Title: POLYETHYLENE GLYCOL CONJUGATES RELEASING NITRIC OXIDE

Abstract: The present invention relates to polyethylene glycol derivatives having a controlled release nitric oxide as well as their use as drugs. It has been surprisingly found that it is possible connect physically or by chemical bonds polyethylene glycol nitrate with pharmacologically active molecules, such as anti-inflammatory, antitumoral drugs, peptides, proteins and oligonucleotides thus obtaining compounds having a better activities profile.
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations” appearing at the beginning of each regular issue of the PCT Gazette.
Polyethylene glycol conjugates releasing nitric oxide

The present invention relates to polyethylene glycol derivatives releasing nitric oxide, their preparation, compositions containing them and their use as drugs.

It is known that polyethylene glycols (PEGs) are linear, flexible polymers, available in a great range of molecular weights and largely employed in many pharmaceutical preparations, for example formulations to be administered by parenteral, topical, opthalmic, oral and rectal route. They correspond to the general formula \( \text{HOCH}_2(\text{CH}_2\text{OCH}_2)_m\text{CH}_3\text{OH} \) or in methoxylated form \( \text{CH}_3\text{OCH}_2(\text{CH}_2\text{OCH}_2)_m\text{CH}_3\text{OH} \), wherein \( m \) represents the average number of the polyoxyethylene moieties. PEGs are stable and show a good compatibility with tissues and mucosas. According to their molecular weight, they can exist in several forms. In this way, PEGs of from 200 Dalton (Da) to 600 Da are liquids, PEGs with molecular weight higher than 1000 Dalton are solids of wax type, whereas with 6000 Da and more are free-flowable powders.

PEGs have a low toxicity in oral, parenteral and topical applications. After intravenous administration in human being, PEGs with molecular weight of from...
1000 Da to 10000 Da are quickly excreted, predominantly by renal route; those having a higher molecular weight with decreasing rate while the molecular weight increases.

PEGs are employed in aqueous solutions as agents for adjusting their viscosity and respectively their consistency. At concentrations of about 30%, they are used also for parenteral solutions. In solid pharmaceutical forms, PEGs with high molecular weight can increase the binder efficiency, thus conferring plasticity to grains. Those with high molecular weight are above all employed also as lubricants (Handbook of excipients 2000, 392-398).

Recently, the unusual PEGs properties have been utilized for ameliorating the characteristics of compounds such as proteins and peptides, to which PEG was bound, thus improving besides their solubility, stability and proteolytic inactivation resistance, pharmacokinetic properties and moreover for diminishing immunogenicity and antigenicity (Delgado C. et al., Critical Rev. Ther. Drug Carrier Syst 1992, 9, 249-304; Adv. Drug reviews 2002, 54, 453-606).

It has been suggested that the mentioned effects are due to the formation, around protein, of a PEG molecules shall with strictly connected water
molecules that sterically prevent proteolitic enzymes from approaching and at the same time contacting the immune system cells. Furthermore, the molecular weight increase reduces the glomerular filtration with consequent increase of plasmatic half-life and improvement of conjugates pharmacokinetics. In literature several examples of compounds of proteic nature having interesting biological properties, obtained by genetic engineering, are described. Among said compounds hemoglobin, insulin, urokinase, alpha-interferon, G-CSF, hGH, asparaginase, adenosin deaminase, superoxide dismutase (metalloenzyme catalysing the dismutation reaction of superoxide radical in hydrogen peroxide and molecular oxygen, later on SOD), catalase, etc, can be mentioned. For example the antioxidant enzymes SOD and catalase could be employed in the treatment of rheumatoid arthritis, ligaments degenerative disease, ischemia and vascular injuries in general. However, the therapeutic strength of native proteins is highly restricted by their short half-life and by possible allergic side reactions. These problems can be overcome conjugating said proteins with PEG, and in fact several pegylated proteins have been approved by FDA, in particular PEG-adenosin deaminase, PEG-interferon, PEG-asparaginase and PEG-G-CSF.
Now it has been assumed that it would be possible to further improve the therapeutic properties of drugs and biotechnological products preparing nitrated derivatives of the above mentioned PEGs that, after conjugation, could join the PEGs advantages with those of nitric oxide controlled release.

In fact, nitric oxide is a recognized endogenous component that is also a strong guanilate cyclase activator, that is the enzyme catalyzing the cyclic guanosine monophosphate synthesis (cyclic GMP), and it shows also many biological activities, such as vasodilatation, gastric function regulation, platelets aggregation inhibition, inhibition of endothelial leukocytes adhesion, further to effects on non adrenergic and non cholinergic neurotransmittion etc., said biological activity being also responsible of a better organ function with high blood flow, such as liver and kidneys.

It is further known that nitric oxide is responsible for many side effects, having at certain doses pro-inflammatory characteristics, effects on blood pressure, increase of smooth muscle cell apoptosis, and generally an ascertained cytotoxicity.

Therefore, it would be important to have at disposal a good control of nitric oxide release, this however
being a difficult aim to achieve in that it is a gas
extremely reactive with a few seconds half-life.

A nitric oxide donor is a compound containing a
nitric oxide moiety and directly releasing or
directly transferring by chemical route nitrogen
monoxide (nitric oxide) to another molecule,
preferably in its positively charged nitrosonium
form. Nitric oxide donors include, but are not
limited to, S-nitrosothiols, nitrites, N-oxo-N-
nitrosoamines and substrates of several forms of
nitric oxide synthase.

Now it has been surprisingly found, and this is
an object of the present invention, that it is
possible to obtain PEG derivatives having a
controlled release of nitric oxide. It has been also
found that by mixing, or with other appropriate
pharmaceutical technologies, it is possible to
physically combine polyethylene glycol nitrate with
active ingredients. At last it has been found that it
is possible to connect by chemical bonds polyethylene
glycol nitrate with pharmacologically active
molecules, such as anti-inflammatory, antitumoral
drugs etc. In said molecules also compounds of
biological nature are included, such as peptides,
proteins and oligonucleotides.
Object of the present application are polyethylene glycol derivatives having the nitrate moiety attached to PEG hydroxyl function(s) both directly and employing so-called spacers. Suitable spacers may be molecules containing aliphatic or aromatic groups. Examples of possible spacers are reported for examples in patent applications EP 609415, EP 670825, EP 722434, EP 759899. Further, it is possible to use spacers having antioxidant properties. The nitrate groups could be inserted also in higher amount into the same product employing polyfunctional branched spacers. The general formula of the derivatives object of the present invention is the following:

\[(R)_{a}-\text{PEG-}[X-(Y-Z-\text{NO}_2)_{q}]_{b}\]

wherein

- a is 0 or 1,
- PEG is polyethylene glycol,
- b is 1 or 2,
- q is the number of the moieties carrying the nitro group and is a number higher than or equal to 1,
- X is a branching function that may also be missing, but when present it may have one or more branchings,
- for example of formula:
or it can be further branched,
\[ n = 0-5 \]
\[ m = 0-5, \]
\[ W = \text{ester, amide, carbamate, ether, thioether, disulphide or another covalent bond,} \]
\[ Y \text{ represents an antioxidant function, that can be also missing,} \]
\[ Z \text{ is a spacer to which the nitro group is attached by an in vivo hydrolizable bond, for example:} \]
\[ -O-(\text{CH}_2)_s-\text{ONO}_2, \text{ with } s = 1-10, \]
\[ R \text{ represents one or more pharmacologically active groups, a drug, peptide or protein, directly connected to PEG or to a branching function, or } R \text{ is } -\text{OH or } -\text{OCH}_3. \]

The different moieties are conjugated, preferably via ester, amide, carbamate, ether, thioether,
disulphide or other covalent bonds, directly or by means of one or more linkers, the linkers being preferably selected among alkyl groups, such as NH₂-(CH₃)ₙ-NH₂ (n = 0-12) or HOOC-(CH₂)ₘ-COOH (m = 0-12), or aromatic groups or cleavable peptides or other biodegradable sequences, such as H-GPLG-OH, H-GLFG-OH.

Examples of compounds of formula (I) are the following:

\[
\begin{align*}
\text{CH}_3 & \text{-O-PEG-} \text{-CO-} \text{NH-CH} \text{-O-} \text{CO-} \text{O-} \left(\text{CH}_4\right)_4 \text{-CONC}_2 \\
\text{O} & \text{-CH} \text{-CH} \text{-CH} \text{-CH} \text{-CO-} \text{O-} \left(\text{CH}_4\right)_4 \text{-CONC}_2 \\
\text{QNO} & \left(\text{CH}_4\right)_4 \text{-O-} \text{CO-} \text{O-} \text{NH-} \text{CO-} \text{NH-} \text{CO-} \text{O-} \left(\text{CH}_4\right)_4 \text{-CONC}_2 \\
\text{O} & \text{-CH} \text{-CH} \text{-CH} \text{-CH} \text{-CO-} \text{O-} \left(\text{CH}_4\right)_4 \text{-CONC}_2 \\
\text{R-PEG-} & \text{-CO-} \text{NH-CH} \text{-O-} \text{CO-} \text{O-} \left(\text{CH}_4\right)_4 \text{-CONC}_2 \\
\text{O} & \text{-CH} \text{-CH} \text{-CH} \text{-CH} \text{-CO-} \text{O-} \left(\text{CH}_4\right)_4 \text{-CONC}_2 
\end{align*}
\]

The derivatives thus obtained may be employed also in combinations with non-covalently connected active ingredients. Depending on the selected pharmaceutical form and the physical characteristics of PEG nitrate derivative, different technologies can be used for preparing said derivatives, and this according to the description reported for example in

On 1977, Abuchowski et al. (J. Biol. Chem. 11, 3578-3581) have first described a method for obtaining proteins attached with covalent bond to PEG. Later on, further procedures have been described (F.M. Veronese. Biomaterials 2001, 22, 405-417; M. J. Roberts et Al. Advance Drug Delivery Reviews 2002, 54, 459-476). Most of them involves the PEG hydroxyl end-group activation to give a functional reactive moiety that was then attached to a specific site on the protein. The common reagents employed for activating PEGs are trichloro-s-triazine, carbonyldimidazole, succinic anhydride, succinimidyl carbonate, pNO₂-phenylchloroformiate, among others.

Activating agent selection, PEG molecular weight, the modified groups percentage, the specific properties of the protein to be modified as well as the spacer type carrying the nitrate function can modify the end characteristics of the product. In fact, it is possible to change anyone of said variables for obtaining the desired biological characteristics.

As mentioned above, object of the present invention are also pharmaceutical compositions containing at least a compound of the present
invention together with the usual adjuvants and/or non toxic carriers usually employed in pharmaceutical field.

The daily dose of active ingredient administered to a host can be a single dose or it can be an effective amount divided into several smaller doses that are to be administered throughout the day. The dosage regimen and administration frequency for treating the mentioned diseases with the compound of the invention and/or with the pharmaceutical compositions of the present invention will be selected in accordance with a variety of factors, including for example age, body weight, sex and medical condition of the patient as well as severity of the disease, route of administration, pharmacological considerations and eventual concomitant therapy with other drugs. In some instances, dosage levels below or above a well established range and/or more frequent may be adequate, and this logically will be within the judgment of the physician and will depend on the disease state.

The compounds of the invention may be administered orally, parenterally, rectally or topically, by inhalation, as spray or aerosol, in dosage unit formulations containing conventional non-
toxic pharmaceutically acceptable carriers, adjuvants and vehicles as desired. Topical administration may also involve the use of transdermal administration such as transdermal patches or iontophoresis devices. The term "parenteral" as used herein includes subcutaneous injections, intravenous, intramuscular, intrarneal injection or infusion techniques.

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent. Among the acceptable vehicles and solvents are water, Ringer’s solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides, in addition fatty acids such as oleic acid find use in the preparation of injectables.

Suppositories for rectal administration of the drug can be prepared by mixing the active ingredient with a suitable non-irritating excipient, such as cocoa butter and polyethylene glycols.
Solid dosage forms for oral administration may include capsules, tablets, pills, powders, granules and gels. In such solid dosage forms, the active ingredient may be admixed with at least one inert diluent such as sucrose, lactose or starch. Such dosage forms may also comprise, as in normal practice, additional substances other than inert diluents, e.g. lubricating agents such as magnesium stearate. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering agents. Tablets and pills can additionally be prepared with enteric coatings.

Liquid dosage forms for oral administration may include pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs containing inert diluents commonly used in the art, such as water. Such compositions may also comprise adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring and the like.

Here below a few examples of methods for preparing the compounds object of the present invention will be described.

**EXAMPLES**

Example 1:

*Preparation of mPEG 5000-butyl nitrate.*
Step 1. Derivatization of mPEG-OH 5000 Da end hydroxyl group to give mPEG 5000-0-CH₂-COOH.

To 1 millimol of mPEG-OH 5000 Da 20 ml toluene were added, and the solution was dehydrated by distillation for removing water strictly bond to PEG.

To the PEG solution, that is the distillation residue, 2 millimol of potassium tert.butylate were added (1M solution in tert.butanol). The solution was maintained under stirring and then refluxed at 50°C.

After 4 hours, to the solution containing PEG-OH 4 millimol bromoacetic acid tert.butyl ester were added, the reaction mixture was refluxed 1 hour, then it was cooled at room temperature and maintained under stirring for 12 hours. The end solution, after filtration on celite to remove potassium bromide, was concentrated to dryness in a rotary evaporator. The residue was treated with 33 ml of a TFA : CH₂Cl₂ : H₂O solution (45.4 : 54.5 : 0.1). After three hours stirring for hydrolyzing the tert.butyl ester, the product was evaporated to dryness and treated with 20 ml CH₂Cl₂.

Step 2. PEG-0-CH₂-COOH monomethoxy was derivatized with butandiol monohydrate to give the desired: mPEG 5000-spacer-ONO₂

To 1 millimol of the PEG-O-acetate dissolved in 10 ml of CH₂Cl₂ 4 millimol EDC and 4 millimol HOBt
were added under stirring. After 1 hour reaction, 4 millimol triethylamine and 4 millimol butandiol mononitrate were added. After 12 hours the esterification product was precipitated under vigorous stirring in 200 ml diethyl ether. The precipitate was collected by filtration, dried under vacuum and recrystallized from CH₂Cl₂-diethyl ether.

Example 2:

Preparation of PEG 5000-dibutyl nitrate.

(Derivatization of HO-PEG-OH 5000 Da at -CH₂-COO-(CH₂)₄-ONO₂) end standing hydroxyl moieties.

HO-PEG-OH was derivatized with an acetic derivative to give carboxyl-PEG-carboxyl (as described in example 1 step 1) that in turn activated via EDC/HOBt, reacts with butandiol monohydrate to give the desired:

\[ O₂N-OE₆spacerg-PEG 5000-spacerg-ONO₂ \]

To 1 millimol HO-PEG-OH 5000Da 20 ml toluene were added, and the solution was dehydrated by distillation for removing the hydrating water of PEG.

To the PEG-diol solution, that is the distillation residue (see above), 4 millimol potassium tert.butylate in 1M solution in tert.butyl alcohol were added. The solution was maintained under stirring and refluxed at 50°C. After 4 hours, to the PEG-OK containing solution 8 millimol of bromoacetic
acid tert-butyl ester were added and reflux was continued for 1 hour, then the reaction mixture was cooled under stirring for 12 hours. The solution thus obtained was filtered on celite for removing potassium bromide, and concentrated to dryness in an evaporator. The residue was taken with 33 ml of a TFA : CH₂Cl₂ : H₂O solution (45.4 : 54.5 : 0.1). After three hours stirring for hydrolyzing the tert-butylic acid ester, the product was dried in an evaporator and treated with 20 ml CH₂Cl₂.

To the HOOC-CH₂-O-PEG-O-CH₂-COOH solution 4 millimol EDC and 4 millimol HOBT were added under stirring. After 1 hour reaction 1, 4 millimol triethylamine and 4 millimol butandiol mononitrate were added. After 12 hours, the esterification product was precipitated in 200 ml diethyl ether under vigorous stirring. The precipitate was collected by filtration, dried under vacuum and recrystallized from CH₂Cl₂-diethyl ether.

Example 3:

Preparation of ketorolac-PEG 10.000-monobutyl-nitrate via amide bond.

(Ketorolac-CO-NH-(CH₃)₆-NH-CO-CH₂-O-PEG-O-CH₂-COO-(CH₂)₄-ONO₂)

Step 1. Derivatization of HO-PEG-OH end hydroxylic groups to give -O-CH₂-COOH (see example 2).
Stadio 2. Derivatization of an end carboxyl group of
HOOC-CH₂-O-PEG-O-CH₂-COOH (1) at H₂N-(CH₂)₆-NH-CO- to
give H₂N-(CH₂)₆-NH-CO-CH₂-O-PEG-O-CH₂-COOH.

One millimol of PEG dicarboxylated (1) having a
MP = 10000 Da was solubilized in 50 ml CH₂Cl₂. To this
solution 4 millimol N,N'-dicyclohexylcarbodiimide
(DCC) and 3 millimol N-hydroxysuccinimide (NHS) were
added under stirring. The reaction was prosecuted for
three hours, then 1 millimol of cadaverine-BOC (1,6-
diaminohexane mono t-butyloxy carbonyl) was added. The
solution was maintained under stirring for 5 hours,
then the product NHS-CO-CH₂-O-PEG-O-CH₂-CO-NH-(CH₂)₆-
NH-BOC (2) together with eventual impurities such as
NHS-CO-CH₂-O-PEG-O-CH₂-CO-NHS (3) and BOC-NH-(CH₂)₆-NH-
CO-CH₂-O-PEG-O-CH₂-CO-NH-(CH₂)₆-NH-BOC (4), was
precipitated under stirring in 1000 ml diethyl ether.
The precipitate was collected by filtration and dried
under vacuum. The residue thus obtained was
solubilized in 25 ml 0.2M borate buffer pH 8.5 and
the product was allowed to hydrolyse in order to
remove all the carboxyl group from N-
hydroxysuccinimide. The solution was then charged in
an anion exchange column so as to remove the
bimodified derivative 4 that was not treated. The
desired monocarboxylated product obtained from
hydrolysis of 2 and the bicarboxylated impurity
obtained from hydrolysis of 3, were eluted with a 0.1M HCl solution. The collected fraction containing the eluted products were concentrated at 25 ml and treated under stirring with 35 ml TFA. After 30 min the hydrolysis of BOC groups was terminated and TFA was removed under vacuum in a rotary evaporator. The solution was charged in a column containing a cation exchange resin, wherein the dicarboxylic derivative coming from 3 was not retained, whereas the desired product, monocarboxylic and monoaminic \( \text{H}_2\text{N}-(\text{CH}_3)_6-\text{NH}-\text{CO-CH}_2-\text{O-PEG-O-CH}_2-\text{COOH} \) (5), was retained and then eluted with 0.1M NaCl solution. The product containing fractions, leaving the cation exchange column, were concentrated in a rotary evaporator and the product was then extracted with \( \text{H}_2\text{Cl}_2 \). From the organic phase, concentrated in an evaporator, the product was precipitated under stirring in 500 ml diethyl ether. The precipitate was collected by filtration, dried under vacuum and recrystallized from warm/cold ethyl acetate.

**Step 3.** Bond of ketorolac to \( \text{H}_2\text{N}-(\text{CH}_3)_6-\text{NH}-\text{CO-CH}_2-\text{O-PEG-O-CH}_2-\text{COOH} \) (5) to give ketorolac-\( \text{CO-NH-(CH}_3)_6-\text{NH}-\text{CO-CH}_2-\text{O-PEG-O-CH}_2-\text{COOH} \) (6).

One millimol of ketorolac, free acid, was dissolved in 25 ml \( \text{CH}_2\text{Cl}_2 \) and to this solution 0.9 millimol DCCI and 0.9 millimol NHS were added under
stirring. After 3 hours, to the solution 0.8 millimol of (5) were added. The reaction was prosecuted for 5 hours, then product (6) was precipitated under stirring in 500 ml diethyl ether.

Step 4. Bond of HO-(CH₂)₄-ONO₂ to ketorolac-CO-NH-(CH₂)₆-NH-CO-CH₂-O-PEG-O-CH₂-COOH (6) to give ketorolac-CO-NH-(CH₂)₆-NH-CO-CH₂-O-PEG-O-CH₂-COO-(CH₂)₄-ONO₂.

The reaction was carried out as described in example 1, step 2.

Example 4

Preparation of ketorolac-PEG3400-monobutyl-nitrate via ester bond.

(Ketorolac-CO-O-PEG-COO-(CH₂)₄-ONO₂)

Step 1. Derivatization of hydroxyl group of HO-PEG-COOH with ketorolac to give ketorolac-CO-O-PEG-COOH.

One millimol of ketorolac, free acid, was dissolved in 25 ml CH₂Cl₂ and to this solution 0.9 millimol DCCI and 0.9 millimol NHS were added under stirring. After 3 hours, to the solution 0.8 millimol of HO-PEG-COOH were added. The reaction was prosecuted for 5 hours, then the product (6) was precipitated under stirring in 250 ml diethyl ether.

Step 2. Bond of HO-(CH₂)₄-ONO₂ to ketorolac-CO-O-PEG-COOH to give ketorolac-CO-O-PEG-COO-(CH₂)₄-ONO₂.

The reaction was carried out as described in example 1, step 2.
Example 5

Preparation of epirubicin-PEG3400-monobutyl-nitrate.

Step 1. Derivatization of hydroxyl group of HO-PEG-COOH with epirubicin to give epirubicin-NH-CO-O-PEG-COOH.

One millimol of HO-PEG-COOH was made anhydrous by toluene distillation. The residue was diluted with 10 ml of CH₂Cl₂ and 3 millimol of 4-nitrophenyl chloroformate and 3 millimol of Et₃N were added under stirring. After 6 hours the solution was filtered and then dropped into 150 ml of diethyl ether under stirring. The precipitated product, 4-nitrophenyl-OCO-PEG-COOH, was recovered by filtration and dried under vacuum. Epirubicin, 1.5 millimol, was dissolved in 10 ml of DMF and 1 millimol of 4-nitrophenyl-OCO-PEG-COOH was added under stirring. The solution was alkalinised by 2.5 millimol of Et₃N. The reaction was allowed to proceed for 12 hours. After that, 8 ml of water were added and the solution was acidified at pH 3 by 0.1N HCl. The product epirubicin-NH-CO-O-PEG-COOH was extracted using CH₂Cl₂ (6x80 ml) and 50 g of anhydrous Na₂SO₄ were added to the organic phase. After 30 minutes the organic solution was filtered and concentrated to small volume and then dropped in 150 ml of diethyl ether. The desired product,
epirubicin-NH-CO-O-PEG-COOH, was recovered by filtration and dried under vacuum.

Step 2. Bond of HO-(CH₂)₄-ONO₂ to epirubicin-NH-CO-O-PEG-COOH to give epirubicin-NH-CO-O-PEG-COO-$(CH₂)₄$-ONO₂.

The reaction was carried out as described in example 1, step 2.

Example 6

Preparation of epirubicin-PEG₃₄₀₀-(monobutyl-nitrate)₂

Step 1. Derivatization of hydroxyl group of HO-PEG-COOH with epirubicin to give epirubicin-NH-CO-O-PEG-COOH.

The reaction was executed as described in example 5, step 1.

Step 2. Derivatization of carboxyl group of epirubicin-NH-CO-CO-PEG-COOH with aminoadipic acid to give epirubicin-NH-CO-O-PEG-aminoadipic-(COOH)₂.

One millimol of epirubicin-NH-CO-CO-PEG-COOH was dissolved in 10 ml of CH₂Cl₂ and then 3 millimol of N,N'-dicyclohexylcarbodiimide and 3 millimol of N-hydroxysuccinimide were added. The reaction was prosecuted for 3 hours and then the reaction mixture was filtered and dropped into 200 ml of diethyl ether under vigorous stirring. The product epirubicin-NH-CO-O-PEG-COOSu was recovered by filtration and dried.
under vacuum. A solution of aminoadipic acid was prepared by dissolving 2 millimol of the aminobicarboxylic acid in 10 ml of a water:acetonitrile mixture (2:1). To the solution 3 millimol of Et₃N were added and then 1 millimol of epirubicin-NH-CO-O-PEG-COOSu was added under stirring. After 1 hour the solution was acidified at pH 3 with 0.1N HCl. The product epirubicin-NH-CO-O-PEG-aminoadipic-(COOH)₂ was extracted using CH₂Cl₂ (6x80 ml) and 50 g of anhydrous Na₂SO₄ were added to the organic phase. After 30 minutes the organic solution was filetered and concentrated to small volume and then dropped into 150 ml of diethyl ether. The desired product, epirubicin-NH-CO-O-PEG-aminoadipic-(COOH)₂ was recoverd by filtration and dried under vacuum.

Step 3. Bond of HO-(CH₂)₄-ONO₂ to epirubicin-NH-CO-O-PEG-aminoadipic-(COOH)₂ to give epirubicin-NH-CO-O-PEG-aminoadipic-(COO-(CH₂)₄-ONO₂)₂.

To one millimol of epirubicin-NH-CO-O-PEG-aminoadipic-(COOH)₂ dissolved in 10 ml for CH₂Cl₂ 4 millimol EDC and 4 millimol HOBT were added under der stirring. After 1 hour reaction, 4 millimol triethylylamine and 4 millimol butandiol mononitrate were added. Twelve hours later, teh esterification product was precipitated under vigorous stirring in
200 ml diethyl ether. The precipitate was collected by filtration, dried under vacuum and recrystallized from CH₂Cl₂/diethyl ether.

Example 7:

5 \textbf{NO in vitro release}

The product, prepared as described in example 1, was tested for evaluating the nitric oxide release.

The product was incubated 6 hours in a medium both in presence and in absence of Caco-2 cells at different concentrations. Caco-2 is a colon tumoral cell line having large metabolic capacities similar to normal cells and can be utilized to evaluate drugs metabolism. Further, a second line of fresh isolated rat hepatocytes was employed. With the aid of these two systems it has been evaluated if and in what rate the compound in object is able to release nitric oxide. Corresponding data are reported in figure 1.

As clear from the graph illustrated in figure 1, the product releases nitric oxide in presence of cells, those are able to hydrolyze the compound. At contrary, in absence of cells no nitric oxide release can be detected, and this as evidence of the stability of the product not releasing spontaneously nitric oxide.

Example 8:
Example of PEG-NO2/prednisolone mixture anti-inflammatory activity.

The derivative obtained as described in example 1 was mixed with prednisolone at a molar ratio of 1/1. The resulting mixture was tested and compared with prednisolone alone and with a mixture of prednisolone and a compound that was usually employed for having nitric oxide release, i.e., sodium nitroprussiate (SNP). After i.p. administration, the anti-inflammatory activity was evaluated in mice in by zimosane induced peritonitis model as described by Getting et al. Br. J. Pharmacol. 120, 1075-1082, 1977.

Results expressed as ED$_{50}$ are shown in the following table I:

Table I

ED$_{50}$ of PEG-ONO$_2$ + prednisolone (1/1); prednisolone; mixture prednisolone + SNP

<table>
<thead>
<tr>
<th></th>
<th>PEG-ONO$_2$ + prednisolone (1/1)</th>
<th>Prednisolone</th>
<th>Mixture prednisolone + SNP (1/1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$6 \mu$mol/kg$^{-1}$</td>
<td>25.8 $\mu$mol/kg$^{-1}$</td>
<td>15.2 $\mu$mol/kg$^{-1}$</td>
<td></td>
</tr>
</tbody>
</table>

These results confirm that with PEG nitrated derivatives in mixture with an active ingredient, it
is possible to obtain an ameliorated biological activity in comparison with that obtained with the active ingredient and its combination with a conventional NO donor (SNP). In animals blood pressure measurements, carried out according to well known procedures, have shown a significant decrease higher than 10% in comparison with the basal value for the group treated with the combination with SNP.

Example 9:

Preparation of methotrexate-CO-HN-(CH₂)₅-NH-CO-CH₂-O-PEG-O-CH₂-COO-(CH₂)₄-ONO₂

Step 1. Esterification of methotrexate carboxylic group with PEG heterobifunctional derivative described in Example 3.

Methotrexate was reacted with thionyl chloride in presence of dimethylformamide, methylene chloride and n-hexane to give the acid chloride. The H₂N-(CH₂)₅-NH-CO-CH₂-O-PEG-O-CH₂-COOH, prepared as described in example 3, step 2, was reacted with methotrexate acylic chloride in presence of methylene chloride at a temperature of 25 °C to give a derivative of carboxylated PEG, conjugated with methotrexate.

Step 2. The derivative obtained in step 1 was dissolved in CHCl₃ and then esterified with butandiol mononitrate as described in example 1 step 2.

Example 10:
Modification of superoxide dismutase

\((\text{O}_2\text{NO-}(\text{CH}_2)_4\text{-OCO-HN-}(\text{CH}_2)_6\text{-NH-CO-CH}_2\text{-O-PEG-O-CH}_2\text{-COOH})\)

having a PEG with a PM of 10000 Da was attached with covalent bond to human superoxide dismutase (SOD)).

**Step 1.** Derivatization of the \(-\text{NH}_3\) group of \(\text{H}_2\text{N-}(\text{CH}_2)_6\text{-NH-CO-CH}_2\text{-O-PEG-O-CH}_2\text{-COOH}\) with \(\text{HO-(CH}_3)_4\text{-ONO}_2\) to give \(\text{O}_2\text{NO-}(\text{CH}_2)_4\text{-OCO-HN-}(\text{CH}_2)_6\text{-NH-CO-CH}_2\text{-O-PEG-O-CH}_2\text{-COOH}\).

\(\text{HO-(CH}_3)_4\text{-ONO}_2\) (1 millimol) was dissolved in 5 ml \(\text{CH}_2\text{Cl}_2\). Under stirring 0.9 millimol \(\text{p-NO}_2\)-chloroformiate were added and the mixture was allowed to react 6 hours, then 0.5 millimol \(\text{H}_2\text{N-}(\text{CH}_2)_6\text{-NH-CO-CH}_2\text{-O-PEG-O-CH}_2\text{-COOH}\) were added. After 4 hours, the product \(\text{O}_2\text{NO-}(\text{CH}_2)_4\text{-OCO-HN-}(\text{CH}_2)_6\text{-NH-CO-CH}_2\text{-O-PEG-O-CH}_2\text{-COOH}\) thus obtained was precipitated under stirring in diethyl ether and purified by recrystallization with \(\text{CH}_2\text{Cl}_2/\text{diethyl ether}\). The carboxyl group of intermediate \(\text{O}_2\text{NO-}(\text{CH}_2)_4\text{-OCO-HN-}(\text{CH}_2)_6\text{-NH-CO-CH}_2\text{-O-PEG-O-CH}_2\text{-COOH}\) was activated by EDC/NHS to give \(\text{O}_2\text{NO-}(\text{CH}_2)_4\text{-OCO-HN-}(\text{CH}_2)_6\text{-NH-CO-CH}_2\text{-O-PEG-O-CH}_2\text{-CO-NHS}\).

To the PEG solution, that is the distillation residue, 2 millimol of potassium tert.butylate were added (1M solution in tert.butanol). The solution was maintained under stirring and then refluxed at 50°C. After 4 hours, to the solution containing PEG-OK 4 millimol of bromoacetic acid tert.butyl ester were added, the reaction mixture was refluxed 1 hour, then...
it was cooled at room temperature and maintained under stirring for 12 hours. The end solution, after filtration on celite to remove potassium bromide, was concentrated to dryness in a rotary evaporator. The residue was treated with 33 ml of a TFA : CH₂Cl₂ : H₂O solution (45.4 : 54.5 : 0.1). After a three hours stirring for hydrolysing the tert.butyl ester, the product was evaporated to dryness and treated with 20 ml CH₂Cl₂.

To the PEG-O-acetate solution 4 millimol EDC and 4 millimol HOBt were added under stirring. After 1 hour reaction, 4 millimol triethylamine and 4 millimol butandiol mononitrate were added. After 12 hours the esterification product was precipitated under vigorous stirring in 200 ml diethyl ether. The precipitate was collected by filtration, dried under vacuum and recrystalized from CH₂Cl₂-diethyl ether.

Example 11
Effect of PEG-NO₂/doxorubicin on neoplastic and normal cells vitality in vitro

Doxorubicin (DOX), an anthracycline antineoplastic agent, is effective in the treatment of many solid tumors and leukemias. DOX is very effective in the treatment of breast cancer, and in the treatment of Hodgkin’s and non-Hodgkin’s lymphomas. Moreover, it has also notable activity
against tumors of the ovaries, lung, testes and cervix. The cardiotoxicity associated with DOX is unique among antineoplastic agents and is a major concern during DOX therapy that may be dose limiting. Indeed, the risk of cardiotoxicity is proportional to the cumulative dose of DOX received, and a total cumulative dose of 550 mg/m² is considered the therapeutic of DOX therapy because the risk vs benefits ratio is no longer favorable.

In an attempt to reduce DOX toxicity we have generated a PEG-NO-DOX and preliminary studies were carried out to investigate if the addition of PEG-NO to doxorubicin (PEG-NO2-dox) maintains antiproliferative activity on neoplastic cells and reduces toxicity of the DOX on healthy tissues, in particular cardiovascular tissues. As showed in Figure 2, PEG-NO-DOX not only maintains antiproliferative activity but was more potent than doxorubicin on either colon tumoral (Caco-2) and hepatocarcinoma (HepG2) cell lines. In contrast, as showed in Figure 3, PEG-NO-DOX exerts much lower toxicity than DOX on HUVEC, a normal endothelial cell line. Taken together, these preliminary results in vitro indicated that PEG-NO-DOX may represent an attractive alternative to standard DOX.
CLAIMS

1. Compounds of general formula
   \[(R)_{a}\lignder{\text{PEG}}-[X-(Y-Z-\text{NO}_2)]_q\]_b

wherein

5  \(a\) is 0 or 1,

PEG is polyethylene glycol,

\(b\) is 1 or 2,

\(q\) is the number of the moieties carrying the nitro group and is higher than or equal to 1,

10 \(X\) represents a branching function, that can be present or missing and, when present, it may have one or more branchings according to the formula:

\[
\begin{array}{c}
\text{W} \quad \text{CH} \\
\text{H}_n \quad \text{H}_m \\
\end{array}
\]

or:

\[
\begin{array}{c}
\text{W} \quad \text{CH} \\
\text{H}_n \quad \text{H}_m \\
\end{array}
\]

or it can be further branched,

\(n = 0-5,\)

\(m = 0-5,\)
Y is missing or is an antioxidant function,

Z represents a spacer to which the nitro group is
attached by means of an in vivo hydrolyzable bond
\(-O-(CH_2)_s-ONO_2\), with \(s = 1-10\),

5 \(W = \text{ester, amide, carbamate, ether, thioether,}
\)
disulphide or another covalent bond,

R represents one or more pharmacologically active
groups, drug, oligonucleotide, peptide or protein,
directly bound to PEG or at a branching function, or

10 R is \(-OH\) or \(-CH_3\), wherein the different moieties are
conjugated, preferably via ester, amide, carbamate,
ether, thioether, disulphide or other covalent bonds,
directly or by means of one or more linkers, the
linkers being preferably selected among alkyl groups,

15 such as \(NH_2-(CH_2)_n-NH_2\) (\(n = 0-12\)) or \(HOOC-(CH_2)_m-COOH\)
(\(m = 0-12\)), or aromatic groups or cleavable peptides
or other biodegradable sequences of H-GFLG-OH, H-
GLFG-OH type.

2. Compound of general formula (I) according to claim

20 1, characterized in that being R methoxy, \(a = b = 1\),
it has the formula:

\[
CH_3-O-PEG-X-(Y-Z-NO_2)_q
\]

wherein the symbols and indices have the meaning
described in claim 1.
3. Compound of formula (I) according to claim 1, characterized in that when a is 0 and b is 2, the compound is

\[(O_2N-Z-Y)_q-X-PEG-X-(Y-Z-NO_2)_q\]

wherein symbols and indices have the meaning mentioned in claim 1.

4. Compound of formula (I) according to claim 1, characterized in that when a is 1 and b is 1 and R is a drug, protein or peptide, the compound is:

\[R-PEG-X-(Y-Z-NO_2)_q\]

wherein the symbols and indices have the meaning reported in claim 1.

5. Compounds of formula (I) according to claims 1-4, characterized in that the function carrying the nitro group is aliphatic, aromatic straight or branched.

6. Compounds of general formula (I) according to claims 1-4, characterized in that the active ingredient is selected from anti-inflammatory or antitumoral compounds, peptides, proteins and oligonucleotides.

7. Compounds of general formula (I) according to claim 1, 4 and 6, characterized in that the active ingredient (R) is attached to polyethylene glycol by a chemical bond.

8. Compounds of general formula (I) according to claims 1, 2, 3, 5, wherein R = 0 and the active
ingredient, selected from anti-inflammatory and antitumoral compound, peptides, proteins and oligonucleotides, is not-chemically bound to PEG.

9. Compounds according to anyone of claims 1, 4, 6, 7 and 8 comprising polyethylene glycol, one or more nitro groups attached directly or by spacers at one or at the polyethylene glycol hydroxylic functions and the drug is chemically or physically attached to said polyethylene glycol.

10. Pharmaceutical composition for oral, parenteral, rectal or topic administration, for inalation or spray or as a transdermic composition comprising a compound of formula I as active ingredient and adjuvants or pharmaceutically acceptable carriers.

11. The compound of claim 1, that is ketorolac-PEG 10,000- or PEG 3400 monobutyl nitrate.

12. The compound of claim 1, that is methotrexate-CONH-(CH₂)₆-NH-CO-CH₂-O-PEG-O-CH₂-COO-(CH₂)₄-ONO₂

13. The compound of claim 1, that is PEG-NO₂-doxorubicin.

14. The compound of claim 1, that is PEG-SOD.

15. The compound of claim 1, that is a PEG-NO₂/prednisolone mixture.

16. The compound of claim 1, characterized in that it is PEG 5000-butyl or dibutyl nitrate.
17. The compound of claim 1, that is epirubicin-PEG 3400 monobutyl nitrate.
Fig. 1: Release of nitric oxide (NO) from compound of example 1
Apoptosis Caco-2

Apoptosis HepG2

Figure 2
Figure 3
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48

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)
IPC 7 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, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>WO 92/18465 A (HARVARD COLLEGE) 29 October 1992 (1992-10-29) abstract page 4, lines 4,5 - page 4, lines 11,12; claim 1</td>
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Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

Date of the actual completion of the international search 2 September 2004

Date of mailing of the international search report 14/09/2004

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Authorized officer
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