0205A231, B. Braun Melsungen AG, Germany).

As a further control, saline solution was intravenously administered.

10

1.4.2 Test results

The results summarized in tables 11-13 (figures 1-4, 13-14) reveal that HES-gemcitabine conjugates show their anti-tumor effect in dramatically lower concentrations compared to the unconjugated drug (3-7.5 mg/kg compared to 60 mg/kg for gemcitabine). The results obtained for conjugates CGtI, CGtIO and CGt7 demonstrate a comparable to slightly better performance of the conjugates with an 8-12 fold reduced dose. A slower releasing ester linker (CGt9) allows the application of more than twice the dose (7.5 mg/kg compared to 3 mg/kg for CGtIO) without signs of toxicity and with a better performance than native gemcitabine.

20

1.5 In vivo testing - Temsirolimus and Everolimus

The athymic nude mouse is immunodeficient, thus enabling the xenotransplantation and growth of human tumors. Subcutaneous tumor implantation is a well-described methodology allowing visualization and quantification of tumor growth.

Specific Information :

30 Mouse strain: NMRI nu/nu, female

Animals supplied by: Charles River, Germany

Age of mice at implantation: 5-7 weeks

35 Animal Health and Monitoring:

All experiments were conducted according to the guidelines of the German Animal Welfare Act (Tierschutzgesetz). Animal health was examined prior to tumor implantation and randomization to ensure that only animals without any symptoms of disease were

selected to enter testing procedures. During the experiments, animals were monitored daily regarding tumor burden, general condition, feed and water supply.

Animal Identification:

- 5 Animals were arbitrarily numbered during tumor implantation using ear clips. At the beginning of the experiments, each cage was labelled with a record card indicating the experiment number, date of tumor implantation, date of randomization, tumor type, tumor number and passage, mouse strain, gender, and individual mouse numbers. After randomization, the group identity, test compound, dosage, schedule, and route of
10 administration were added.

Housing Conditions

- The animals were housed in autoclaved individually ventilated cages (TECNIPLAST Sealsafe™-IVC, TECNIPLAST, Hohenpeissenberg, Germany). Depending on group size,
15 they were housed in either type III cages or type II long cages. Dust-free bedding Lignocel® PS 14 was used (ssniff Spezialdiäten GmbH, Soest, Germany). The cages including the bedding were changed weekly. The temperature inside the cages was maintained at $25 \pm 1^\circ\text{C}$ with a relative humidity at $60 \pm 10\%$. The animals were kept under a natural daylight cycle.

20

Diet and Water Supply

- The animals were fed autoclaved ssniff NM complete feed for nude mice (ssniff Spezialdiäten GmbH, Soest, Germany) and had access to sterile filtrated and acidified (pH 2.5) tap water. Bottles were autoclaved prior to use; they were changed twice a week. Food
25 and water were provided ad libitum.

1.5.1 Tumor models

- The tumor xenografts LXFL-529 (Fiebig HH, Berger DP, Dengler WA, Wallbrecher E, Winterhalter BR: Combined In Vitro/In Vivo Test Procedure with Human Tumor Xenografts for New Drug Development. Contrib. Oncol., Basel, Karger, 1992, Vol. 42, pp 321-351) used in this study were derived from surgical specimen from patients treated at the University Hospital in Freiburg, Germany, and directly implanted into nude mice. Prior to surgery, most of the patients had not received any chemotherapy.

35

Following their primary implantation into nude mice (passage 1), the tumor xenografts were passaged until establishment of stable growth patterns. Master stocks of early passage xenografts were then frozen in liquid nitrogen. Usually, a particular master stock batch

itself is only used for maximally 30 passages. Therefore, the xenografts closely reflect the initial primary histology.

Tumor fragments were obtained from xenografts in serial passage in nude mice. After removal from donor mice, tumors were cut into fragments (4-5 mm diameter) and placed in PBS until subcutaneous implantation. Recipient mice were anaesthetized by inhalation of isoflurane. A small incision was made in the back and one tumor fragment per animal was transplanted with tweezers. The mice were monitored daily.

At randomization, tumor-bearing animals were stratified according to tumor volume into treatment and vehicle (control) groups. Only animals carrying one tumor of appropriate size (approximately 50-250 mm³) were considered for randomization. Mice were randomized when the required number of mice qualified for randomization. The day of randomization was designated as day 0, which was also the first day of dosing.

1.5.2 Sample preparations

All test items were formulated in 0.9% NaCl solution and given as i.v. bolus injection despite everolimus, which was given *per oral*.

Everolimus (Lot. 1101012750e was purchased from Sequoia Research Products. Individual treatment schedules and results can be extracted from table 14.

1.5.2 Therapeutic evaluation

Measurement of tumor volume and body weight as well as calculations of relative tumor volumes were carried out analogue to the procedure described in 1.3.

1.5.3 Test results:

The results compiled in table 14 reveal that both HES-everolimus conjugates perform comparably to the native drug without any signs of toxicity. In the first 7 days of the experiments, both conjugates result in a higher tumor growth inhibition than the native drug. As for temsirolimus, all conjugates show a distinct advantage in tumor growth inhibition without any observable toxicity. Compared to the faster releasing conjugate CTm1, both slower releasing conjugates CTm2 and CTm3 show a slight additional advantage.

Table 11: Summary of the results for the CGtI -conjugate

Substance	Mice n	Treatment (Day)	Dose (mg/kg/inj.)	Group sacrif. (at day)	Tumor volume cm ³ /d44	RTV T/C (%) Optimum (at day)	WBC x10 ⁶ /ml (at day 13)	Platelets x10 ⁶ /ml (at day 13)
Saline	8	9,13,16,20,23, 27,30,34,40	-	44	0,577+/-0,246	-	7,84+/-1,40	1221+/-87
Gemcitabine	8	9,13,16,20,23, 27,30,34,40	60	44	0,272+/-0,119*	41,4 (36)	5,22+/-1,47*	915+/-105*
CGtI	8	9,16,23,30, 34, 40	7,5	44	0,307+/-0,107*	43,4 (27)	6,66+/-2,78	918+/-77*

WBC = white blood cell count

Table 12: Summary of the results for the conjugates CGt2-CGt6

Substance	Mice n	Dose (mg/kg/inj.)	Days of Treatment	Number of treatments	Group sacrif. (at day)	Toxic death (at day)	BWC [%] (at day)	Tumor volume cm ³ /d28	RTV T/C (%) Optimum (at day)
Saline	8	-	7,10,12,14,17,19,21,25	8	28			0.674+/-0.270	-
Gemcitabine (Gemzar®)	8	60	7,10,12,14,21,25,28,31	8	34	2 (d15, d18)	-15 (17)	0.159+/-0.058*	23.6 (28)
CGt2	8	7.5	7,10,12,14,17,19,21, 25,28	9	34		0	0.281+/-0.101*	41.8 (28)
CGt3	8	7.5	7,10,17,21,25,28,31	7	34		-6 (12)	0.146+/-0.037*	21.7 (28)
CGt5	8	7.5	7,10,12,14,17,19, 25,28,31	9	34		-6 (21)	0.147+/-0.050*	21.7 (28)
CGt6	8	7.5	7,10,21,25,28	5	34	1 (d14)	-21 (14)	0.225+/-0.145*	33.4 (28)

* significantly different to saline, p<0.05

Table 13: Summary of the results for the conjugates CGtIO, CGt 7 and CGt9

Substance	Mice n	Dose (mg/kg/inj.)	Days of Treatment	Number of treatments	Mortality (at day)	BWC [%] (at day)	Tumor volume cm ³ /d27	RTV T/C (%) d27
Saline	9				-	-	0.873 +/- 0.319	-
Gencitabine (Gemzar®)	9	60	7,14,21	3	-	-2.4 (9)	0.480 +/- 0.317	55.1
CGtIO	9	3	7,14,21	3	-	-1.2 (9)	0.464 +/-0.246	53.3
CGt7	9	3	7,14,21	3	-	-3.7 (9)	0.354 +/-0.106	40.7
CGt9	9	7,5	7,14,21	3	-	-2.9 (9)	0.238 +/-0.090	27.3

FK101 1IPC

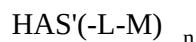
Fresenius Kabi Deutschland GmbH

Table 14: Summary of the results for the everolimus and temsirolimus conjugates

Substance	Mice n	Days of Treatment (dose mg/kg)	Mortality (at day)	BWC [%] (at day)	Tumor volume mm ³ (d14)	RTV T/C optimum [%]
Saline	5	0,3,7,14	1 (14)	-	1927.7	-
Everolimus	5	0,3 (10 mg/kg); 7,10, 14 (15 mg/kg) p.o.	1 (14)	-	926.1	53.9 (d14)
CEv1	5	0,3 (10 mg/kg); 7,10, 14 (15 mg/kg) i.v.	-	-	767.1	55.9 (d14)
CEv2	5	0,3 (10 mg/kg); 7,10, 14 (15 mg/kg) i.v.	-	-	892.1	46.2 (d14)
Temsirolimus	5	0,3,7,10 (20 mg/kg)	1 (13)	-	1073.7	57.1 (d10)
CTm1	5	0,3,7,10 (20 mg/kg)	-	-	651.8	37.2 (d14)
CTm2	5	0,3,7,10 (20 mg/kg)	-	-0.4	572.2	31.8 (d14)
CTm3	5	0,3,7,10 (20 mg/kg)	-	-	928.3	33.5 (d14)

Claims

1. A hydroxyalkyl starch (HAS) conjugate comprising a hydroxyalkyl starch derivative and a cytotoxic agent, said conjugate having a structure according the following formula



wherein

M is a residue of a cytotoxic agent, wherein the cytotoxic agent comprises a primary hydroxyl group,

L is a linking moiety,

HAS' is a residue of the hydroxyalkyl starch derivative,

n is greater than or equal to 1,

wherein the hydroxyalkyl starch derivative has a mean molecular weight MW above the renal threshold, preferably MW greater than or equal to 60 kDa,

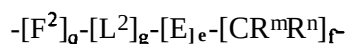
and a molar substitution MS in the range of from 0.6 to 1.5,

and wherein the linking moiety L is linked to a primary hydroxyl group of the cytotoxic agent.

2. The conjugate according to claim 1, wherein the hydroxyalkyl starch conjugate is a hydroxyethyl starch (HES) conjugate comprising a hydroxyethyl starch derivative.
3. The conjugate according to claim 1 or 2, wherein the hydroxyalkyl starch derivative has a mean molecular weight MW in the range of from 80 to 1200 kDa, preferably in the range of from 90 to 800 kDa.
4. The conjugate according to any of claims 1 to 3, wherein the hydroxyalkyl starch derivative has a molar substitution MS in the range of from 0.70 to 1.45, preferably in the range of from 0.80 to 1.40, more preferably in the range of from 0.90 to 1.35.
5. The conjugate according to any of claims 1 to 4, wherein the linking moiety L has a structure $-\text{L}'-\text{F}^3-$, wherein F^3 is a functional group linking L' to M via the group $-\text{O}-$ derived from the primary hydroxyl group of the cytotoxic agent, thereby forming a group $-\text{F}^3-\text{O}-$, preferably wherein F^3 is a $-\text{C}(=\text{Y})-$ group, with Y being O, NH or S, preferably with Y being O or S, and wherein L' is a linking moiety.

6. The conjugate according to claim 5, wherein the bond between the functional group -F³- and the functional group -O- of the residue of the cytotoxic agent M is a cleavable linkage, which is capable of being cleaved *in vivo* so as to release the cytotoxic agent, wherein the functional group -O- is derived from the primary hydroxyl group of the cytotoxic agent.

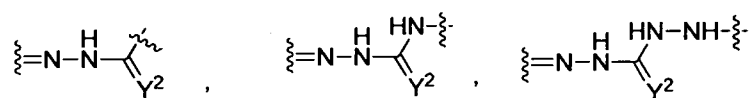
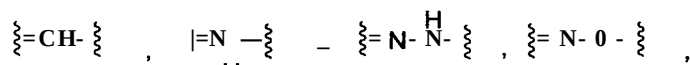
7. The conjugate according to claim 5 or 6, wherein L' has a structure according to the following formula



wherein E is an electron-withdrawing group, preferably selected from the group consisting of -O-, -S-, -SO-, -SO₂-, -NR^e-, succinimide, -C[^]Y*), -NR^e-C(=Y^e)-, -C(=Y^e)-NR^e-, -NO₂ comprising groups, -CN comprising groups, aryl moieties or an at least partially fluorinated alkyl moiety, wherein Y^e is either O, S or NR^e, and R^e is hydrogen or alkyl,

preferably wherein E is selected from the group consisting of -NH-C(=O)-, -C(=O)-NH-, -NH-, -O-, -S-, -SO-, -SO₂- and -succinimide-,

F² is selected from the group consisting of -Y¹-, -C(=Y²)-, -C(=Y²)-NR^{F2}-,



and -CH₂-CH₂-C(=Y²)-NR^{F2}-,

wherein Y¹ is selected from the group consisting of -S-, -O-, -NH-, -NH-NH-, -CH₂-CH₂-SO₂-NR^{F2}-, -CH₂-CHOH-, and cyclic imides, such as succinimide, and

wherein Y² is selected from the group consisting of NH, S and O, and wherein R^{F2} is selected from the group consisting of hydrogen, alkyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl group,

L² is a linking moiety, preferably an alkyl, alkenyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl group,

f is in the range of from 1 to 20,

g is 0 or 1,

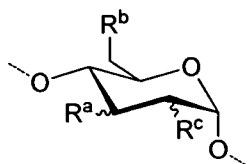
q is 0 or 1,

e is 0 or 1,

and wherein R^m and R^n are, independently of each other, H, alkyl, aryl or a side chain of a natural or unnatural amino acid, preferably H or alkyl, more preferably H or methyl.

5

8. The conjugate according to any of claims 1 to 7, wherein the hydroxyalkyl starch derivative comprises at least one structural unit according to the following formula (I)



(I)

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wherein R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS", $-[O-(CR^wR^xHCR^yR^z)]_x-OH$, $-[O-(CR^wR^x)-(CR^yR^z)]_y-X$, $-[O-(CR^wR^xMCR^yR^z)]_y-[F^1]_p-L^1-X$,

wherein R^w , R^x , R^y and R^z are independently of each other selected from the group consisting of hydrogen and alkyl,

15

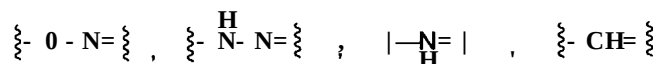
y is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4, and

x is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4,

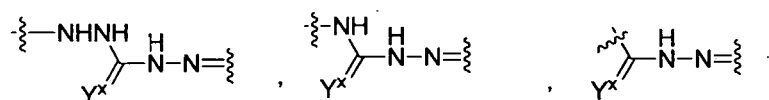
and wherein at least one of R^a , R^b and R^c is $[O-(CR^wR^x)-(CR^yR^z)]_y-X$ or

$-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^1-X$,

wherein X is selected from the group consisting of $-Y^{5x}$, $-C(=Y^{5x})$, $-C(=Y^{5x})-NR^{xx}$,



20



and $-\text{CH}_2-\text{CH}_2-\text{C}(=Y^{5x})-NR^{xx}$,

wherein Y^{**} is selected from the group consisting of -S-, -O-, -NH-, -NH-NH-,

$-\text{CH}_2-\text{CH}_2-\text{S}O_2-NR^{xx}$, and cyclic imides, and wherein Y^x is selected from the group

consisting of NH, S and O, and wherein R^{**} is selected from the group consisting of

25

hydrogen, alkyl, alkenyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl and heteroarylalkyl groups,

X preferably being -S-,

F^1 is a functional group, preferably selected from the group consisting of $-Y^7$,

$-Y^7-C(=Y^6)-$, $-C(=Y^6)-$, $-Y^7-C(=Y^6)-Y^8-$,

30

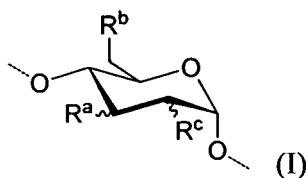
with Y^7 and Y^8 being, independently of each other, selected from the group consisting of -NH-, -O- and -S-, and wherein Y^6 is O, NH or S,

and wherein p is 0 or 1,

L^1 is a linking moiety,

and wherein HAS'' is a remainder of HAS.

- 5 9. The conjugate according to any of claims 1 to 8, wherein the hydroxyalkyl starch derivative comprises at least one structural unit according to the following formula (I)



wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS''$, $-[O-CH_2-CH_2]_s-OH$, $-[O-CH_2-CH_2]_t-X$ and

10 $-tO-CH_2-CH_2-r[F^1]_p-L^1-X$,

and wherein s is in the range of from 0 to 4,

and wherein t is in the range of from 0 to 4,

p is 0 or 1,

wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-X$ or $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X$,

15

and wherein HAS'' is a remainder of HAS.

10. The conjugate according to claim 8 or 9, wherein the linking moiety L^1 is an alkyl, alkenyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl group.

20

11. The conjugate according to claim 9 or 10, wherein at least one of R^a , R^b and R^c is

(i) $-[O-CH_2-CH_2]_t-X$, or

(ii) $-[O-CH_2-CH_2]_t-r[F^1]_p-L^1-X$, and wherein p is 1 and $-F^1$ is $-O-$, or

(iii) $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-X$, and wherein p is 1 and $-F^1$ is $-O-C(=O)-NH-$.

25

wherein X is S,

and wherein t is in the range of from 0 to 4.

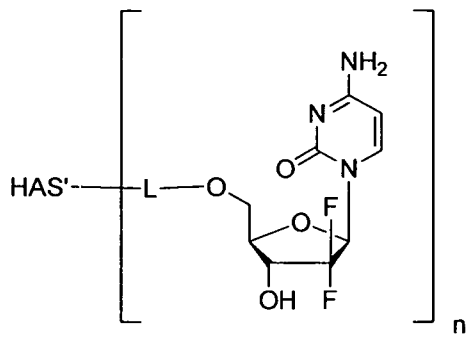
12. The conjugates according to any of claims 1 to 11, wherein the cytotoxic agent is an antimetabolite, more preferably a nucleoside analogue, most preferably a cytidine analogue.

5

13. The conjugates according to any of claims 1 to 12, wherein the cytotoxic agent is selected from the group consisting of clofarabine, nelarabine, cytarabine, cladribine, decitabine, azacitidine, floxuridine, pentostatin and gemcitabine.

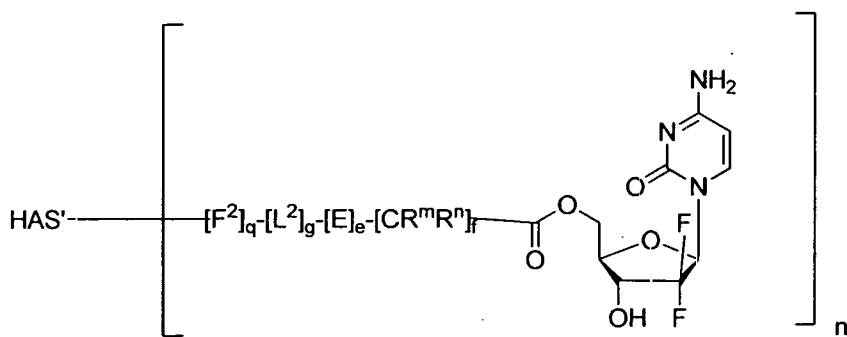
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14. The conjugate according to claims 1 to 13, wherein the conjugate has a structure according to the following formula:



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15. The conjugate according claim 7, said conjugate having a structure according to the following formula

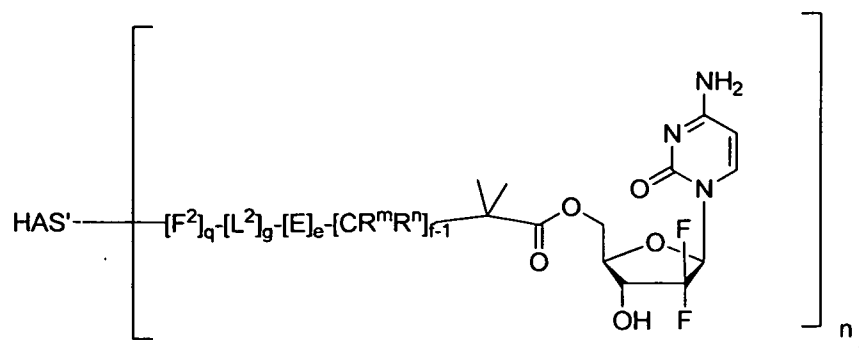


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16. The conjugate according to claim 15, wherein at least one of R^m or R^n of at least one repeating unit of the structural unit $-\left[\text{CR}^m\text{R}^n \right]_r$ is an alkyl group.

25

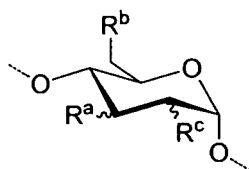
17. The conjugate according to claim 16, the conjugate having a structure according to the following formula



wherein R^m and R^n are, independently of each other, H or alkyl.

5

18. The conjugate according to any of claims 14 to 17, wherein HAS' comprises at least one structural unit according to the following formula (I)



(I)

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wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS''$, $-[O-CH_2-CH_2]_s-OH$ and $-[O-CH_2-CH_2]_t-X-$,

wherein s is in the range of from 0 to 4,

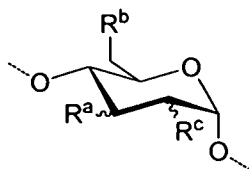
wherein t is in the range of from 0 to 4

wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-X-$, wherein X is $-S-$,

15

and wherein HAS'' is a remainder of HAS.

19. The conjugate according to any of claims 14 to 18, wherein HAS' comprises at least one structural unit according to the following formula (I)



(I)

20

wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS''$, $-[O-CH_2-CH_2]_s-OH$, and $-[O-CH_2-CH_2]_r[F']_p-L'-X-$, and wherein

s is in the range of from 0 to 4,

t is in the range of from 0 to 4,

p is 0 or 1,

and wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_p-[F^1]_p-L^1-X$,

5 wherein F^1 is $-O-$,

wherein L^1 is a linking moiety having a structure according to the following formula

$-[CR^{dR^f}]_h-[F^4]_u-[CR^{ddR^f}]_z$ alpha- wherein F^4 is a functional group, preferably selected from the group consisting of $-S-$, $-O-$ and $-NH-$, in particular $-S-$, wherein

z is in the range of from 0 to 20, more preferably of from 0 to 10, more preferably of

10 from 0 to 3, and most preferably of from 0 to 2,

h is in the range of from 1 to 5, preferably in the range of from 1 to 3, more preferably 3,

u is 0 or 1,

integer alpha is in the range of from 1 to 10,

15 and R^d , R^f , R^{dd} and R^f are, independently of each other, selected from the group consisting of H, alkyl, hydroxyl, and halogene, preferably selected from the group consisting of H, methyl and hydroxyl, and wherein each repeating unit of $-[CR^{dR^f}]_h-$ may be the same or may be different, and wherein each repeating unit of $-[CR^{ddR^f}]_z-$ may be the same or may be different and wherein each repeating unit of F^4 may be the same or may be different,

20 wherein, more preferably, L^1 has a structure selected from the group consisting of

$-CH_2-CHOH-CH_2-$, $-CH_2-CHOH-CH_2-S-CH_2-CH_2-$,

$-CH_2-CHOH-CH_2-S-CH_2-CH_2-CH_2-$, $-CH_2-CHOH-CH_2-NH-CH_2-CH_2-$,

$-CH_2-CHOH-CH_2-NH-CH_2-CH_2-CH_2-$, $-CH_2-$, $-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-$,

25 $-CH_2-CH_2-CH_2-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-CH_2-$,

$-CH_2-CH(CH_2OH)-$, $-CH_2-CH(CH_2OH)-S-CH_2-CH_2-$,

$-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-$,

$-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-S-CH_2-CH_2-$,

$-CH_2-CH_2-CH_2-S-CH_2-CH_2-$, $-CH_2-CH_2-S-CH_2-CH_2-$ and

30 $-CH_2-CH_2-O-CH_2-CH_2-$, more preferably from the group consisting of

$-CH_2-CHOH-CH_2-$, $-CH_2-CHOH-CH_2-S-CH_2-CH_2-$,

$-CH_2-CHOH-CH_2-S-CH_2-CH_2-CH_2-$, $-CH_2-CHOH-CH_2-NH-CH_2-CH_2-$ and

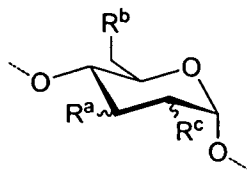
$-CH_2-CHOH-CH_2-NH-CH_2-CH_2-CH_2-$, more preferably from the group consisting of $-CH_2-CHOH-CH_2-$, $-CH_2-CHOH-CH_2-S-CH_2-CH_2-$ and

35 $-CH_2-CHOH-CH_2-S-CH_2-CH_2-CH_2-$,

wherein X is S,

and wherein HAS'' is a remainder of HAS.

20. The conjugate according to any of claims 14 to 18, wherein HAS' comprises at least one structural unit according to the following formula (I)



(I)

- 5 wherein R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS'', $-[O-CH_2-CH_2]_s-OH$, and $-[O-CH_2-CH_2]_t-[F^1]_p-L'-X-$, and wherein

s is in the range of from 0 to 4,

t is in the range of from 0 to 4,

- 10 p is 0 or 1,

and wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-[F^1]_p-L'-X-$,

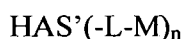
wherein F^1 is $-O-(C=O)-NH-$,

wherein L^1 is an, optionally substituted, alkyl group,

wherein X is -S-,

- 15 and wherein HAS'' is a remainder of HAS.

21. A method for preparing a hydroxyalkyl starch (HAS) conjugate comprising a hydroxyalkyl starch derivative and a cytotoxic agent, said conjugate having a structure according to the following formula



wherein

M is a residue of a cytotoxic agent, and wherein the cytotoxic agent comprises a primary hydroxyl group,

- 25 L is a linking moiety,

HAS' is a residue of the hydroxyalkyl starch derivative,

and n is equal to or greater than 1,

said method comprising

- (a) providing a hydroxyalkyl starch (HAS) derivative having a mean molecular weight MW above the renal threshold, preferably a mean molecular weight MW greater than or equal to 60 kDa, and a molar substitution MS in the range of from 0.6 to 1.5, said HAS derivative comprising a functional group Z^1 ; and providing a cytotoxic agent comprising a primary hydroxyl group;
- 30

(b) coupling the HAS derivative to the cytotoxic agent via an at least bifunctional crosslinking compound L comprising a functional group K^1 and a functional group K^2 , wherein K^2 is capable of being reacted with Z^1 comprised in the HAS derivative and wherein K^1 is capable of being reacted with the primary hydroxyl group comprised in the cytotoxic agent.

22. The method according to claim 21, wherein the functional group K^1 comprises the group $-C(=Y)-$, with Y being O, NH or S, wherein K^1 is preferably a carboxylic acid group or a reactive carboxy group.

23. The method according to claim 21 or 22, wherein the cytotoxic agent is reacted with the crosslinking compound L prior to the reaction with the HAS derivative.

24. The method according to any of claims 21 to 23, wherein the crosslinking compound L has a structure according to the following formula



wherein K^1 comprises the group $-C(=Y)-$, with Y being O, NH or S,

and L' is a linking moiety.

25. The method according to any of claims 21 to 24, wherein K^2 is reacted with the functional group Z^1 , comprised in the HAS derivative, wherein Z^1 is selected from the group consisting of an aldehyde group, a keto group, a hemiacetal group, an acetal group, an alkynyl group, an azide, a carboxy group, an alkenyl group, a thiol reactive group, $-SH$, $-NH_2$, $-O-NH_2$, $-NH-O-alkyl$, $-(C=G)-NH-NH_2$, $-G-(C=G)-NH-NH_2$, $-NH-(C=G)-NH-NH_2$, and $-SO_2-NH-NH_2$, where G is O or S and, if G is present twice, it is independently O or S.

26. The method according to claim 25, wherein upon reaction of the primary hydroxyl group comprised in the cytotoxic agent with K^1 , a functional group $-F^3-O-$ is formed, wherein

F^3 comprises the functional group $-C(=Y)-$, with Y being O, NH or S, in particular wherein F^3 is the functional group $-C(=Y)-$, with Y being O.

27. The method according to any of claims 21 to 26, wherein the at least one crosslinking compound L has a structure according to the following formula:



wherein E is an electron-withdrawing group, preferably selected from the group consisting of $-C(=O)-NH-$, $-NH-C(=O)-$, $-NH-$, $-O-$, $-S-$, $-SO-$, $-SO_2-$ and $-succinimide-$,

L^2 is a linking moiety, preferably an alkyl, alkenyl, alkylaryl, arylalkyl, aryl, heteroaryl, alkylheteroaryl or heteroarylalkyl group,

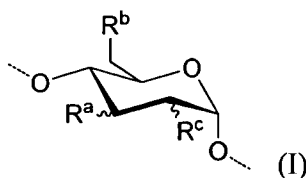
f is in the range of from 1 to 20,

g is 0 or 1,

e is 0 or 1,

and wherein R^m and R^n are, independently of each other, H, alkyl, aryl or a residue of a natural or unnatural amino acid, preferably H or alkyl, more preferably H or methyl, in particular H.

28. The method according to any of claims 21 to 27, wherein the hydroxyalkyl starch derivative provided in step (a) comprises at least one structural unit according to the following formula (I)



wherein at least one of R^a , R^b or R^c comprises the functional group Z^1 , preferably consisting of $-O-HAS''$, $-[O-(CR^wR^xHCR^yR^z)]_x-OH$, $-[O-(CR^wR^x)-(CR^yR^z)]_y-Z^1$ and $-[O-(CR^wR^x)-(CR^yR^z)]_y-[F^1]_p-L^1-Z^1$,

wherein R^w , R^x , R^y and R^z are independently of each other selected from the group consisting of hydrogen and alkyl,

y is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4, and

x is an integer in the range of from 0 to 20, preferably in the range of from 0 to 4,

F^1 is a functional group,

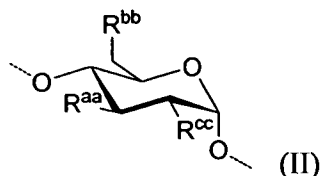
p is 0 or 1,

L^1 is a linking moiety,

wherein HAS'' is a remainder of HAS,

and wherein step (a) comprises

- (a1) providing a hydroxyalkyl starch having a mean molecular weight MW greater than or equal to 60 kDa and a molar substitution MS in the range of from 0.6 to 1.5 comprising the structural unit according to the following formula (II)

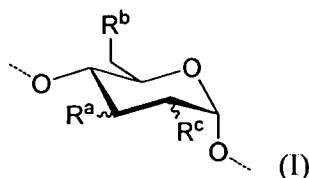


- 5 wherein R^{aa} , R^{bb} and R^{cc} are, independently of each other, selected from the group consisting of $-O-HAS''$ and $-[O-(CR^wR^x)-(CR^yR^z)]_x-OH$,
 wherein R^w , R^x , R^y and R^z are independently of each other selected from the
 group consisting of hydrogen and alkyl,
 and x is an integer in the range of from 0 to 20, preferably in the range of from 0
 10 to 4;

- (a2) introducing at least one functional group Z^1 into **HAS** by

- 15 (i) coupling the hydroxyalkyl starch via at least one hydroxyl group comprised in **HAS** to at least one suitable linker comprising the functional group Z^1 or a precursor of the functional group Z^1 , or
 (ii) displacing at least one hydroxyl group comprised in **HAS** in a substitution reaction with a precursor of the functional group Z^1 or with a suitable linker comprising the functional group Z^1 or a precursor thereof.

- 20 29. The method according to claim 28, wherein the **HAS** derivative formed in step (a2) comprises at least one structural unit according to the following formula (I)



- 25 wherein R^a , R^b and R^c are, independently of each other, selected from the group consisting of $-O-HAS''$, $-[0-CH_2-CH_2]_s-OH$, $-[0-CH_2-CH_2]_t-Z'$ and $-[0-CH_2-CH_2]_t-[F^1]_p-L^1-Z^1$, and wherein

and wherein s is in the range of from 0 to 4,

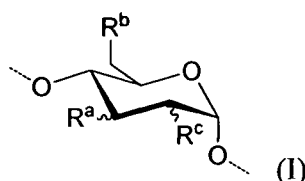
and wherein t is in the range of from 0 to 4,

p is 0 or 1,

- 30 wherein at least one of R^a , R^b and R^c is $-[0-CH_2-CH_2]_t-X-$ or $-[0-CH_2-CH_2]_t-[F^1]_p-L^1-\zeta^1$,

and wherein HAS" is a remainder of HAS.

30. The method according to claim 28 or 29, wherein in (a2)(i), the hydroxyalkyl starch is reacted with a suitable linker comprising the functional group Z^1 or a precursor of the functional group Z^1 , and comprising a functional group Z^2 , the linker preferably having the structure $Z^2-L^1-Z^1$ or $Z^2-L^1-Z^{1*}-PG$, with Z^2 being a functional group capable of being reacted with the hydroxyalkyl starch, thereby forming a hydroxyalkyl starch derivative comprising at least one structural unit according to the following formula (I)

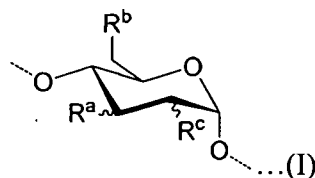


wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-Z^1$ or $-[O-CH_2-CH_2]_t-[F^1]_p-L^1-Z^{1*}-PG$ with PG being a suitable protecting group and Z^{1*} being the protected form of the functional group Z^1 ,

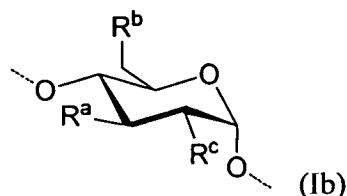
wherein Z^1 is preferably $-SH$, Z^{1*} is preferably $-S-$ and PG is preferably a suitable thiol protecting group, more preferably a protecting group forming together with Z^{1*} a group selected from the group consisting of thioethers, thioesters and disulfides, and wherein in case the linker comprises the protecting group PG , the method further comprises deprotection of Z^{1*} to give Z^1 .

31. The method according to claim 30, wherein step (a2)(i) comprises

(aa) activating at least one hydroxyl group comprised in the hydroxyalkyl starch with a reactive carbonyl compound having the structure $R^{**}-(C=O)-R^*$, wherein R^* and R^{**} may be the same or different, and wherein R^* and R^{**} are both leaving groups, wherein upon activation a hydroxyalkyl starch derivative comprising at least one structural unit according to the following formula (1)



preferably (lb)



is formed, in which R^a , R^b and R^c are independently of each other selected from the group consisting of -O-HAS", $-[0-\text{CH}_2-\text{CH}_2]_s-\text{OH}$, $-[0-\text{CH}_2-\text{CH}_2]_t-0-\text{C}(=\text{O})-\text{R}^*$,

5

wherein s is in the range of from 0 to 4,

and wherein t is in the range of from 0 to 4,

and wherein at least one of R^a , R^b and R^c comprises the group $-[0-\text{CH}_2-\text{CH}_2]_t-0-\text{C}(=\text{O})-\text{R}^*$, and

10

(bb) reacting the activated hydroxyalkyl starch according to step (aa) with the suitable linker comprising the functional group Z^1 or a precursor of the functional group Z^1 .

32. The method according to claim 31, wherein the reactive carbonyl compound having the structure $\text{R}^{**}-(\text{C}=\text{O})-\text{R}^*$ is selected from the group consisting of phosgene, diphosgene, triphosgene, chloroformates and carbonic acid esters, preferably wherein the reactive carbonyl compound is selected from the group consisting of p-nitrophenylchloroformate, pentafluorophenylchloroformate, N,N'-disuccinimidyl carbonate, sulfo-N,N'-disuccinimidyl carbonate, dibenzotriazol-1-yl carbonate and carbonyldiimidazol.

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33. The method according to claim 31 or 32, wherein in step (bb), the activated hydroxyalkyl starch derivative is reacted with a linker comprising the functional group Z^2 and the functional group Z^1 or a precursor of the functional group Z^1 , the linker preferably having the structure $Z^2-L^1-Z^1$ or $Z^2-L^1-Z^1-PG$,

25

wherein Z^{1*} is the protected form of Z^1 and PG is a protecting group, preferably wherein Z^1 is -SH and Z^{1*} is -S-,

Z^2 is a functional group capable of being reacted with the

$-[0-\text{CH}_2-\text{CH}_2]_t-0-\text{C}(=\text{O})-\text{R}^*$ group,

L^1 is an alkyl group,

30

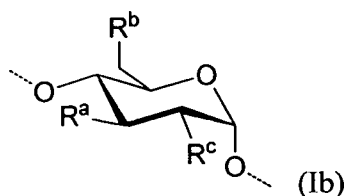
wherein upon reaction of the $-0-\text{C}(=\text{O})-\text{R}^*$ group with the functional group Z^2 , the functional group F^1 is formed, and

Z^2 is preferably $-\text{NH}_2$.

34. The method according to claim 33, wherein the linker has the structure $Z^1L^1-Z^1PG$, wherein Z^1 is -S- and PG is a thiol protecting group, more preferably a protecting group forming together with Z^1 a group selected from the group consisting of thioethers, thioesters and disulfides, and wherein the method further comprises deprotection of Z^1 to give Z^1 .

35. The method according to claim 28 or 29, wherein step (a2)(i) comprises
(I) coupling the hydroxyalkyl starch via at least one hydroxyl group comprised in the hydroxyalkyl starch to a first linker comprising a functional group Z^2 , Z^2 being capable of being reacted with a hydroxyl group of the hydroxyalkyl starch, thereby forming a covalent linkage, the first linker further comprising a functional group W, wherein the functional group W is an epoxide or a group which is transformed in a further step to give an epoxide.

36. The method according to claim 35, wherein the first linker has a structure according to the formula Z^2-L^W-W , wherein Z^2 is a functional group capable of being reacted with a hydroxyl group of the hydroxyalkyl starch, L^W is a linking moiety, wherein upon reaction of the hydroxyalkyl starch with the first linker, a hydroxyalkyl starch derivative is formed comprising at least one structural unit according to the following formula (Ib)



wherein R^a , R^b and R^c are, independently of each other, selected from the group consisting of -O-HAS", $-[O-CH_2-CH_2]_s-OH$, and $-[O-CH_2-CH_2]_t-[F^1]_p-L^W-W$,

wherein s is in the range of from 0 to 4,

and wherein t is in the range of from 0 to 4,

and wherein at least one of R^a , R^b and R^c is $-[O-CH_2-CH_2]_t-[F^1]_p-L^W-W$,

and wherein F^1 is the functional group being formed upon reaction of Z^2 with a hydroxyl group of the hydroxyalkyl starch, wherein F^1 is preferably -O- or $-CH_2-CHOH-$, preferably -O-,

and wherein HAS" is a remainder of HAS.

37. The method according to claim 35 or 36, wherein W is an alkenyl group and the method further comprises
 (II) oxidizing the alkenyl group W to give the epoxide, wherein as oxidizing agent, potassium peroxymonosulfate is preferably employed.

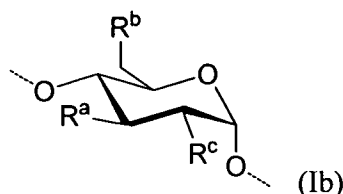
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38. The method according to any of claims 35 to 37, wherein Z^2 is a halogene (Hal) or an epoxide, and wherein the linker Z^2 -L^w-W preferably has the structure Hal-CH₂-CH=CH₂.

10

39. The method according to claim 37, the method comprising
 (III) reacting the epoxide with a nucleophile comprising the functional group Z¹ or a precursor of the functional group Z¹, wherein the nucleophile is preferably a dithiol or a thiosulfate, thereby forming a hydroxyalkyl starch derivative comprising at least one structural unit, preferably 3 to 100 structural units, according to the following formula (Ib)

15



wherein R^a, R^b and R^c are independently of each other selected from the group consisting of -O-HAS", $-[0-\text{CH}_2-\text{CH}_2]_s-\text{OH}$, and $-[0-\text{CH}_2-\text{CH}_2]_t-[\text{F}^1]_p-\text{L}^1-\text{Z}^1$,

wherein s is in the range of from 0 to 4,

20

and wherein t is in the range of from 0 to 4,

P is 1,

at least one of R^a, R^b and R^c comprises the group $-[0-\text{CH}_2-\text{CH}_2]_t-[\text{F}^1]_p-\text{L}^1-\text{Z}^1$, and wherein Z¹ is -SH.

25

40. The method according to claim 41, wherein the nucleophile is ethanedithiol or sodium thiosulfate.

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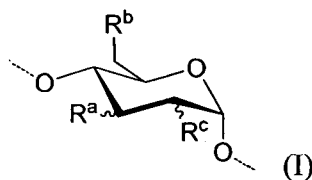
41. The method according to claim 28, wherein in step (a2)(ii), prior to the displacement of the hydroxyl group, a group R^L is added to at least one hydroxyl group thereby generating a group -O-R^L, wherein -O-R^L is a leaving group, in particular an -O-Mesyl (-OMs) or -O-Tosyl (-OTs) group.

42. The method according to claim 28 or claim 41, wherein Z¹ is -SH, and wherein in step (a2)(ii) at least one hydroxyl group comprised in the hydroxyalkyl starch is displaced

by a suitable precursor of the functional group Z^1 , the method further comprising converting the precursor after the substitution reaction to the functional group Z^1 .

43. The method according to claim 42, wherein in step (a2)(ii) the at least one hydroxyl group comprised in the hydroxyalkyl starch is displaced with thioacetate giving a precursor of the functional group Z^1 having the structure $-S-C(=O)-CH_3$, wherein the method further comprises the conversion of the group $-S-C(=O)-CH_3$ to give the functional group Z^1 , preferably wherein the conversion is carried out using sodium hydroxide and sodium borohydride.

44. The method according to any of claims 41 to 43, wherein the hydroxyalkyl starch derivative obtained according to step (a2)(ii) comprises at least one structural unit according to the following formula (I)



wherein R^a , R^b and R^c are independently of each other selected from the group consisting of $-O-HAS''$, $-[O-CH_2-CH_2]_s-OH$, and $-[O-CH_2-CH_2]_t-Z^1$, wherein

wherein s is in the range of from 0 to 4,

wherein t is in the range of from 0 to 4,

wherein at least one of R^a , R^b and R^c comprises the group $-[O-CH_2-CH_2]_t-Z^1$,

wherein Z^1 is $-SH$,

and wherein HAS'' is a remainder of HAS .

45. The method according to any of claims 28 to 44, wherein in step (b) the hydroxyalkyl starch derivative obtained according to step (a) is coupled to a crosslinking compound L having a structure according to the formula $K^2-[L^2]_g-[E]_e-[CR^mR^n]_f-K^1$, wherein E is an electron-withdrawing group, L^2 is a linking moiety, and wherein
- g and e are 0,
 - f is in the range of from 1 to 20,
 - R^m and R^n are, independently of each other, H or alkyl, preferably H or methyl,
 - and K^2 is a halogene,

and wherein upon reaction of Z^1 with K^2 the covalent linkage $-X-[CR^mR'']_r-$ is formed;

or

— g and e are 0,

5 f is in the range of from 1 to 20,

R^m and R'' are, independently of each other, H or alkyl, preferably H or methyl, in particular H,

and K^2 is maleimide,

10 and wherein upon reaction of Z^1 with K^2 the covalent linkage $-X$ -succinimide- is formed.

46. The method according to claim 45, wherein Z^1 is $-SH$, and X is $-S-$.

15 47. The method according to any claims 21 to 46, wherein the cytotoxic agent is an antimetabolite, more preferably a nucleoside analogue, in particular wherein the cytotoxic agent is selected from the group consisting of clofarabine, nelarabine, cytarabine, cladribine, decitabine, azacitidine, fludarabine, floxuridine, doxifluridine, pentostatin and gemcitabine.

20 48. A hydroxyalkyl starch conjugate obtained or obtainable by a method according to any of claims 21 to 47.

49. A pharmaceutical composition comprising a conjugate according to any of claims 1 to 20 or according to claim 48.

25

50. A hydroxyalkyl starch conjugate according to any of claims 1 to 20 or according to claim 48, or a pharmaceutical composition according to claim 49 for use as medicament.

30 51. A hydroxyalkyl starch conjugate according to any of claims 1 to 20 or according to claim 48, or a pharmaceutical composition according to claim 49 for the treatment of cancer, preferably for the treatment of cancer selected from the group consisting of biliary cancer, bladder cancer, breast cancer, cervical cancer, colorectal cancer, gastrointestinal cancer, head and neck cancer, leukaemia, lymphoma, malignant
35 melanoma, mesothelioma, non-small cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, sarcoma and small cell lung cancer.

52. Use of a hydroxyalkyl starch conjugate according to any of claims 1 to 20 or according to claim 48, or a pharmaceutical composition according to claim 49 for the

5 manufacture of a medicament for the treatment of cancer, preferably, wherein the cancer is selected from the group consisting of biliary cancer, bladder cancer, breast cancer, cervical cancer, colorectal cancer, gastrointestinal cancer, head and neck cancer, leukaemia, lymphoma, malignant melanoma, mesothelioma, non-small cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, sarcoma and small cell lung cancer.

10 53. A method of treating a patient suffering from cancer comprising administering a therapeutically effective amount of a hydroxyalkyl starch conjugate according to any of claims 1 to 20 or according to claim 48, or a pharmaceutical composition according to claim 49, preferably wherein the patient suffers from a cancer being selected from the group consisting of biliary cancer, bladder cancer, breast cancer, cervical cancer, colorectal cancer, gastrointestinal cancer, head and neck cancer, leukaemia, lymphoma, malignant melanoma, mesothelioma, non-small cell lung cancer, ovarian
15 cancer, pancreatic cancer, prostate cancer, sarcoma and small cell lung cancer.

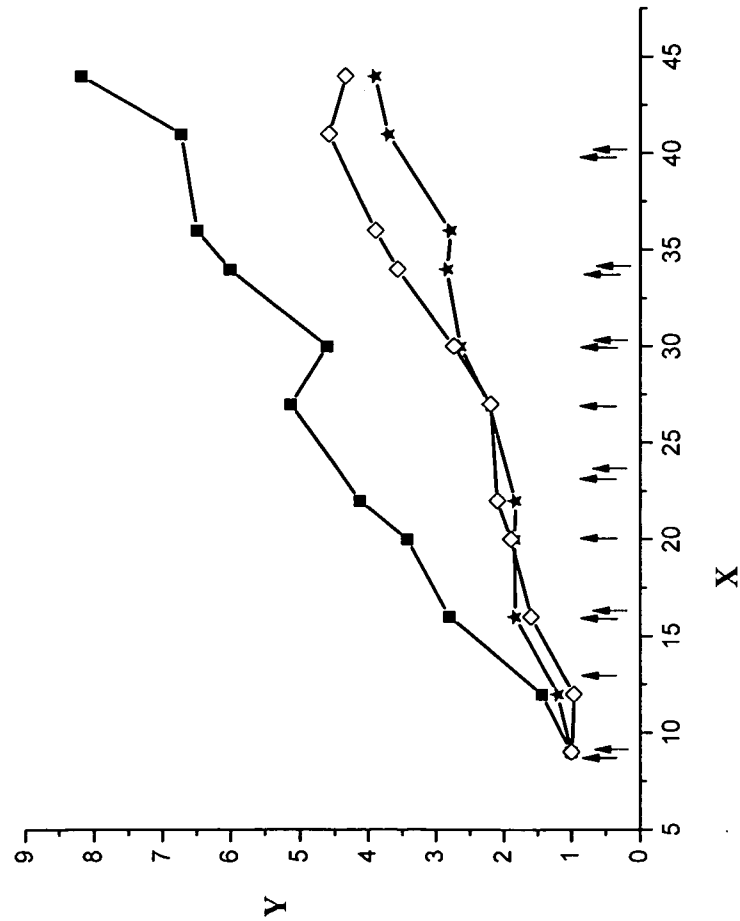
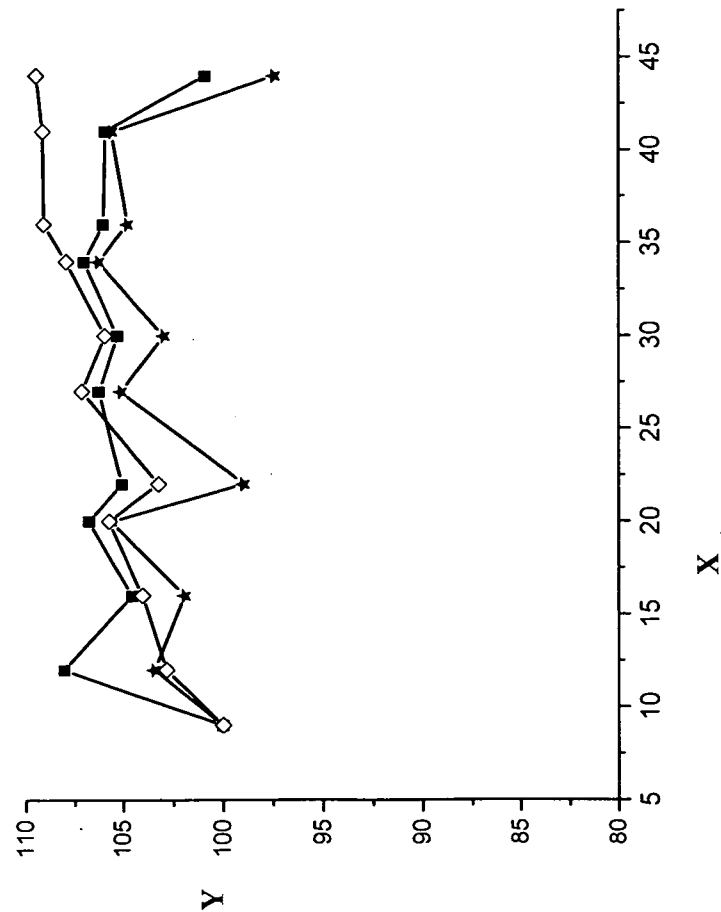


Fig. 1:

Fig. 2:



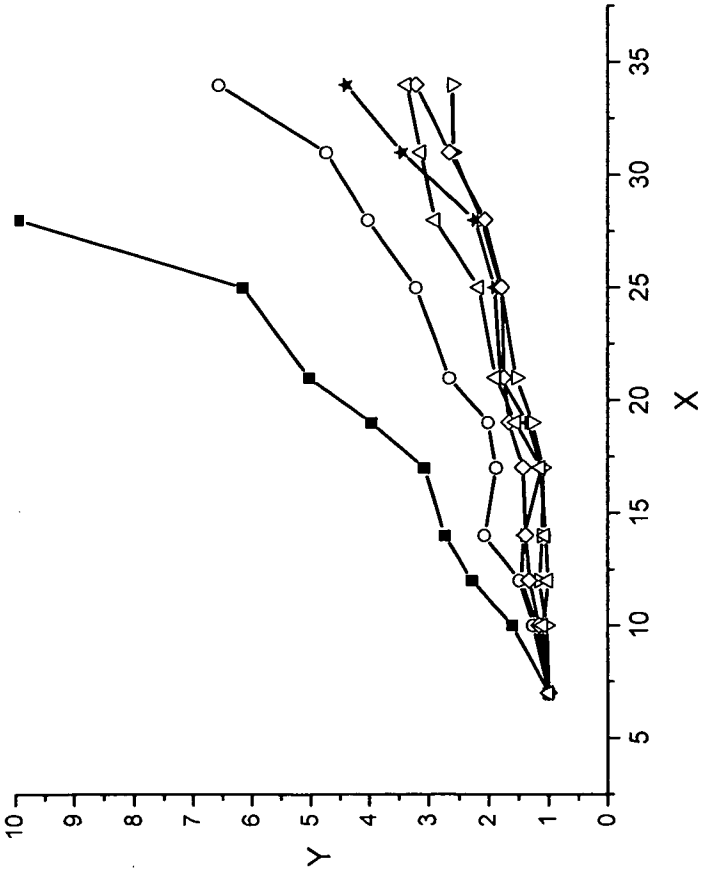


Figure 3:

Figure 4:

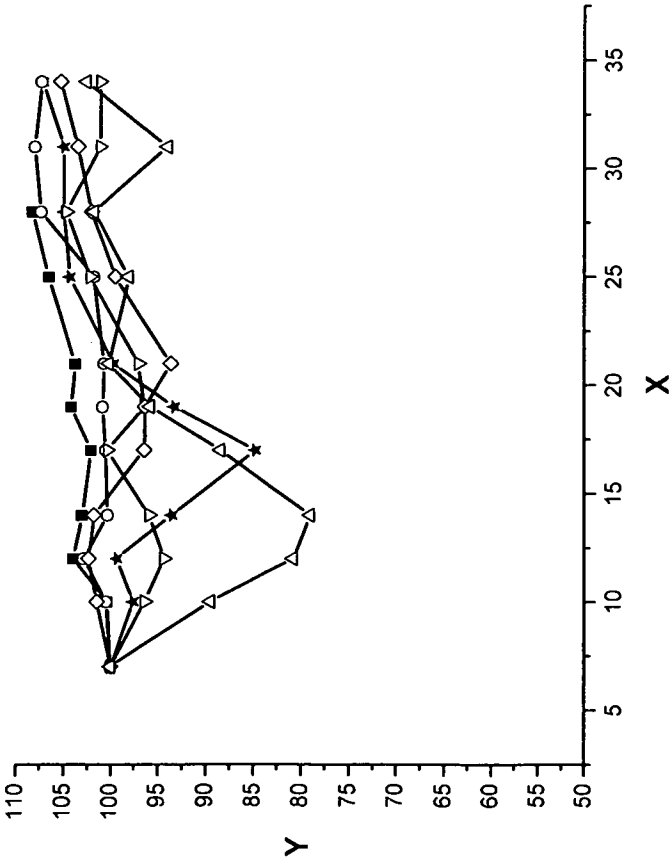


Figure 5:

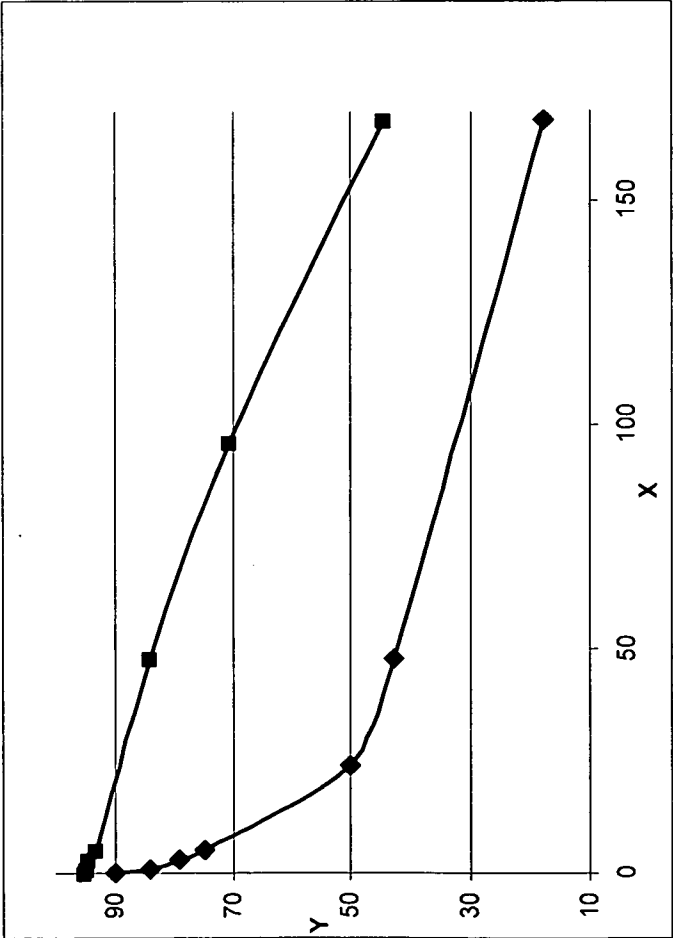


Figure 6:

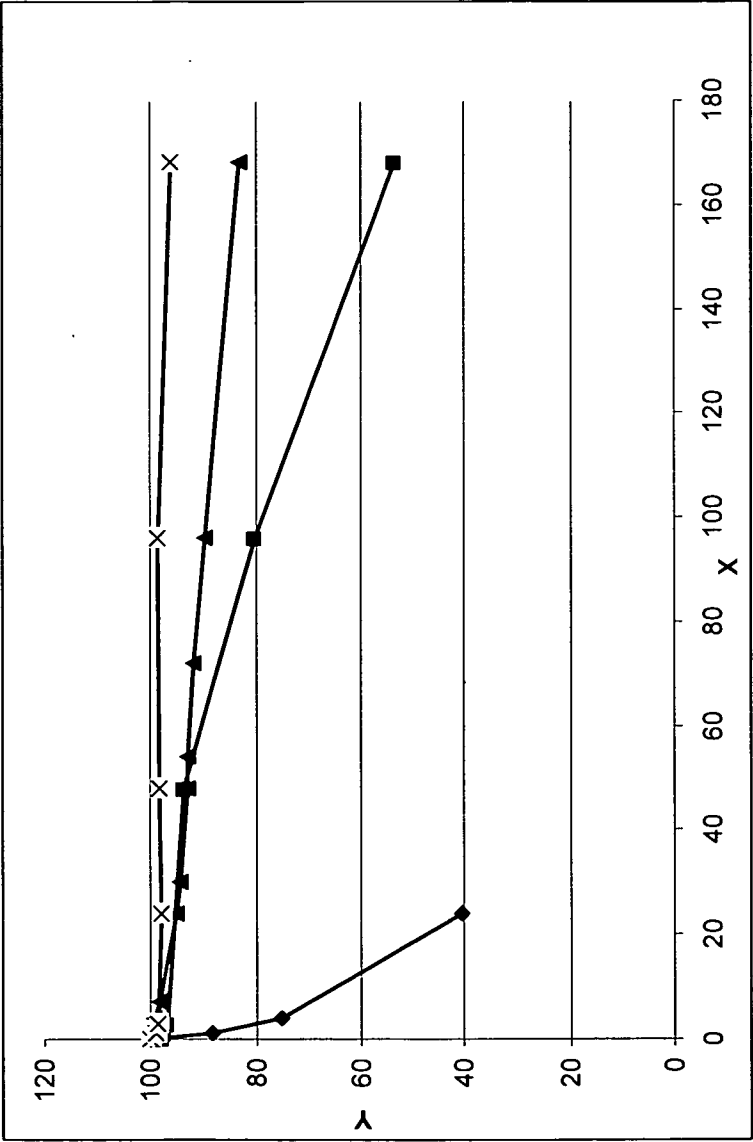


Figure 7:

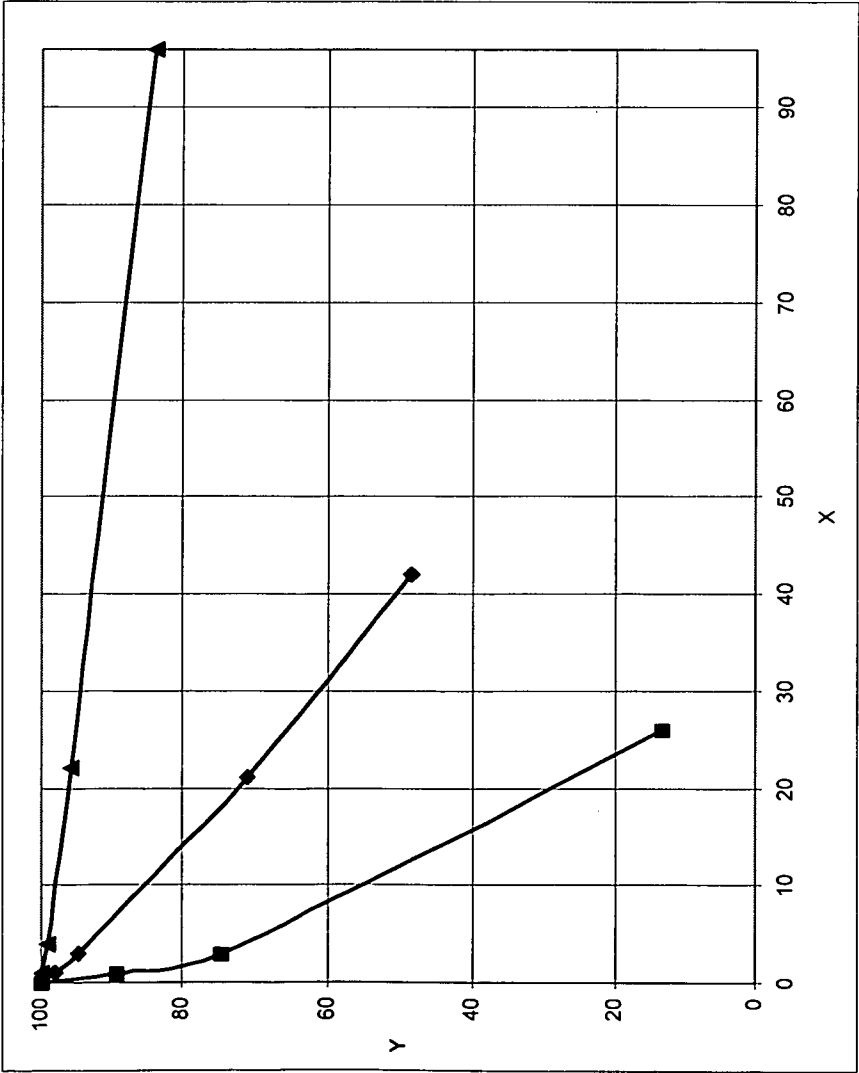


Figure 8:

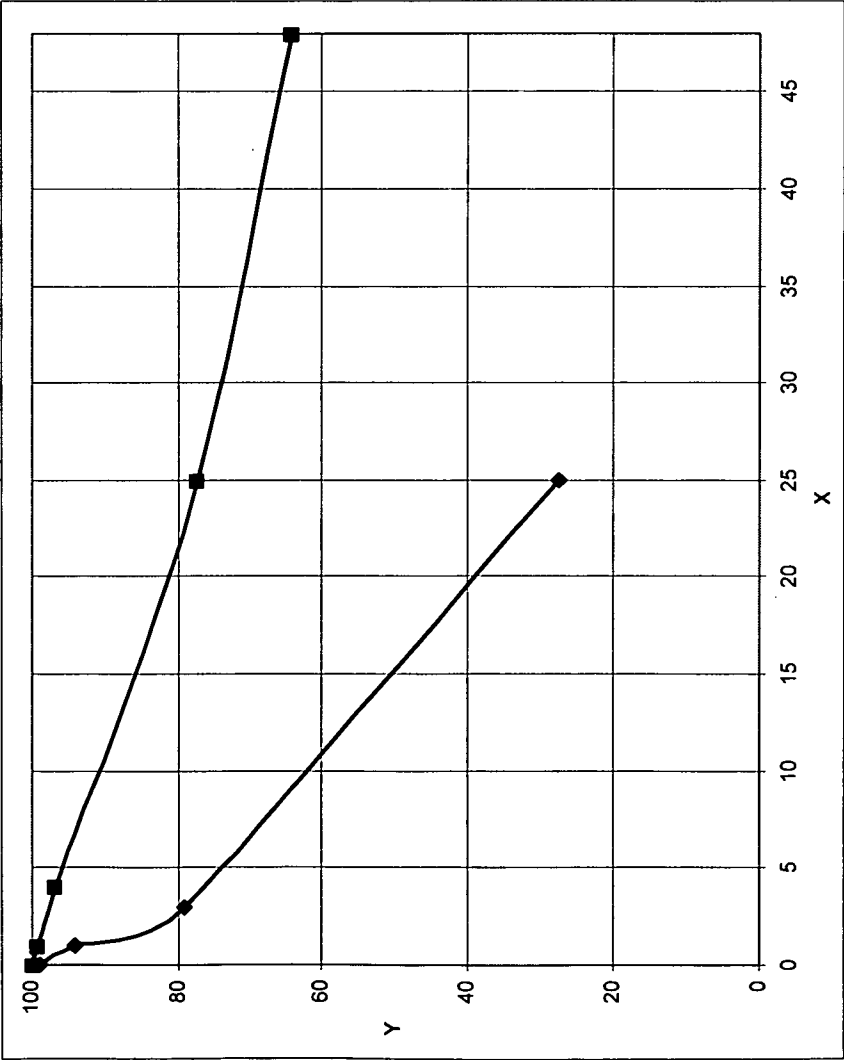


Figure 9:

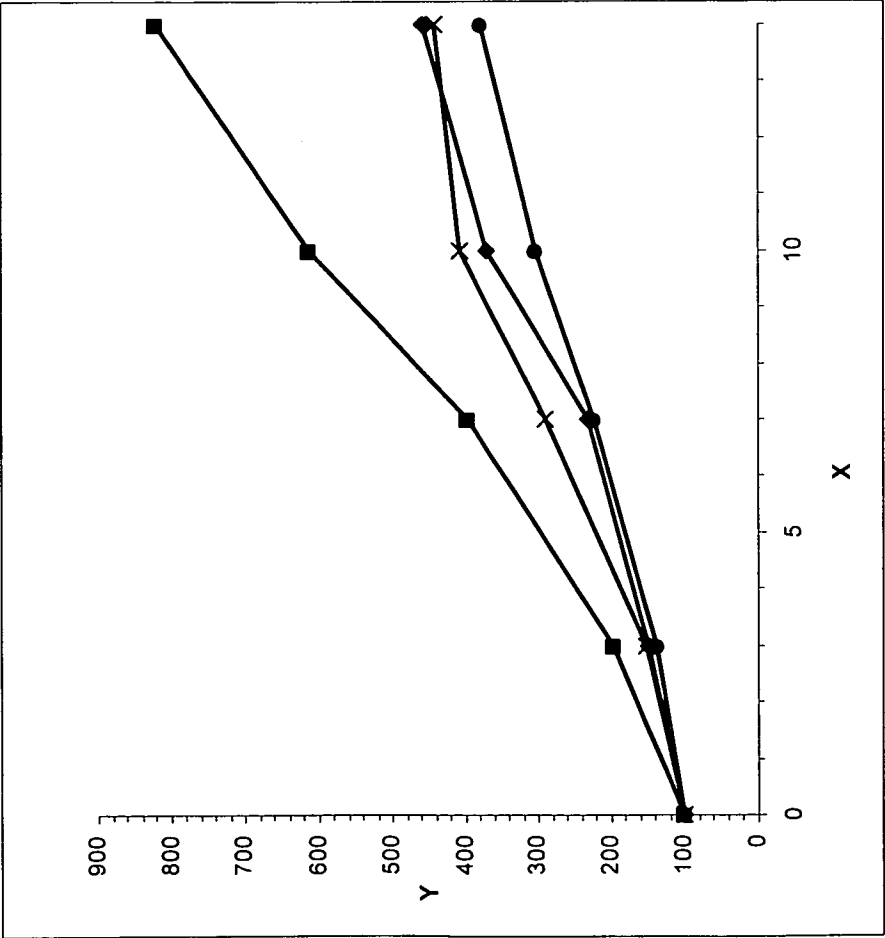


Figure 10:

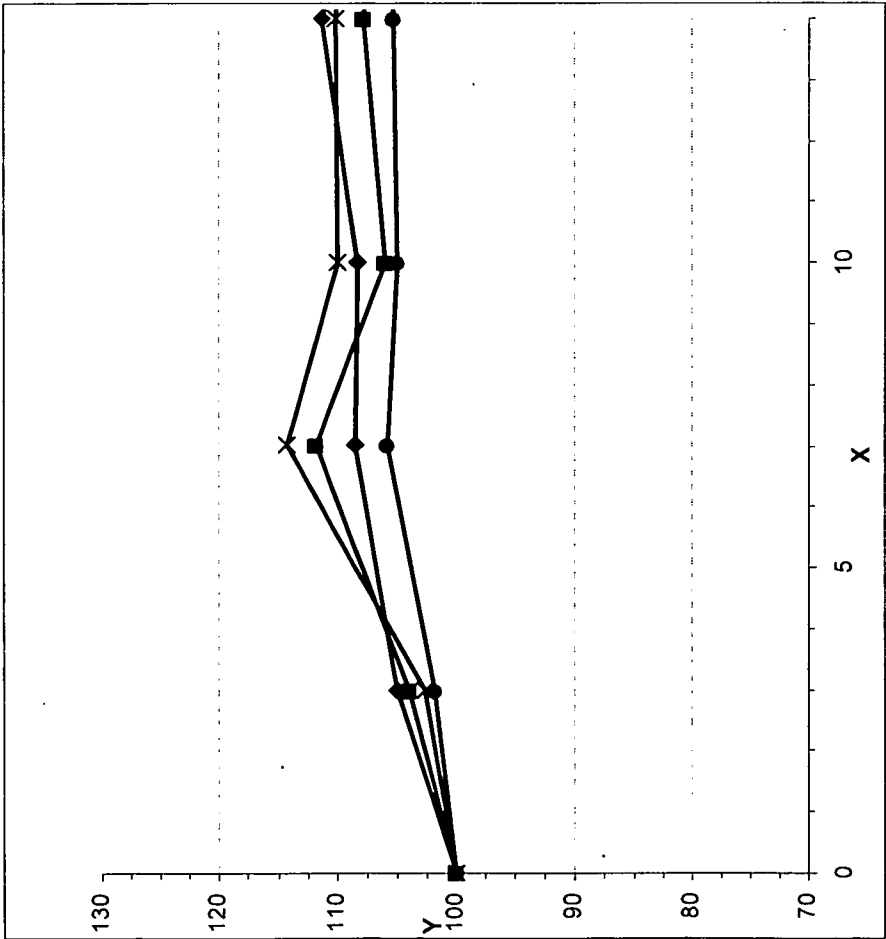


Figure 11:

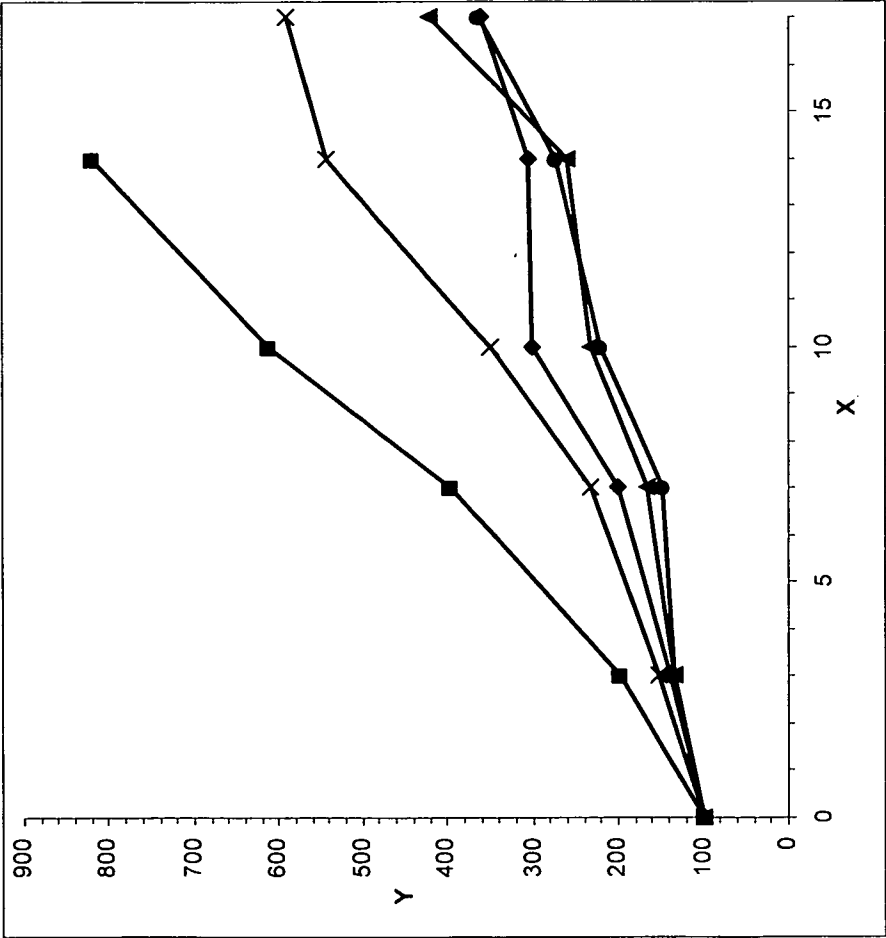


Figure 12:

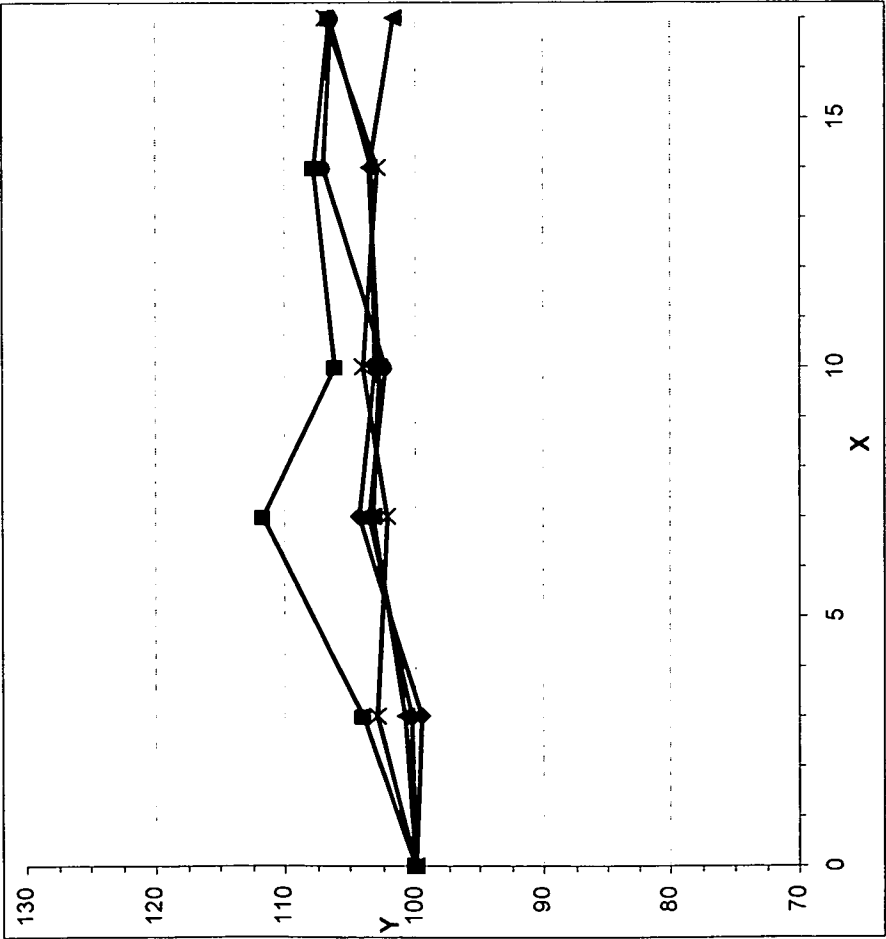


Figure 13

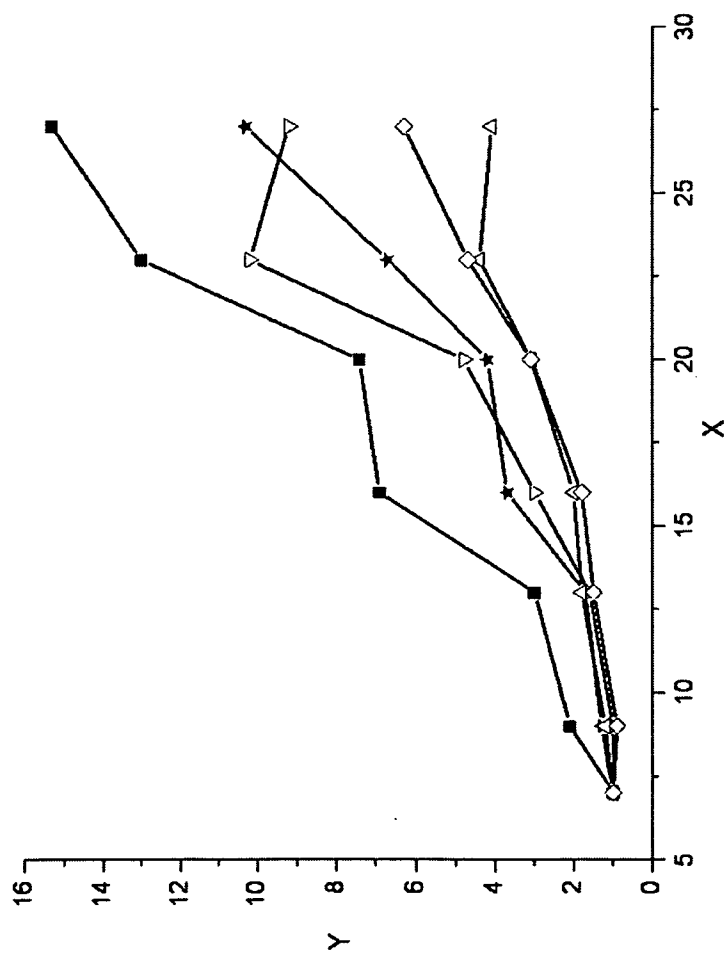
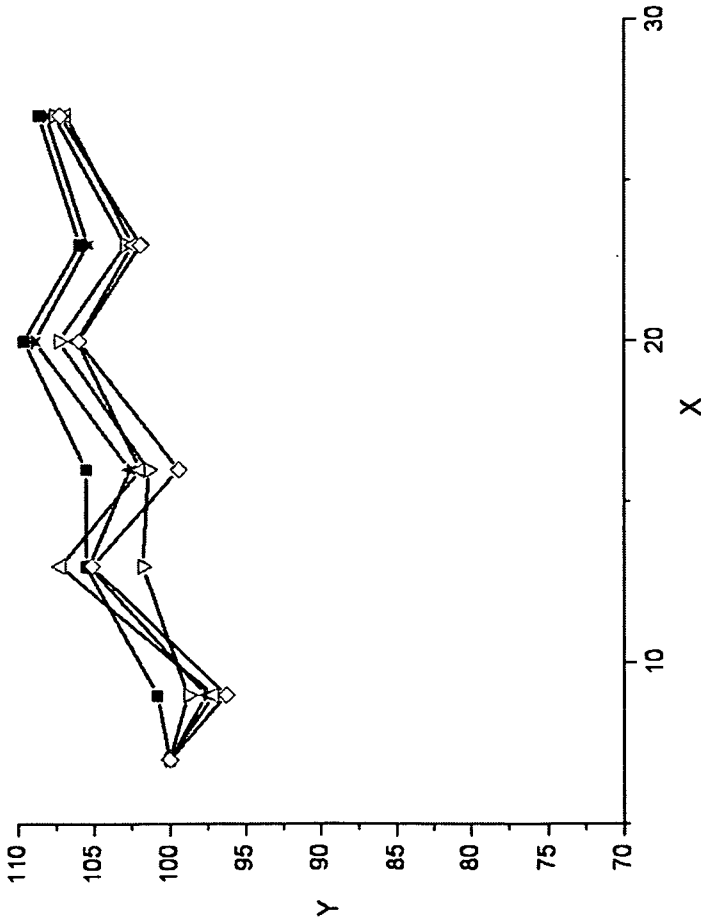


Figure 14



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2011/003462

A. CLASSIFICATION OF SUBJECT MATTER
 INV. A61K47/48 A61P35/00
 ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal , WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	us 5 981 507 A (JOSEPHSON LEE [US] ET AL) 9 November 1999 (1999-11-09) examples 2, 7, 17, 18, 36, 38 figures 2, 3 column 9, line 63 - line 66 -----	1-14, 21-53
A	us 2005/238723 A1 (ZANDER NORBERT [DE] ET AL) 27 October 2005 (2005-10-27) table 1 examples 1-12 -----	1-53
A	Wo 2010/042638 A2 (LEE YOUNG BOK [US] ; AHN CHANG HO [US] ; KIM DEOG JOONG [US] ; GHANDEHARI) 15 April 2010 (2010-04-15) page 25, paragraph 2 - page 26, paragraph 1 tables 1-2 ----- -/-	1-53



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

30 August 2011

Date of mailing of the international search report

07/09/2011

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Monami , Amel i e

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2011/003462

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CAVALLARO G ET AL: "Folate-mediated targeting of polymeric conjugates of gemcitabine", INTERNATIONAL JOURNAL OF PHARMACEUTICS, ELSEVIER BV, NL, vol. 307, no. 2, 13 January 2006 (2006-01-13), pages 258-269, XP025113454, ISSN: 0378-5173, DOI: DOI: 10.1016/J.IJPHARM.2005.10.015 [retrieved on 2006-01-13] abstract compounds 3-4,6-7 -----	1-53
A	PASUT G ET AL: "Antitumoral activity of PEG-gemcitabine prodrugs targeted by folate", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 127, no. 3, 8 May 2008 (2008-05-08), pages 239-248, XP022617739, ISSN: 0168-3659, DOI: DOI: 10.1016/J.JCONREL.2008.02.002 [retrieved on 2008-02-15] abstract compounds 2,5 -----	1-53

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Information on patent family members

International application No

PCT/EP2011/003462

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