(54) Title: NOVEL INHIBITORS OF P-GLYCOPROTEIN

(57) Abstract: The invention relates to inhibitors for p-glycoprotein, being compounds that comprise or consist of a tripeptide represented by the sequence XAB, wherein X, A, and B are 7-amino acids, wherein X can be any amino acid and A and B are basic amino acids, for use in the treatment of a disorder. The invention further relates to compositions comprising such compounds, and to the use of such compounds or compositions in the treatment of a disorder. The invention further relates to a method for inhibiting p-glycoprotein in its ability to export substances from a cell, and to a method of treatment of a disorder, said methods comprising administration of a compound or of a composition according to the invention to a subject. The invention also relates to combination therapy using compounds or compositions according to the invention in combination with state of the art compounds or compositions.
Novel inhibitors of p-glycoprotein.

FIELD OF THE INVENTION

The present invention relates to the field of medicine, specifically the field of cancer treatment. A new class of molecules is provided that inhibits P-glycoprotein activity.

BACKGROUND OF THE INVENTION

The P-glycoprotein (pGP) is a transmembrane protein belonging to the class of ABC transporters. The protein acts as a cytoplasmic pump to efflux substances from the cell, and its efflux mechanism requires energy derived from the hydrolysis of ATP to ADP. In many cases, the substrates are potentially toxic xeno-metabolites (xenobiotics). pGP is almost ubiquitous in the human body, as it is expressed in the lower gastrointestinal tract, in organs such as the liver, pancreas, small and large intestines, jejunum, colon, proximal convoluted tubules of the kidney, endothelial cells of the capillaries constituting the blood-brain-barrier, lungs, spleen, skeletal muscles, and the heart (Thiebaut et al, 1987). With this broad distribution, pGP is considered one of the most effective lines of defense against xenobiotics. Despite being the most prominent, pGP is not the only protein in the body with this type of transport function; other proteins belonging to the class of ABC transporters, for example, are also involved, directly or indirectly, in mediating the efflux of xenobiotics from the cytoplasm. Such molecules, along with pGP, are often recognized as the cause of multidrug resistance (MDR). Even though these transporters share many structural features and can recognize similar substrates, each member of the family shows a distinct preference for some particular classes of molecules (Sharom, 2008). pGP shows a preferential transport of amphipathic compounds that are positively charged at physiological pH; other transporters, such as MRPI or ABCG2, are less specific and show a preference towards cationic, hydrophilic compounds, usually in the form of conjugates (Cole et al, 1994; Ishikawa et al, 2005; Staud and Pavek, 2005).

Next to its role in the elimination of xenobiotics, the high efficiency of pGP in exporting molecules from the cell is also considered to be a major limiting factor in the current use of different classes of drugs, and particularly of anticancer agents. Upregulation of pGP expression following chemotherapeutic therapy is frequently seen in later stages of cancer (Krishna and Mayer, 2000). Due to the rapid export of molecules and thus...
insufficient intracellular accumulation, the bioavailability of many therapeutics is frequently lowered to subtherapeutic levels. Moreover, export from pGP-overexpressing tumors also results in higher concentrations in the bloodstream and faster overall clearance, or in changes in the biodistribution.

Inhibition or modulation of pGP activity has therefore long been recognized as a means to modulate drug activity and in particular to enhance the activity of chemotherapeutics towards pGP-overexpressing tumors. Similarly, such pGP inhibition or modulation would improve the efficiency of therapeutics for the central nervous system or the cardiovascular system, and of antimicrobials that are known substrates for pGP (Hunter and Hirst, 1997; Schinkel, 1999).

The development of pGP inhibitors has encountered multiple hurdles that have been hampering their clinical use. pGP inhibitors were initially identified among existing drugs that were found to be substrates for pGP. These substrates are pharmacological agents with a wide array of indications: from the immunosuppressant cyclosporin, to the chemotherapeutic vincristine, from the protease inhibitors lopinavir and ritonavir, to the antifungal ketoconazole. Although these compounds show a high affinity to pGP, their toxicological profile at pGP-inhibitory concentrations caused them to fail in clinical trials (Dantzig et al, 2003). Moreover, most of these compounds are also substrates for cytochrome P450 (CytP450), which may lead to drug-drug interactions (Guengerich, 2008, Morrissey et al, 2012; Zhang and Benet, 2001).

A second generation of pGP inhibitors was developed in the attempt to overcome the drawbacks of the first generation. Parent drugs underwent small chemical modifications. Dexverapamil and the cyclosporin-analogue valspodar (PSC-833) are potent pGP inhibitors, but suffer from low affinity to the protein, therefore requiring a large therapeutic dose that inevitably elicits side effects. All the second-generation inhibitors failed in clinical trials, due to high toxicity, undesired pharmacokinetics, and again high drug-to-drug interaction via the CytP450 pathway (Darby et al, 2011; Thomas and Coley, 2003).

A third generation of inhibitors has been developed by extensive QSAR and molecular modeling, driven by the intention to identify the structural requirements to design potent compounds with limited toxicity and virtually no drug-to-drug interaction. However, early attempts failed at identifying such uniform structural requirements. pGP inhibitors belong to different molecular classes and they share the ability to interact with, and
eventually cross, the plasma membrane. Such structural dissimilarities reflect the broad specificity of pGP and make it almost impossible to fully predict the behavior of a compound from in silico modeling. It was, however, proposed that an ideal inhibitor should possess spatially separated hydrophilic and hydrophobic moieties that can arrange themselves in a way suitable for interaction with pGP (Higgins and Gottesman, 1992). As a result, various potential candidates were designed and synthesized, among which the polyheterocyclic tariquidar with a molecular weight of 646 Da was the only one to enter advanced clinical trials. It was suspended in phase III due to unfavorable toxicity reports (Ozben, 2006). Elacridar, an acridonecarboxamide derivative developed at Glaxo Smith Kline, was originally described to be pGP selective, but is today also recognized as an inhibitor of other members of the ABC transporter family (Matsson et al, 2009). A new class of peptidomimetics, the lead compound of which is known as Reversin 121, which is Boc-Asp(Obzl)-Lys(Z)-OtBu, a fully protected dipeptide that has no accessible basic moieties and no charges at physiological conditions, has shown high potency and low toxicity in vitro. Sold as a "for laboratory use only" peptide chemosensitizer and inhibitor of pGP, these compounds never entered clinical trials mainly because of their very limited solubility in water (Arnaud et al, 2010). The lack of solubility of Reversin 121 in water is strongly detrimental to its practical in vivo application. The broad structural differences between pGP-active compounds are also reflected in their different molecular weight, ranging from 324 Da (quinidine) to 1.2 kDa (cyclosporine and derivatives). Next to these synthetic molecules, several natural products derived from green tea, garlic, grapefruit, or orange are also claimed to possess an intrinsic activity as modulators or inhibitors of pGP (Bailey et al, 1991; Bansal et al, 2009; Yuan et al, 2008). However, only a few compounds (curcumin, ginsenosides, gomisin A, lamellarin D, and terpenoids) were isolated, characterized and tested, both, in vitro and in vivo (Kim et al, 2003; Vanhuyse et al, 2005; Wan et al, 2006). There is no report of any of these molecules having entered clinical trials as pGP inhibitors, as they suffer from the same drawbacks as the first generation inhibitors. Of further interest is Kendarimide A, a complex, heavily modified peptide isolated from the marine sponge of Haliclona sp. Its structure is not fully resolved, though it appears to be a highly N-methylated, linear peptide, comprising an intramolecular C-terminal cysteinyl-cysteine 8-membered ring.
(Aoki et al., 2004; Kotoku et al., 2005). There is no evidence of any follow up to the original study.

There are various problems in this field of technology. The compounds currently indicated, but not approved by the FDA, as in vivo substrates or inhibitors of pGP belong to therapeutic classes other than pGP inhibitors (antibiotics, antihypertensive, antiarrhythmic, anti-HIV, anticancer, antigout, beta-blocker, antihistaminic, antifungal, immunosuppressor). The concentrations required to act as inhibitors of pGP cause adverse effects and drug-to-drug interactions, especially via the interaction with the CytP450 pathway, limiting their use in combination therapy.

Moreover, due to their complex chemical structure many present synthetic, isolation, and formulation hurdles. The synthesis and/or isolation of such molecules involves numerous intermediate steps. Difficulties in scaling up the production at affordable costs is a challenge. Solubility in water is often limited, which can greatly hamper clinical development and application.

The ideal pGP inhibitor should preferably not have an activity as a drug on its own. This eliminates all the FDA-approved drugs with pGP-inhibitory activity as possible candidates. Furthermore, the molecule should be small, easy to synthesize by robust procedures, non-toxic, not metabolized by metabolizing enzymes interfering with the activity of the drug for which export by pGP is to be inhibited, and compatible with standard pharmacological routes of application and with formulation protocols. The present invention provides a solution to these problems.

Human lactoferrin (hLF) is a 77 kDa iron-binding glycoprotein of 692 amino acids that constitutes 15% of the amount of protein contained in human mother milk and can also be found in low concentrations in blood plasma (Nemet and Simonovits, Haematologia (Budap.) 18, 3-121985). The bovine homologue (bLF) consists of 688 amino acids and shares 68% amino acid identity with hLF (Crichton, Adv. Protein Chem. 40, 281-363, 1990). However, only 0.5-1% of bovine milk protein is bLF. For both proteins antimicrobial (Orsi, Biometals 17, 189-196, 2004; Ward and Conneely, Biometals 17, 203-208, 2004), antifungal, LPS binding (Vogel et al., Biochem. Cell Biol. 80, 49-63, 2002) and antiviral properties (Berkhout et al., Biometals 17, 291-294, 2004) have been reported as well as several enzymatic activities like DNase, RNase, ATPase and phosphatase activity (Kanyshkova et al., Eur. J. Biochem. 270, 3353-3361, 2003). The
murine equivalent of hLF is referred to as mLF. Lactoferrin (LF) proteins also act as
transcription factors (He and Furmanski, Nature 373, 721-724, 1995) and have an impact
on immune regulation by inducing the secretion of interleukins (Sorimachi et al, Biochem. Mol. Biol. Int. 43, 79-87, 1997; Vogel et al, 2002).

Efflux pumps are surface proteins that can prevent antimicrobial drugs from
accumulating in a bacterial cell by identifying and pumping the drugs out of the cell.
They are not limited to bacterial cells, but also operate in eukaryotic cells: in eukaryotic
cells, efflux pumps can e.g. prevent other drugs from accumulating in diseased cells in a
subject by pumping those drugs out of the cell. This efflux of drugs prevents the drugs
from achieving therapeutically effective concentrations inside cells. Inhibition of efflux
activity would allow drugs to reach an effective concentration inside cells, and would
thus potentiate the drug's activity. Various otherwise perfectly suitable drug candidates
are not viable for clinical use due to their efflux, caused by efflux pumps. Strategies that
administer drugs together with fragments of said drugs have been reported, where
specific fragments can competitively inhibit an efflux pump to improve the effect of their
It is also known that pGP in its capacity as an efflux pump is present on the wall of the
intestinal tract and inhibits the absorption of orally administered drugs. When an
anticancer agent is orally administered, its absorption and thus its bioavailability is
significantly inhibited by the action of pGP (Schinkel, et al, Cell 11, 491-502, 1994). In
a similar way, expression of pGP in cancer cells counteracts the activity of anticancer
drugs leading to multi-drug resistant cancer cells. Accordingly, when a pGP inhibitor is
administered in combination with an anticancer agent, it may be possible to facilitate the
treatment of a malignant tumor by way of allowing the agent to accumulate in multi-drug
resistant cancer cells, due to the inhibitor's activity blocking the activity of pGP as an
efflux pump.
An aspect of this invention is a new approach to suppressing drug efflux where such
efflux counteracts the accumulation of a drug to therapeutic doses, including newly
developed drugs. Therefore, there is a need in drug treatment for methods that prevent a
drug from being exported by efflux pumps. There have been numerous prior attempts to
improve the efficacy of anticancer agents using pGP inhibitors in cancer cells. For
example, verapamil (a calcium channel inhibitor) and cyclosporin A (an
immunosuppressive agent) are known to be effective in reversing the multi-drug-
resistance of cancer cells against anticancer agents. However, these compounds exhibit
low binding affinities to pGP and as described above, the administration thereof in high
dosage may pose a high risk of toxicity. Furthermore, the administration of known pGP
inhibitors often induces serious adverse effects such as blood pressure decline and
Publication No: WO 94/07858 discloses VX-710, a compound that shows pGP-
inhibitory activity at a μM concentrations. However, VX-710 also inhibits the activity of
CytP450. When administered together with either paclitaxel or vinblastin in clinical
trials, VX-710 inhibits the CytP450-mediated metabolism of these drugs, leading to an
undesirable increase of the cytotoxic agent in the serum and over-exposure of the patient
to the cytotoxic agent.

In an attempt to overcome the disadvantages listed here above, strategies that do not
affect normal pGP function have been investigated. These strategies aim to avoid contact
of a drug with pGP. For example, drugs such as Paclitaxel have been chemically
conjugated to carriers such as a cell-penetrating peptide in an effort to avoid the negative
effect of efflux by pGP (Vargas, J.R., Mol Pharm. 11, 2553-2565, 2014). Similarly, the
encapsulation of a drug into nanoparticulate carriers, including polymer particles and
liposomes with the potential to enter cells, has been shown to reduce the effect of drug
resistance (Wang et al, J Control Release 192, 47-56, 2014; Dong and Mumper,
Nanomedicine (Lond). 5, 597-615, 2010). However, both these strategies can only
operate on a per-drug basis, and often require chemical modification of the drug
molecule, for example through conjugation to targeting or carrier moieties. As a result,
so far only very few drugs have been successfully formulated as conjugates and into
nanocarriers.

Accordingly, there exists a continued need to develop novel effective agents that can be
used to sensitize or resensitize drug resistant cells or microbes to therapeutic or
prophylactic drugs, and/or to prevent or overcome the development of drug resistance in
cancer cells without adverse side effects. The present invention solves this problem.
SUMMARY OF THE INVENTION

In an aspect, the invention provides for compounds for use in the treatment, delay, prevention, cure, or stabilization of a disorder of a subject in need thereof, comprising or existing of tripeptides of the general structure XAB, wherein A and B represent basic amino acids. A and B can either be different or identical basic amino acids. These tripeptides act as substrates for pGP and can therefore competitively inhibit the export of drugs from the cell. These peptides show this activity when added to cells, and even higher such activity when import into the cytoplasm is enhanced, which can either occur by conjugation to a cell-penetrating peptide or by formulation into a carrier such as liposomes or such as (albumin) nanoparticles. On the basis of these findings we can conclude that the basic tripeptide can also independently enter cells. These tripeptides are smaller than most pGP inhibitors reported so far, and have an excellent solubility that does not limit possible applications. Due to their peptidic nature they are neither metabolized by CytP450, nor do they induce the expression of CytP450. The concentrations at which these peptides show activity fall well within the lower micromolar range previously reported for other pGP inhibitors. These peptides are therefore ideally suited for use in the treatment, delay, prevention, cure, or stabilization of a disorder, preferably cancer, of a subject in need thereof.

In a second aspect, the invention relates to compounds as defined above, and their pharmaceutically acceptable salts. In a third aspect, the invention relates to compositions that comprise such compounds. These compositions can comprise further excipients. A fourth aspect relates to the use of the compounds or compositions described above. In a fifth aspect, the invention relates to a method for inhibiting pGP in cells, said method comprising contacting the cells with a compound or composition according to the invention. In a sixth aspect, the invention relates to a method of treatment of a disorder, comprising administration of a compound or composition according to the invention to a subject. In a further aspect, the invention relates to combination therapy involving the compound or composition according to the invention and further therapeutic compounds. Combination therapy is beneficial and can be synergistic because the pGP-inhibiting effect of compounds or compositions according to the invention can potentiate the effect of further pharmaceutical compounds.
DETAILED DESCRIPTION OF THE INVENTION

Surprisingly, it has now been demonstrated that a novel compound is a potent inhibitor of p-glycoprotein and has low toxicity yet high solubility compared to state of the art p-glycoprotein inhibitors.

Accordingly, in a first aspect the invention provides for a compound comprising or consisting of a tripeptide represented by the sequence XAB, wherein X, A, and B are amino acids, wherein X can be any amino acid and A and B are basic amino acids, for use in the treatment, delay, prevention, cure or stabilization of a disorder of a subject in need thereof, comprising administration to the subject an effective dose of the compound, wherein the compound inhibits p-glycoprotein in its ability to export substances from a cell. Said compound is herein referred to as a compound according to the invention. In an embodiment, the compound according to the invention is not conjugated to a drug, preferably not conjugated to a drug selected from the group consisting of Paclitaxel, Methotrexate and Doxirubicin, more preferably the compound according to the invention is not Paclitaxel conjugated to octaarginine or to nonaarginine; Doxirubicin conjugated to penetratin, pegelin, a tat or Tat cell penetrating peptide or a Vectocell peptide; or Methotrexate conjugated to a phage display identified cell penetrating peptide (Vargas, J.R., Mol Pharm. 11, 2553-2565, 2014).

A tripeptide, as defined herein, is a molecule that comprises three amino acid residues, said three residues forming a single chain. Herein, the use of the term peptide or tripeptide should be so construed that next to otherwise featureless tripeptides where each residue is a naturally occurring proteinogenic amino acid linked to its neighbor through a backbone amide bond, also peptides comprising non-natural amino acids, peptidomimetics, unconventional linkages, and many common variations are encompassed. This includes peptides comprising alkylated bonds, inverted bonds, or other types of bonds, such as esters, triazoles, carbamates, ureas, thioureas, imides, imines, halogenated bonds, alpha-halogenated bonds, ketones, or peptides comprising beta-amino acids, other extended amino acids, or peptoids where side chains of residues are attached to the backbone amide bonds instead of to the corresponding alpha carbon atoms, or bonds that involve side chains instead of backbone functional groups. Peptides can comprise amino acids of any chirality, such as L-amino acids or D-amino acids, or mixtures thereof. Accordingly, the term 'amino acid' as used in this invention should be
interpreted as any moiety that can constitute a residue in a peptide as defined above. Most often, an amino acid is a molecular acid, preferably featuring a carboxylic acid, said amino acid featuring an amine at the alpha-carbon next to the carboxylic acid. However, the amine can also be more distant from the carboxylic acid. The most common naturally occurring proteinogenic amino acids and their three-letter abbreviations and one-letter codes are the following: Alanine (Ala, A); Arginine (Arg, R), Asparagine (Asn, N); Aspartic acid (Asp, D); Cysteine (Cys, C); Glutamic acid (Glu, E); Glutamine (Gin, Q); Glycine (Gly, G); Histidine (His, H); Isoleucine (Ile, I); Leucine (Leu, L); Lysine (Lys, K); Methionine (Met, M); Phenylalanine (Phe, F); Proline (Pro, P); Serine (Ser, S); Threonine (Thr, T); Tyrosine (Tyr, Y); Tryptophan (Trp, W); Valine (Val, V). In the context of this invention, naturally occurring amino acids are also called natural amino acids. Natural amino acids are often proteinogenic, which means that they are used by organisms in the biosynthesis of proteins. In some cases, natural amino acids can also be non-proteinogenic. Natural amino acids are those amino acids that can be found in nature, without further limiting their role or function. As known to a person skilled in the art, amino acids are often characterized by the nature of their side chains. Amino acids that are considered to be basic amino acids are lysine, arginine, and histidine. Amino acids that are considered to be acidic amino acids are aspartic acid, glutamic acid, and tyrosine. Amino acids that are considered to be polar uncharged amino acids are serine, threonine, cysteine, asparagine, and glutamine. Amino acids that are considered to be hydrophobic amino acids are alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, proline, and tryptophan. Proline is considered to be a conformationally restrained amino acid. Glycine is achiral yet can be part of both D-peptides and L-peptides. Within the embodiments of this invention, tripeptides can be comprised in larger peptides, or in larger molecules. Peptides are understood to possibly comprise capping groups such as terminal amides, acetamides, methyl esters, other terminating esters, or other terminal moieties that are known to a person skilled in the art. Peptides are further understood to possibly feature protecting groups such as t-butyl carbamate, 9-fluorenymethyl carbamate, benzyl carbamate, benzyl ester, t-butyl ester, methyl ester, or other protecting groups known in the art.

Within the embodiments of the invention, the term "treating" preferably means that there is a detectable change in a parameter associated with a disorder in a subject, which can be an improvement in a parameter of the subject itself, or a deterioration of a parameter
associated with a parasite, infectious organism, or microbe or other possible factor. Within the embodiments of the invention, the term "delaying" preferably means that a parameter associated with a disorder in a subject is only at a level similar to such a level in an untreated subject after at least 1, 2, 3, 6, 12, 18, 24, 30, 36, 48, 60 or more months.

Within the embodiments of the invention, the term "preventing" preferably means that during at least one, two, three, four, five, or more years no parameters associated with a disorder are found to be at levels corresponding to a disorder, meaning the disorder is not or not yet detected. Within the embodiments of the invention, the term "curing" preferably means that there is a change in parameters associated with a disorder in a subject to such an extent that said disorder can no longer be detected. Within the embodiments of the invention, the term "stabilizing" may mean that a parameter associated with a disorder in a subject is unchanged after at least 1, 2, 3, 6, 12, 18, 24, 30, 36, 48, 60 or more months. Therefore, in the context of the invention, preventing, treating, curing, stabilizing and/or delaying a condition may mean that:

- at least a symptom of this condition has been improved, and/or
- at least a parameter associated with this disease or condition has been improved.

In the context of the invention, a symptom is preferably said to have been improved when a parameter associated with said symptom has changed at least 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%, or more, said change being towards parameter levels found in healthy subjects, or in subjects not in need of treatment. If a parameter has changed 100%, and the change is a decrease, this may mean that the parameter can no longer be detected.

In the context of this invention, a disorder can be any type of disorder with which pGP activity is associated and/or in which it might hinder treatment. As described above, this might be through unwanted export of beneficial drugs from a subject's cells, or through unwanted export of antimicrobial or antibiotic drugs from a microbe or similar subject. A preferred disorder is thus an infection by an organism that could be treated were it not for pGP activity in that organism. A preferred disorder is tuberculosis.

Within the embodiments of the invention, a preferred disorder is cancer. In preferred embodiments of this aspect, a compound is provided comprising a tripeptide represented by the sequence XAB, wherein X, A, and B are amino acids, wherein X can be any amino acid and A and B are basic amino acids, for use in the treatment, delay, prevention, cure
or stabilization of a cancer of a subject in need thereof, comprising administration to the
subject an effective dose of the compound, wherein the compound inhibits p-
5 glycoprotein in its ability to export substances from a cell. In this context, cancers include
a cancer of epithelial origin or neuronal origin or a carcinoma or a solid tumor or a
sarcoma or a liquid tumor such a leukemia or a lymphoma. Cancer cells may be from the
bladder; brain; breast; colon; esophagus; gastrointestinal; head; kidney; liver; lung;
nasopharynx; neck; ovary; prostate; skin; stomach; testis; tongue; neuron or uterus. In
addition, the cancer may specifically be of the following histological type, though it is
not limited to these: neoplasm; malignant; carcinoma; carcinoma undifferentiated; giant
and spindle cell carcinoma; small cell cell carcinoma; pilomatrix carcinoma; transitional
cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma;
malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular
carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic
carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma; familial polyposis
coli; solid carcinoma; carcinoid tumor; malignant; branchiolo-alveolar carcinoma;
papillary carcinoma; squamous cell carcinoma; basal adenocarcinoma; papillary
adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic
adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell
carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma;
nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid
carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous
adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma;
cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous
cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet
ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular
carcinoma; inflammatory carcinoma; paget's disease of the breast; acinar cell carcinoma;
adenosquamous carcinoma; adenocarcinoma with squamous metaplasia; ovarian stromal
tumor, malignant; and rhabdostoma, malignant; Sertoli cell carcinoma. A cancer may be
neuroblastoma.

In this context a leukemia includes any of: Acute lymphoblastic leukemia (ALL) such as
precursor B acute lymphoblastic leukemia, precursor T acute lymphoblastic leukemia,
Burkitt's leukemia, and acute biphenotypic leukemia, Chronic lymphocytic leukemia
(CLL) such as B-cell prolymphocytic leukemia, a more aggressive disease, Acute
myelogenous leukemia (AML) such as acute promyelocyte leukemia, acute myeloblasts leukemia, and acute megakaryoblastic leukemia, Chronic myelogenous leukemia (CML) such as chronic monocytic leukemia, Hairy cell leukemia (HCL), T-cell prolymphocyte leukemia (T-PLL), Large granular lymphocytic leukemia and Adult T-cell leukemia.


Any cancer that is known to feature upregulated pGP activity, or where drug options are limited due to pGP activity, is encompassed within the scope of the invention. Such cancers are in particular ovarian carcinoma and breast cancer, but high expression of pGP is also observed in hepatic carcinomas and some forms of blood cancers. In the context of the invention, the treatment of a cancer may also be the inhibition of tumor cell proliferation, the induction or increased induction of tumor cell death, the prevention or delay of the occurrence of metastases, the prevention or delay of tumor cell migration, an inhibition or prevention or delay of the increase of a tumor weight or growth, and/or a prolongation of patient survival of at least one month, several months or more (compared to those not treated or treated with a control or compared with the subject at the onset of the treatment) and/or improvement of the quality of life and observed pain relief.
In the context of the invention, a patient may survive and/or may be considered as being disease free. Alternatively, the disease or condition may have been cured or delayed. In the context of the invention, an improvement of quality of life and observed pain relief may mean that a patient may need less pain relief drugs than at the onset of the treatment. Alternatively or in combination with the consumption of less pain relief drugs, a patient may be less constipated than at the onset of the treatment. "Less" in this context may mean 5% less, 10% less, 20% less, 30% less, 40% less, 50% less, 60% less, 70% less, 80% less, 90% less, or 100% less. In this latter case a patient may no longer need any pain relief drug.

This improvement of quality of life and observed pain relief may be seen, detected or assessed after at least one week, two weeks, three weeks, four weeks, one month, two months, three months, four months, five months, six months or more of treatment in a patient and compared to the quality of life and observed pain relief at the onset of the treatment of said patient.

Within the embodiments of the invention, inhibition of the proliferation of tumor cells is preferably at least 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, or more. Proliferation of cells may be assessed using known techniques, such as FACS or resazurin assays, for example as described in the examples, or by MRI, PET, SPECT, or CT, or by otherwise determining changes in tumor volume or metabolic activity. The proliferation and the status of tumor cells may be assessed through biopsies and (immuno-)histological characterization of the tumor and its surrounding tissue. An induction of tumor cell death may be at least 1%, 5%, 10%, 15%, 20%, 25%, or 25 more. Tumor growth may be inhibited at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 55%, 60%, 65%, 70%, or 75%, or more. Tumor cell death may be assessed using techniques known to the skilled person. Tumor cell death may be assessed using MRI, PET, SPECT, or CT, or by otherwise determining changes in tumor volume or metabolic activity. The death or decrease in activity of tumor cells may be assessed through biopsies and (immuno-)histological characterization of the tumor and its surrounding tissue. In certain embodiments, tumor weight increase or tumor growth may be inhibited at least 20%, 30%, 40%, 50%, 55%, 60%, 65%, 70% or 75%, or more. Tumor weight or tumor growth may be assessed using techniques known to the skilled person. The detection of tumor growth or the detection of the proliferation of tumor cells may be assessed in vivo by measuring changes in glucose utilization by positron emission tomography with the
glucose analogue 2-[18F]-fluor-2-deoxy-D-glucose (FDG-PET) or [18F]-3-fluoro-3-deoxy-L-thymidine PET. An ex vivo alternative may be staining of a tumor biopsy with Ki67.

Within the embodiments of the invention, a subject can be any living entity. Preferably, a subject is a human being. Within the embodiments of the invention, a subject may be a microorganism or a microbe or a parasite. Within the embodiments of the invention, subjects may be animals, plants, or fungi of any kind. Within the embodiments of the invention, a subject may be a single cell, or a culture of cells, either cultured in vitro, or obtained from a subject and maintained ex vivo. Subjects are in need of treatment when parameters associated with a disorder can be detected, or when such parameters are expected to become detectable, or when such parameters are feared to become detectable. Administration of a compound or composition according to the invention can be achieved by any method known in the art, as further defined later in this text.

Within the embodiments of the invention, an effective dose of a compound or composition is a dose that can assert a desired effect, such as improving a symptom of a disorder, or changing a parameter associated with a disorder. In the context of the invention, inhibition of p-glycoprotein (pGP) comprises a detectable reduction in the activity of pGP as an efflux pump as measurable by an assay, or as concluded from an experiment. Such an assay can be one where accumulation of fluorescent products, or the absence thereof, is determined, for example as described in the examples. It can also comprise a detectable reduction in the ATPase activity of pGP. Reduction preferably means that activity has been reduced by at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100% as compared to activity in an untreated reference sample, assayed under identical conditions where a reduction of 100% may mean that no activity can be detected.

In the context of the invention, the ability of pGP to export substances from a cell should be interpreted as its ability to neutralize substances, or to remove them from bioavailability, where substances should be read as molecules, complexes of multiple molecules, oligomers, polymers, polypeptides, proteins, or particles. Neutralization should be construed as the removal of a substance from its available status. Preferably, this happens through transport, which is preferably from inside a cell to outside a cell, which is generally depicted as efflux or export. In the context of this invention, when pGP is said to export a substance from a cell, it is to be understood that such export can
be export of a substance from the cell's lumen including but not being restricted to the cytoplasm, from the plasma membrane lipid bilayer, or from any other compartment of the cell.

Compounds as described in this aspect of the invention, may be the compounds as such or may be a pharmaceutically acceptable salts of such a compound or of a related compound according to the invention.

Preferably, within the compound according to the invention, B and/or X are preferably terminal amino acids. In the context of the invention, a terminal amino acid is the last or the first amino acid of a sequence of amino acids, so that a terminal amino acid only has either a preceding or a subsequent amino acid, not both. A terminal amino acid is not connected to any other moiety, except to the sequence of which it is a terminal amino acid, and possibly except to any capping moieties or protecting groups such as known in the art and/or described above. In an embodiment of the invention, X is a terminal amino acid. In another embodiment of the invention, B is a terminal amino acid. In a further embodiment of the invention, X and B are both terminal amino acids.

Preferably, within the compound according to the invention, at least one of X, A, and B is a natural amino acid, preferably an L-amino acid. In a preferred embodiment of the invention, at least two of X, A, and B are natural amino acids, preferably L-amino acids. In a more preferred embodiment of the invention, each of X, A, and B are natural amino acids, preferably L-amino acids. In a more preferred embodiment of the invention, each of X, A, and B are natural L-amino acids.

Preferably, within the compound according to the invention, either A or B are selected from the group consisting of Arginine, Lysine and Histidine. In a preferred embodiment, A and B are each independently selected from the group consisting of Arginine, Lysine and Histidine. Preferably, A and/or B are each independently selected from Arginine or Lysine.

Preferably, within the compound according to the invention, X is selected from the group consisting of Asparagine, Tryptophan, and any amino acid with a side chain that consists of only carbon and hydrogen atoms. In a preferred embodiment of the invention, X is
selected from the group consisting of Asparagine, Tryptophan, Alanine, Valine, Isoleucine, Leucine, and Phenylalanine. In a more preferred embodiment, X is selected from the group consisting of Alanine, Isoleucine, Asparagine, and Tryptophan.

Preferably, within the compound according to the invention, X is selected from the group consisting of Alanine, Isoleucine, Asparagine, and Tryptophan, and A and B are each independently selected from the group consisting of Arginine, Lysine, and Histidine. In a preferred embodiment of the invention, X is selected from the group consisting of Alanine, Isoleucine, Asparagine, and Tryptophan, and A and B are each independently selected from Arginine or Lysine.

In an embodiment of this aspect of the invention, the compound preferably consists of a tripeptide represented by the sequence XAB. In a preferred embodiment, XAB is IKR, being Ile-Lys-Arg. In a further preferred embodiment, XAB is AKR, being Ala-Lys-Arg. In a further preferred embodiment, XAB is WKK, being Trp-Lys-Lys. In a further preferred embodiment, XAB is NRR, being Asn-Arg-Arg.

Within the compounds according to the invention, tripeptides according to the invention are a preferred embodiment. Compounds according to the invention comprising tripeptides are advantageous over previously existing pGP-inhibitor candidates due to their excellent solubility in water at physiological conditions. Physiological conditions are known to a person skilled in the art, and comprise atmospheric pressure, pH-values between 6 and 8, a temperature ranging from room temperature to about 37° C (from about 20° C to about 40° C), and a suitable concentration of buffer salts or other components. Additionally, compounds according to the invention can easily be produced, e.g. synthetically in well under ten reaction steps using standard techniques, reactants, and reagents. Another advantage of compounds according to the invention is that in some embodiments the compounds can independently enter cells to more effectively assert their therapeutic effect. Previously existing pGP-inhibitor candidates were often found to lack solubility or to otherwise lack bioavailability. This problem is solved by the invention.

Preferably, within the compound according to the invention, any of the compounds as defined above further comprises a peptide, a drug, and/or a label. In an embodiment, the
compound according to the invention is not conjugated to a drug selected from the group consisting of Paclitaxel, Methotrexate and Doxirubicin, more preferably the compound according to the invention is not Paclitaxel conjugated to octaarginine or to nonaarginine; Doxirubicin conjugated to penetratin, pegelin, a tat or Tat cell penetrating peptide or a Vectocell peptide; Methotrexate conjugated to a phage display identified cell penetrating peptide (Vargas, J.R., Mol Pharm. 11, 2553-2565, 2014).

In a preferred embodiment, XAB is comprised in a peptide. In a more preferred embodiment, XAB is at the terminus of a peptide. In an even more preferred embodiment, the compound is the peptide represented by KCFQWQRNMRKVRGPPVSCIKR (SEQ ID NO: 2), which is referred to as hLF. In a further even more preferred embodiment, the compound is the peptide represented by RQIKIWFQNRRMKWKK (SEQ ID NO: 5), which may be referred to as penetratin or antennapedia. As is the case for all peptides and other compounds according to the present invention, it is to be understood that penetratin or antennapedia can be used with or without various end group modifications, such as for example and not limited to:

H-KCFQWQRNMRKVRGPPVSCIKR-OH, or
H-KCFQWQRNMRKVRGPPVSCIKR-NH2, or
Ac-KCFQWQRNMRKVRGPPVSCIKR-OH, or
Ac-KCFQWQRNMRKVRGPPVSCIKR-NH2, or similar for other peptide sequences.

In a further embodiment of this aspect, a compound according to the invention comprises both a first peptide and the tripeptide XAB in such a manner that XAB is not comprised in the first peptide, such as in R₉-ahx-IKR (SEQ ID NO: 17), which is a preferred embodiment. As defined herein, and as known to the person skilled in the art, ahx represents 6-aminohexanoic acid, which is also known as aminocaproic acid, which in turn is abbreviated as Acp. Ahx is considered to be a linker moiety that links two further moieties together. In addition to ahx, other linkers can be used, such as, but not limited to, beta-alanine (also known as beta-aminopropionic acid, bAla), 4-Aminobutyric acid (also known as piperidinic acid, 4Abu), 3-Aminoisobutyric acid (bAib), or other linking moieties known in the art. Further preferred embodiments of the invention are Rs-IKR (SEQ ID NO: 12), R₉-IKR (SEQ ID NO: 13), R₉-ahx-IKR (SEQ ID NO: 16), tat-IKR (SEQ ID NO: 10), Tat-IKR (SEQ ID NO: 11), tat-ahx-IKR (SEQ ID NO: 14), and Tat-ahx-IKR (SEQ ID NO: 15), wherein tat is a peptide known to be a cell penetrating peptide that comprises the amino acid sequence RKKRRQRRR (SEQ ID NO: 6), and wherein
Tat is a longer version of tat, comprising the amino acid sequence GRKRRQRRRPQ (SEQ ID NO: 7). The XAB moiety can also be linked to a peptide through grafting of XAB to the side chain of a residue, resulting for example in amino acid residues such as -Lys(XAB-OH)-. Examples of such a preferred embodiment would be H-RRRRRRRRK(IKR)-OH, or H-K(IKR)RRRRRRR-R-OH. A further preferred further embodiment is a fusion protein comprising a molecule according to the invention.

In a further embodiment, the compound according to the invention further comprises a label. A label is understood to be any moiety that facilitates detection using a method for detection, whereby such a label is a fluorophore, a chromophore, a radioactive tracer, a specific isotope, a diagnostic marker, or a hapten, wherein a hapten is preferably biotin.

In a preferred embodiment, the compound is radioactively labeled, preferably by having incorporated a radioactively labeled amino acid, whereby more preferably the radioactively labeled amino acid is a tritium-labelled amino acid. If a label is a diagnostic marker, it may be a fluorogenic substrate to detect the activity of a pathologically relevant protease, for example a caspase involved in the initiation and execution of apoptosis in a cell. Non-limiting examples of labeled compounds according to the invention are fluorescein-fiLF, fluorescein-mLF (where mLF represents a peptide known to be a cell penetrating peptide derived from murine lactoferrin), fluorescein-penetratin, fluorescein-ahx-IKR, fluorescein-IKR, fluorescein-Rs-ahx-IKR.

In a preferred embodiment, the compound according to the invention further comprises a drug or a drug candidate. A drug can be a small molecule, a peptide, a depsipeptide, an acyldepsipeptide, an antibiotic, an antimicrobial, a polypeptide, a protein, a protein fragment, a nucleic acid, a nucleic acid analogue, or parts thereof, a chemotherapeutic, a decoy molecule, or any other entity or combination thereof. A nucleic acid can be selected from the group comprising DNA molecules, RNA molecules, PNA molecules, oligonucleotides, siRNA molecules, antisense molecules, ribozymes, aptamers, and spiegelmers. A drug is understood to be any entity that can assert a therapeutic effect, which can also be for vaccination. Non-limiting examples of preferred drugs are daunorubicin and doxorubicin.

In a preferred embodiment of this aspect is provided a compound comprising or consisting of a tripeptide represented by the sequence XAB, wherein X, A, and B are amino acids, wherein X can be any amino acid and A and B are basic amino acids, for use in increasing the residence time of a drug inside a cell. As explained earlier herein,
various drugs lose efficacy due to their export from a cell. Increased intracellular residency of such drugs accordingly increases their efficacy. Intracellular residence time of a drug can be determined through known techniques, for example through fluorescence correlation spectroscopy or through suitable mass spectrometry or high-performance liquid chromatography/mass spectrometry protocols. A drug preferably has an increased residence time in a cell when more of the drug can be detected inside a cell after a given amount of time when a compound according to the invention has also been administered, as compared to when only the drug has been administered. In this context, preferred amounts of time after which to determine residency are 30 minutes, one hour, two hours, three hours, four hours, or five hours, preferably four hours.

In a second aspect of the invention, a compound is provided that is defined as any of the compounds described above, which includes compounds named above. Such compounds comprise a tripeptide represented by the sequence XAB, wherein X, A, and B are amino acids, wherein X can be any amino acid and A and B are basic amino acids. Within this aspect, X and/or B can be terminal amino acids. Within this embodiment, X and/or A and/or B can be natural amino acids, preferably L-amino acids. Within this aspect, A and B can each be independently selected from the group consisting of Arginine, Lysine and Histidine, preferably selected from Arginine and Lysine. Within this aspect, X can be selected from the group consisting of Asparagine, Tryptophan, or any amino acid with a side chain that consists of only carbon and hydrogen atoms, preferably X is selected from the group consisting of Alanine, Isoleucine, Asparagine, and Tryptophan. In an embodiment of this aspect, X is selected from the group consisting of Alanine, Isoleucine, Asparagine, or Tryptophan and A and B are each independently selected from the group consisting of Arginine, Lysine and Histidine, preferably selected from Arginine and Lysine. In a further embodiment, the compound consists of a tripeptide represented by the sequence XAB, wherein XAB preferably is IKR or AKR, more preferably IKR. Within this aspect, the compound can further comprise a peptide, a drug and/or a label as specified above. Compounds as described in this aspect of the invention, may be the compounds as such or may be a pharmaceutically acceptable salt of such a compound or of a related compound according to the invention.
In a third aspect of the invention, a composition comprising a compound according to the invention is provided. In an embodiment of this aspect, the composition further comprises an excipient, preferably a pharmaceutically acceptable excipient. Preferred in this aspect is a composition that comprises a compound according to the invention, a pharmaceutically acceptable excipient, and further comprises a therapeutic compound. Within this aspect are compositions that comprise liposome formulations as an excipient. Preferred liposomal formulations are based on DPPC or on DOPE or on combinations thereof. A preferred formulation is DPPC/DOPE/CHEMS/Cholesterol 4:2:2:1. A more preferred formulation is DPPC/Cholesterol 2:1. Liposomes may further feature acylated peptides, other acylated functional molecules, and may carry further cargo molecules, in particular liposomes may feature a shell of polyethyleneglycols to enhance circulation time in a subject.

Similarly within this aspect are compositions that comprise nanoparticle formulations as an excipient. Preferred nanoparticle formulations are albumin nanoparticle formulations. Albumins are globular proteins that are easy to purify, biodegradable, and that have low toxicity and little potential to trigger immune responses. Albumin nanoparticles comprise crosslinked albumin proteins. A preferred albumin is human serum albumin, more preferably recombinant serum albumin, because this further decreases the chance of an undesired immune response.

In an embodiment of the third aspect of the invention, the composition is for use in the treatment, delay, prevention, cure or stabilization of a disorder, preferably cancer, of a subject in need thereof, said use comprising administration to the subject of an effective dose of the composition, wherein the composition inhibits p-glycoprotein in its ability to export compounds from a cell. In an embodiment, the composition is for use in increasing the residence time of a drug in a cell. Terms, features and definitions are preferably as provided above.

In a fourth aspect of the invention, the use is provided of either a compound according to a previous aspect of the invention, or of a composition according to a previous aspect of the invention. Said use is for the treatment, delay, prevention, cure or stabilization of a disorder of a subject in need thereof, and comprises administration to the subject of an effective dose of a compound according to the invention or of a composition according
to the invention, wherein the compound or composition inhibits p-glycoprotein in its ability to export substances from a cell.

In an embodiment of this aspect, the use is provided of either a compound according to the invention, or of a composition according to the invention, for the treatment, delay, prevention, cure or stabilization of a cancer of a subject in need thereof, and comprises administration to the subject of an effective dose of a compound according to the invention or of a composition according to the invention, wherein the compound or composition inhibits p-glycoprotein in its ability to export substances from a cell.

In an embodiment of this aspect, the use is provided of either a compound according to the invention, or of a composition according to the invention, for increasing the residence time of a drug in a cell. Terms, features and definitions are preferably as provided above.

A fifth aspect of the invention provides a method for inhibiting p-glycoprotein in its ability to export substances from a cell, said method comprising contacting the cell with a compound according to the invention, or with a composition according to the invention. In the context of the invention, contacting a cell with a compound or a composition can comprise adding such a compound or composition to a medium in which a cell is cultured. Contacting a cell with a compound or a composition can also comprise adding such a compound or composition to a medium, buffer, or solution in which a cell is suspended, or which covers a cell. Other preferred methods of contacting a cell comprise injecting a cell with a compound or composition, or exposing a cell to a material comprising a compound or composition according to the invention.

In an embodiment of this aspect, the method is an in vitro method. In a further embodiment of this aspect, the method is an ex vivo method. In a further embodiment of this aspect, the method is an in vivo method. In a preferred embodiment of this aspect, the method is an in vitro or an ex vivo method.

Within the embodiments of this aspect, the cell can be a cell from a sample obtained from a subject. Such a sample can be a sample that has been previously obtained from a subject. Within the embodiments of this aspect, samples can have been previously obtained from a human subject. Within the embodiments of this aspect, samples can have been obtained from a non-human subject. In a preferred embodiment of this aspect, obtaining the sample is not part of the method according to the invention.
In a specific embodiment of the method of the invention, the method is for increasing the residence time of a drug inside a cell.

In a further aspect of the invention a method of treatment is provided. Said method can be used to treat, delay, prevent, cure or stabilize a disorder of a subject in need thereof, and comprises administration to the subject of an effective dose of either a compound according to the invention, or of a composition according to the invention, wherein the compound or composition inhibits p-glycoprotein in its ability to export substances from a cell. In an embodiment of this aspect, the disorder is a cancer. Terms, features and definitions are preferably as provided above.

In a further aspect of the invention, combination therapy is provided. Within embodiments of this aspect, a compound for use according to the first aspect of the invention is administered together with an effective dose of a further therapeutic compound or composition. Within further embodiments of this aspect, a composition for use as defined above is administered together with an effective dose of a further therapeutic compound or composition. It is within embodiments of this aspect that a use as described is combined with administration of an effective dose of another therapeutic compound or composition. It is within embodiments of this aspect that a method of this invention further comprises administration of an effective dose of another therapeutic compound or composition.

Encompassed by this aspect are embodiments wherein a compound, composition, use, or method according to this invention is effectuated simultaneously with the administration of said further therapeutic compound or composition, which is referred to as simultaneous administration. Also encompassed by this aspect are embodiments where the administration of said further therapeutic compound or composition can take place either before or after the use or method according to this invention, which is referred to as separate administration. In preferred embodiments, separate administration can comprise administrations that are separated by a minimal amount of time, or by 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, or more minutes, or by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more hours, or by 1, 2, 3, 4, 5, or more days, or by a week or by a month or by a longer duration of time.
In the context of this invention, further therapeutic compounds or compositions are preferably compounds or compositions that comprise substrates of pGP. More preferred further therapeutic compounds or compositions are such entities that are limited in their therapeutic effect as a result of pGP-activity, or that have undesirable distribution profiles, metabolic profiles, excretion profiles, or toxicity profiles, as a result of pGP-activity. Combination of such a compound or composition with a compound or composition according to the invention can help overcome, or otherwise ameliorate or obviate these undesirable characteristics.

Non-limiting examples of preferred further therapeutic compounds or compositions are, or comprise, Actinomycin, All-trans retinoic acid, Azacitidine, Azathioprine, Bleomycin, Bortezomib, Carboplatin, Capecitabine, Cisplatin, Chlorambucil, Cyclophosphamide, Cytarabine, Daunorubicin, Docetaxel, Doxifluridine, Doxorubicin, Epirubicin, Epothilone, Etoposide, Fluorouracil, Gemcitabine, Hydroxyurea, Idarubicin, Imatinib, Irinotecan, Mechloretamine, Mercaptopurine, Methotrexate, Mitoxantrone, Oxaliplatin, Paclitaxel (Taxol), Pemetrexed, Teniposide, Tioguanine, Topotecan, Valrubicin, Vinblastine, Vincristine, Vindesine, or Vinorelbine, more preferably Daunorubicin or Doxorubicin, most preferably Daunorubicin.

In the context of this invention, a peptide can be obtained by isolating it from a natural product. Peptides can be obtained through isolation from a digest of a larger protein. Preferably, peptides according to the invention are of synthetic origin. A preferred method for peptide synthesis is solid phase peptide synthesis (SPPS), which is well-known to a person skilled in the art. Advantages of obtaining short peptides through SPPS are the ease of synthesis, the low component cost, the speed of synthesis, and the possibility for automation using synthesis robots, synthesizers, semi-automatic synthesizers, or automatic synthesizers. Peptides or peptide analogues according to the invention are therefore more readily accessible than many other molecules. Compounds according to the invention that comprise peptides featuring a XAB motif embedded in an encompassing peptide sequence are especially accessible using this strategy. SPPS strategies known in the art allow both N-terminal and C-terminal modification, such as alkylation, amidation, or labeling.
It is within the scope of the invention that the compounds or compositions according to the invention are preferably delivered to a specific type of cells or tissue or organ comprising such specific type of cells. In a preferred embodiment such specific delivery is mediated through a targeting moiety or a targeting molecule that may be comprised in the molecule or composition according to the invention.

Compositions and pharmaceutical compositions according to the invention may be manufactured by processes well known in the art; e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes, which may result in liposomal formulations, coacervates, oil-in-water emulsions, nanoparticulate/microparticulate powders, or any other shape or form. Compositions for use in accordance with the invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent on the route of administration chosen.

When a composition or a pharmaceutical composition according to the invention is encapsulated, all components of said composition are preferably encapsulated in the same resulting particle. Coencapsulation of a compound according to the invention with a further therapeutic compound ensures simultaneous delivery and thus mitigates differences in pharmacodynamics properties that might reduce the positive effect of a compound according to the invention on said further therapeutic agent.

For injection, the compounds according to the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Formulations that promote penetration of the epidermis are known in pharmacology, and can find use in the treatment of many skin conditions, such as, but not limited to, psoriasis and fungal infections. Formulations that promote penetration of the epidermis and underlying layers of skin are also known, and can be used to apply compositions of the invention to, for example, underlying muscle or joints. In some preferred therapeutic embodiments, formulation comprising compositions according to the invention that
deliver compounds for alleviation of rheumatoid- or osteo-arthritis can be administered by applying a cream, ointment, or gel to the skin overlying the affected joint. Oral and parenteral administration may be used where the compound and/or composition is stable enough to endure the harsh proteolytic environment of the gut. If so, the compound or composition according to the invention can be formulated readily by combining a compound or composition according to the invention with pharmaceutically acceptable carriers well known in the art, or by using a compound or composition according to the invention as a food additive. Such strategies enable the compounds or compositions according to the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. Preparations or pharmacological preparations for oral use can made with the use of a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Additionally, coformulations may be made with uptake enhancers known in the art, or with proteolytic inhibitors known in the art.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, PVP, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solution, and suitable organic solvents or solvent mixtures. Polymethacrylates can be used to provide pH-responsive release profiles so as to pass the stomach. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which can be administered orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with a filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active
compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compounds or compositions according to the invention may be administered in the form of tablets or lozenges formulated in a conventional manner.

For administration by inhalation, the compounds and compositions according to the invention can be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and optionally a suitable powder base such as lactose or starch.

The compound or composition according to the invention may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. In this way it is also possible to target a particular organ, tissue, tumor site, site of inflammation, etc. Formulations for infection may be presented in unit dosage form, e.g., in ampoules or in multi-dose container, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Compositions or pharmaceutical compositions for parenteral administration include aqueous solutions of the compositions in water soluble form. Additionally, suspensions of the compositions may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compositions to allow for the preparation of highly concentrated solutions.
Alternatively, one or more components of the composition may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use. The compositions may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the composition according to the invention may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compositions may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil), or as part of a solid or semi-solid implant that may or may not be auto-degrading in the body, or ion exchange resins, or one or more components of the composition can be formulated as sparingly soluble derivatives, for example, as a sparingly soluble salt. Examples of suitable polymeric materials are known to the person skilled in the art and include PLGA and polylactones such as polycaproic acid.

The compositions or pharmaceutical compositions according to the invention also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Compounds, compositions and/or pharmaceutical compositions for use in the invention include compounds and compositions wherein the active ingredients are contained in an amount effective to achieve their intended purposes. More specifically, a therapeutically effective amount means an amount of compound effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated. Determination of a therapeutically effective amount is within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any compound or composition used in the methods according to the invention, the therapeutically effective amount or dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC50 as determined in cell culture (where inhibitor
molecules are concerned). Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of a compound or composition according to the invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio between LD50 and ED50. Compounds or compositions exhibiting high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for human use. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics" Ch. 1 p. 1).

The amount of compound or composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

A pharmaceutical composition that comprises a compound or a composition according to the invention in combination with a further therapeutic compound can be supplied such that the compound and one or more of the composition components, and the further therapeutic compound are in the same container, either in solution, in suspension, or in powder form. The compound or composition according to the invention can also be provided separately from one or more of the further molecules, and can be mixed with one or more of the further molecules prior to administration. Various packaging options are possible and known to the ones skilled in the art, depending, among others, on the route and mechanism of administration. For example, where the compound according to the invention is supplied separately from one or more of the further therapeutic compounds, the compositions may, if desired, be presented in a pack having more than one chamber, and in which a barrier can be ruptured, ripped, or melted to provide mixing of the compound or composition according to the invention with the further therapeutic compound. Alternatively, two separately provided elements can be mixed in a separate container, optionally with the addition to one or more other carriers, solutions, etc. One
or more unit dosage forms containing the further therapeutic compound can be provided in a pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound according to the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Suitable conditions indicated on the label may include any disease which may be treated or prevented or diagnosed using the compositions according to the invention. In particular, the invention is ideally suited to cancer therapy or chemotherapy.

Compounds according to the invention can be compounds that are also cell penetrating peptides. However, in certain preferred embodiments of the invention, the compounds according to the invention are specifically not for use as a cell penetrating peptide, and are as such specifically not used as cell penetrating peptides. This means that in certain preferred embodiments of the invention, the compounds according to the invention are for example not used to promote entry into a cell of the compound itself, or of further compounds, or to induce ceramide formation in a cell. In this context, it is not relevant whether the compound of the invention is linked to another molecule or whether it is used in a composition that further comprises additional therapeutic compounds.

Good therapeutic results can be achieved through adoption of a dosage regime or of incidental administration of low doses of compounds or compositions according to the invention. The invention allows for the prevention of side effects through its use of low doses. pGP-inhibitors known in the prior art are often only effective at doses that incur severe side effects. In relation to this, the invention allows for dosage regimes that involve an intake schedule featuring intake moments that occur multiple times a day, daily, once every four days, weekly, twice a week, preferably six, five, four, or three times a week, more preferably even less often, thus relieving the burden on the patient.

In the context of the invention, an improvement of a parameter may be measured immediately after a single treatment, or after an hour, two hours, four hours, twelve hours, or after one day, two days, four days, or after at least one week, one month, six months of treatment or more. A symptom may be any symptom known in the art. A parameter may be the assessment of pGP activity as explained herein, or the proliferation of cancer cells as explained herein.
The experience of symptoms by the patient may be assessed by interview or anamnesis, or alternately by known tests that assess condition or fatigue levels as appropriate. When appropriate, a parameter can be considered to be improved or deteriorated on the indication of the subject.

Proliferation of cells and/or cell viability may be assessed using known techniques such as FACS techniques or other techniques, including techniques as described in the examples. Proliferation of cells and/or cell viability may also be assessed using other known techniques, such as resazurin assays, for example as described in the examples, or by MRI, PET, SPECT, or CT, or by otherwise determining changes in cell metabolic activity. The proliferation and the status of cells may be assessed through biopsies and (immuno-)histological characterization of the cells and possible surrounding tissue. When FACS is used to assess cell proliferation, a detected reduction in cell numbers can indicate that the compound or composition according to the invention has a therapeutic effect. Enzyme expression and/or activity can be assessed using known techniques. Preferably, a decrease or increase of a parameter to be assessed means a change of at least 5% of the value corresponding to that parameter. More preferably, a decrease or increase of the value means a change of at least 10%, even more preferably at least 20%, at least 30%, at least 40%, at least 50%, at least 70%, at least 90%, or 100%. In this latter case, it can be the case that there is no longer a detectable value associated with the parameter.

General definitions
In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

The word "about" or "approximately" when used in association with a numerical value (e.g. about 10) preferably means that the value may be the given value (of 10) more or less 0.1%, of the value.

In the context of this invention, a cell can be a cell from a sample obtained from a subject. Such a sample can be a sample that has been previously obtained from a subject.
Such a sample can be obtained from a human subject. Such a sample can be obtained from a non-human subject.

All patent and literature references cited in this specification are hereby incorporated by reference in their entirety.

Table 1. Sequences

<table>
<thead>
<tr>
<th>SEQ NO</th>
<th>Gene / Polypeptide</th>
<th>Sequence</th>
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<td>Human lactoferrin</td>
<td>MKLVFLVLLFLGALGLCLAGRRRSVQWCAVSQPEATKC FQWQRNMRKVGRPPVSCIKRDSPICQICAIENRADAVTL DGGFIYEGALAPYKLRPVAEEVTERQPRTHYAVAV VKKGGSFQLNELQGLKSCHTGLRRRAGWNVPIGTLRPFL NWTGPEPPIEAAVARFFSASCVPAGDKGQFPNLRCRCAG TGENKCAFSSQEPYFSYSKAFKCLRDGDAGDVAFIRESTVF EDLSDEAERDEYELLCPDNTRKPVDKFKDCMCLRVP SHA VVARSVNGKEDAIIWLLRQAQEKEFKDKSPKFQLFSPS GQKDLLFKDSAIIGFSRPVPRISGLYLGSYGFTAIQNLKRS EEEVAARRARVWCACGEQELRKCQWSGLSEGVTCS SASTTEDCIALVLKGEADAMSLDDGGYVYTAGKCGLVPV LAENYKSSQSSDPNCVDRPVGYALAVAVVRRDSTSLT WNSVKGKKSCHTAVDRTAGWNIMPMLLFNQTSCKFDE YFSQSCAPGPSDLACICIGDEQGENKCVPNSERYY GYTGAFRCALAENAGDVAFKDVTVLQNTDGNNEAWA KDLKLADFALLCLDGKRPVTEARSCHLAMAPNHAVVS RMDKVERLKQVLLLHQAKFGRNGSDCPDKFCLFQSETK NLLFNDNTECLARLHGTKTTYEKYLGPQYVAGITNLKKCS TSPLLEACEFLRK</td>
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FIGURE LEGENDS

5  **Figure 1.**
In the presence of the hLF peptide, daunorubicin shows a time and dose-dependent cell killing. Cell viability measured by mitochondrial activity on MES1977 cells (resazurin assay) incubated with DNR and hLF (5 μM) for A) 2 hours, B) 2 hours followed by 24 hours recovery in the absence of drug and peptide, and C) incubated with DNR and peptide for 24 hours.

10  **Figure 2.**
MES1977 cells were incubated for 4 hours with daunorubicin (250 μg/ml), alone or in combination with hLF, D-hLF, R9, D-R9, and Penetratin (5 μM). A) Propidium iodide positive cells, B) Annexin V positive cells, as measured by flow cytometry.

15  **Figure 3.**
MES1977 cells were incubated for 4 hours with daunorubicin (250 μg/ml), alone or in combination with H-IKR-OH or with H-IKR-NH2 (5 μM). A) Annexin V positive cells, B) Propidium iodide positive cells as measured by flow cytometry.

20  **Figure 4.**
hLF is a substrate for pGP. MES1977 cells incubated for 1 hour with DNR (250 μg/ml), or fluorescein-labelled analogs of any one of hLF, mLF, R9, or penetratin (2.5 μM), in
the presence or absence of PSC-833 (5 µM). Ratios of median fluorescein fluorescence intensity obtained by flow cytometry.

Figure 5.
The C-terminal IKR motif causes inhibitory activity. This activity can be transplanted onto a cell-penetrating peptide which by itself does not act as a pGP inhibitor. MES1977 cells were incubated for 24 hours with DNR (250 µg/ml) and different peptides carrying or lacking the IKR motif (5 µM). Penetatin does not carry the IKR motif, but carries a WKK and an NRR motif. A) Propidium iodide-positive cells, B) Annexin V-positive cells, as measured by flow cytometry.

Figure 6.
The C-terminal IKR motif is sufficient to induce cell death and caspase activation. MES1977 cells were incubated for 5 hours with DNR (250 µg/ml) and different peptides carrying or lacking the IKR motif (5 µM). A) Lactate dehydrogenase activity as an assay to probe for disruption for membrane integrity and B) caspase activity in treated samples, normalized to the activity of non-treated cells. LDH activity was measured in cell lysate as a positive control. Staurosporin was used as a positive control for the induction of caspases.

Figure 7.
The IKR motif is a substrate for pGP. This activity can be transferred onto other peptides. Fluorescein-labeled peptides (100 µM) were incubated with pGP-outside/in vesicles in the presence of either AMP or ATP. pGP can only transport substrates in the presence of ATP; the higher the ratio, the more efficient the transport.

Figure 8.
Effect of isoleucine/alanine substitution on IKR activity. Testing of liposomal formulations of DNR, IKR, and/or AKR on MES1977 cancer cell viability (resazurin assay). Cells were incubated for 2 hours with free DNR (25 µg/ml), or with free DNR + either Liposomal (IKR) or Liposomal (AKR), or with liposomal (DNR) + either liposomal (IKR) or liposomal (AKR), or with liposomal (DNR+IKR), or with liposomal (DNR+AKR), followed by 24 hours recovery in the absence of drug and peptide.
Figure 9.
Structure-activity relationship of the IKR peptide and the impact of liposomal coformulation on activity. Substitution of either cationic amino acid by alanine greatly reduces activity.

Figure 10.
Structure-activity relationship of the IKR peptide and the impact of liposomal coformulation on activity. Retro- and inverted IKR tripeptides did not significantly potentiate the effect of DNR, and neither did the dipeptides IK and KR.

Figure 11.
The cytotoxic effect of doxorubicin on pGP-expressing cell lines is enhanced by the presence of Rs-ahx-IKR. Cell viability was measured by mitochondrial activity after 24 hours incubation with DOX and Rs-ahx-IKR (5 µM). A) Jurkat cells, B) HL-60/VCR cells, C) SH-SY5Y cells.

Figure 12.
The cytotoxic effect of liposomal doxorubicin on pGP-expressing cell lines is enhanced by the presence of IKR. DOX and IKR were co-encapsulated in the same vesicles. Cell viability was measured by mitochondrial activity after 24 hours incubation with DOX and IKR (5 µM). A) Jurkat cells, B) HL-60/VCR cells, C) SH-SY5Y cells.

Figure 13.
The cytotoxic effect of doxorubicin-loaded nanoparticles on pGP-expressing cell lines is enhanced by the presence of IKR. DOX and IKR were co-loaded in the same albumin nanoparticles. Cell viability was measured by mitochondrial activity after 24 hours incubation with DOX and IKR (5 µM). A) Jurkat, B) HL-60/VCR, C) SH-SY5Y cells.

Figure 14.
The growth inhibition induced by paclitaxel on pGP-expressing cell lines is enhanced by the presence of Rs-Ahx-IKR. Cell viability was measured by mitochondrial activity after 24 hours incubation with PTX and Rs-IKR (5 µM). A) Jurkat, B) HL-60/VCR, C) SH-SY5Y cells.
Figure 15.
The growth inhibition induced by paclitaxel-loaded nanoparticles on pGP-expressing cell lines is enhanced by the presence of IKR. PTX and IKR were co-loaded in the same albumin nanoparticles. Cell viability was measured by mitochondrial activity after 24 hours incubation with PTX and IKR (5 µM). A) Jurkat, B) HL-60/VCR, C) SH-SY5Y cells.

Figure 16.
Doxorubicin (DOX) accumulates in HL-60/VCR in presence of Rs-Ahx-IKR or IKR. A) Free DOX (50µg/ml) can enter HL-60/VCR cells, but its intracellular levels drops over time. The effect is reversed by co-adminstration of Rs-Ahx-IKR (5 µM). B) Administration of liposomes comprising both doxorubicin and IKR results in an increased intracellular residence time for the doxorubicin.

EXAMPLES
The invention is further described by the following examples which should not be construed as limiting the scope of the invention.


Example 1. hLF potentiates daunorubicin activity.
MES 1977 cells were exposed to increasing concentrations of daunorubicin (DNR) in the absence or presence of 5 fM hLF (Figure 1). Cell viability as determined by the resazurin
assay was determined after incubation for A) 2 hours, B) 2 hours followed by 24 hours recovery in the absence of drug and peptide, and C) incubated with DNR and peptide for 24 hours.

A resazurin assay measures mitochondrial activity. If cells are exposed to any form of stress, they can temporarily switch off the mitochondria and this results in a false positive reading for cell toxicity. Cells can restore their mitochondrial activity when the stress is removed, i.e. upon removal of DNR. Positive readings after a long recovery (24 hours is considered to be a sufficiently long recovery by a very safe margin) in the absence of DNR will show indubitably that mitochondrial activity is irreversibly compromised, and that cells are thus indeed dead or close to death. When a recovery time was included, daunorubicin only showed toxicity in the presence of peptide (Figure 1B compared to Figure 1C). The peptide by itself thus either potentiates the import of daunorubicin, or interferes with the pGP-mediated export of daunorubicin - the examples following this one confirmed that at least the latter is the case.

Example 2. Potentiation of daunorubicin activity is not a general characteristic of cationic cell-penetrating peptides.

The drug-activity-enhancing effect of hLF could hypothetically be attributed to the activity of hLF as a cell-penetrating peptide. One could conceive as a potential mechanism-of-action that the peptide enhances the rate of endocytosis, which would concurrently lead to an enhanced uptake of the drug. However, neither the highly active CPP nonaarginine, nor its D-amino acid counterpart enhanced daunorubicin toxicity. Some activity could be observed for the D-analog of hLF in the induction of phosphatidylserine exposure as probed by Annexin V staining, and also the CPP penetratin showed an enhancing effect (Figure 2). Thus, mere activity as a CPP is not sufficient to enhance the activity of daunorubicin. In contrast to the stereochemistry, the nature of the C-terminus had no impact on activity as a peptide with a C-terminal amidation was also active (Figure 3).
Example 3. The hLF peptide interferes with the activity of pGP.
The peptide was found to interfere with pGP-mediated export of the drug. To do so, the peptide could either serve as a substrate for pGP and thus compete with the drug for export, or it could interfere with the expression of the transporter on the cell surface, or thirdly, it could disturb pGP functionality without being a substrate.

To address the first possibility, cells were incubated with fluorescein-labeled analogs of either the hLF peptide, the murine analog mLF, R₉ and Penetratin, each in the presence or absence of pGP-inhibitor PSC-833 (also known as valsodar). As shown in Figure 4, the inhibitor strongly increased the cell-associated fluorescence of hLF, a peptide that comprises an IKR peptide, but not of R₉, which does not comprise such a peptide. This result is consistent with the daunorubicin-enhancing activity shown by hLF but not by R₉. Also, PSC-833 caused a higher increase in cell-associated fluorescence for penetratin than for R₉ (figure 4). Penetratin comprises a WKK peptide and an NRR peptide. This result is consistent with the penetratin activity that is shown in example 2 and figure 2.

Example 4. For hLF, the terminal IKR motif is responsible for activity.
After having obtained evidence that the hLF peptide acts as a substrate for pGP (Example 3) the structural element within hLF responsible for this activity was identified. The C-terminal IKR motive of the hLF was found to act as a pGP substrate. Next to testing this IKR peptide alone, it was investigated whether truncation of the C-terminal motif would indeed abolish the pGP-inhibiting activity of the hLF peptide and whether C-terminal elongation of R₈ by IKR would impart Rs with pGP-inhibiting activity and thus with daunorubicin toxicity-enhancing activity. Fully consistent with IKR as the relevant structural motive, the truncated hLF peptide lost pGP-inhibiting activity, while IKR alone, and also the C-terminally elongated Rs, were active inhibitors. The latter result shows that through transfer of the C-terminal IKR motif the activity of the hLF CPP as a pGP inhibitor can be transplanted onto a CPP that by itself does not act as a pGP inhibitor. This observation was confirmed with four independent assays of cell killing (Figure 5 and figure 6). Qualitatively, all results were fully consistent.

Example 5. Cell-free assessment of IKR activity.
Further, conclusive evidence for activity of the IKR-containing peptides as pGP substrates was obtained through an in vitro pGP transport assay. In this assay, peptides
were incubated with pGP-containing vesicles derived from pGP-expressing cells in the presence of either AMP or ATP. Only ATP can serve as an energy source for pGP. IKR and R$_8$-ahx-IKR showed a strong ATP-dependent fluorescence increase. For hLF this increase was still significant in comparison to the control. Truncated hLF did not show an increase (Figure 7). Vesicles were prepared, and measurements were performed, as previously described (Wittgen H. G. et al, (2011, Cannabinoid type 1 receptor antagonists modulate transport activity of multidrug resistance-associated proteins MRP1, MRP2, MRP3, and MRP4. Drug Metab. Dispos. 39, 1294-1302.) and Gozalpour E. et al, (2012, Interaction of Digitalis-Like Compounds with P-Glycoprotein, Tox. Sci., 113(2): Pp. 502-511, both references are incorporated herein by reference) with minor modifications, i.e. by measuring fluorescence instead of radioactivity.

**Example 6. Structure-activity relationships and drug co-formulations**

Next to proving the importance of the IKR motive, a number of previous assays had shown that Rs-conjugated IKR had an even higher activity than IKR alone. Combination with the observations that IKR acts as a pGP substrate yields a model according to which the peptide enters the cells and then competes with drugs such as daunorubicin for export. To further explore the structure-activity relationship of the IKR motive and to define means to deliver the peptide in combination with a drug such as daunorubicin, cells were exposed to a liposomal formulation of the peptide in combination with non-liposomal daunorubicin, or to a combination of two types of liposomes loaded with either daunorubicin or peptide, or to a single species of liposomes in which both substances were jointly included. Activity only depended on the presence of the peptide but not on a particular formulation. However, the liposomal coformulation of both compounds was the most active in compromising cell activity, as determined by a resazurin assay (Figure 8).

Substitution of the isoleucine comprised in IKR by alanine to obtain AKR only had a small effect on activity. In contrast, substitution of either cationic amino acid with alanine greatly reduced activity (Figure 9). This observation is in line with the fact that the mLFL analogue terminates on a C-terminal VKK motive and penetratin on a C-terminal WKK motive. Interestingly, also N-terminal acylation of IKR was suboptimal for activity. The retro tripeptide (RKI) and the chirally inverted tripeptide (D-IKR, which can also be denoted as ikr) were not as efficient in enhancing the efficacy of daunorubicin, consistent
with the limited potentiating effect observed for D-hLF. Moreover, a tripeptide is the shortest possible sequence to show activity, as the dipeptides IK and KR did not significantly potentiate the cytotoxic effect of DNR when in coformulation with DNR (Figure 10).

Example 7. Calculated LogP values to quantify solubility

Tripeptides according to the invention, such as IKR, are convenient to handle and are expected to have excellent (bio)adsorption and (bio)distribution parameters, owing to their excellent solubility in water and aqueous buffer solutions. This example provides calculated LogP values that corroborate and quantify the previously obtained data.

Solubility is commonly expressed as a partition ratio (LogP), which is the ratio of concentrations of a compound in a mixture of two immiscible phases at equilibrium (here: water and 1-n-octanol). LogP is a measure of the difference in solubility of the compound in question in these two phases. More precisely, LogP expresses the ratio of concentrations of the compound in question in the two phases of water and 1-n-octanol at equilibrium. Therefore, LogP is often seen as a measure of how hydrophilic or hydrophobic a compound can be assumed to be.

LogP values can be estimated using computational chemistry (Tetko et al, *J. Comput. Aid. Mol. Des.*, 2005, 19, 453-63.) Table 2 below provides calculated LogP values for various compounds, including the tripeptide IKR. Calculations were performed using VCCLAB, Virtual Computational Chemistry Laboratory, accessible on the internet at www.vcclab.org, 2005. It can be concluded that IKR is exceptionally well-soluble in water. This characteristic of IKR combines well with its previously demonstrated ability to independently enter cells.

Table 2. Calculated LogP values for IKR and other compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Calculated LogP value</th>
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<tbody>
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<td>Hexane</td>
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<td>Reversin 121</td>
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<td>Glutathione</td>
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<tr>
<td>Resazurin</td>
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Example 8. Liposomal formulations

Liposomal formulations as used in some aspects or embodiments of the invention (see for example figures 8, 9, and 10) can be based on DPPC or on DOPE or on combinations thereof. A preferred formulation is DPPC/DOPE/CHEMS/Cholesterol 4:2:2:1, which was shown to encapsulate approximately 48 μg of DNR per mg of lipid. A more preferred formulation is DPPC/Cholesterol 2:1, which was shown to encapsulate approximately 65 μg DNR per mg of lipid.

Example 9. IKR potentiates doxorubicin activity in pGP-expressing cells.

Doxorubicin (DOX) was administered to three different cell lines: to Jurkat cells, to SH-SY5Y cells, and to HL-60/VCR cells. Each of these cell lines expresses pGP. Doxorubicin was administered in a range of concentration from 0 to 100 μg/ml, in the presence and absence of Rs-ahx-IKR (5 μM). Cell viability was measured by determining the mitochondrial activity of the cells after 24-hours incubation.

In the absence of the IKR-peptide (comprised in Rs-ahx-IKR), cell viability was not greatly affected by the addition of DOX, even at high concentration. This effect was most evident for SH-SY5Y and HL-60/VCR cells. At the highest concentration, administration of doxorubicin resulted in a reduction of mitochondrial activity of only 25% for HL-60/VCR, up to 50% for Jurkat. In contrast, coadministration of Rs-IKR caused an increased reduction in mitochondrial activity. This potentiating effect was already evident at the lowest concentrations, and manifested in a dose-dependent manner. At higher concentration, cell viability was effectively halved (Figure 11).

To assess the effect of IKR on liposomal doxorubicin formulations, DOX was encapsulated into liposomes. A set of DOX-liposomes with and without coencapsulated IKR (initial IKR concentration: 20 μM) was prepared. The liposomal formulations were administered to Jurkat cells, to SH-SY5Y cells, and to HL-60/VCR cells, at equivalent DOX concentrations ranging from 0 to 50 μg/ml. Cell viability was measured by determining the mitochondrial activity of the cells after 24-hours incubation.

<p>| | |</p>
<table>
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<tr>
<td>Ile-Lys-Arg (IKR)</td>
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Here, too, cell viability decreased in a dose-dependent manner for all cell lines. The potentiating effect of IKR was evident for all concentrations, particularly for 25 µg/ml and 50 µg/ml, in all cell lines (Figure 12).

Example 10. Coencapsulation of IKR and DOX in albumin nanoparticles causes an enhanced cell death, compared to DOX alone.

Albumin-based nanoparticles are an emerging class of drug delivery vehicles that provide a good pharmacological and toxicological alternative to liposomes and polymeric nanoparticles. They consist of cross-linked albumin proteins instead of from lipids or synthetic polymers. Albumin nanoparticles can be produced with albumins from different sources (bovine, human), obtained by purification from blood or via molecular biology tools such as recombinant expression. The synthesis of albumin nanoparticles is low-cost and convenient. Particle size and drug loading can be tuned by changing synthesis parameters such as pH, concentration of crosslinkers, and annealing times.

Albumin nanoparticles sometimes possess more favourable physicochemical properties compared to liposomes. For initial tests as reported here, bovine serum albumin was used; however, future formulations will be based on recombinant human serum albumin, to further decrease chances of inducing undesired immune responses.

Doxorubicin (DOX) was encapsulated into albumin nanoparticles, with a set of albumin nanoparticles also comprising IKR (initial IKR concentration: 20 µM; expected effective IKR concentration when particles are administered to cells: 5 µM), and another set only comprising doxorubicin. The formulations were administered to Jurkat cells, to SH-SY5Y cells, and to HL-60/VCR cells, at equivalent DOX concentrations ranging from 0 to 50 µg/ml. Cell viability was measured by determining the mitochondrial activity of the cells after 24-hours incubation.

The profile of the potentiating effect of IKR was similar to the effect that was observed for the liposomal formulations, both experiments showing a dose-dependent drop in cell viability. For albumin nanoparticles, the potentiating effect of IKR became evident at even lower concentrations (Figure 13).
Example 11. Coadministration of IKR and PTX reduces cell proliferation, compared to PTX alone.

Paclitaxel (PTX) was administered to Jurkat cells, to SH-SY5Y cells, and to HL-60/VCR cells, in a range of concentration from 1 to 100 nM, in the presence or absence of Rs-ahx-IKR (5 µM). Cell viability was measured by determining the mitochondrial activity of the cells after 24-hours incubation.

Free PTX caused a moderate reduction of viability only in Jurkat cells at the highest concentration; other cell lines remained mostly unaffected. However, the presence of Rs-ahx-IKR resulted in a reduced viability, throughout the range of concentrations, for all cell lines (Figure 14).

To assess the effect of IKR on paclitaxel delivery by nanoparticles, PTX was encapsulated into albumin nanoparticles, with one set of nanoparticles also encapsulating IKR (initial IKR concentration: 20 µM; expected effective IKR concentration when particles are administered to cells: 5 µM), and another set just encapsulating the PTX.

The formulations were administered to Jurkat cells, to SH-SY5Y cells, and to HL-60/VCR cells, at equivalent PTX concentrations ranging from 0 to 50 nM. Cell viability was measured by determining the mitochondrial activity of the cells after 24-hours incubation.

PTX-nanoparticles were able to reduce cell growth in a dose-dependent manner. The inclusion of IKR in the nanoparticles significantly inhibited cell growth. At the highest concentration, cell growth was, in practical terms, halted. Suspension cells appeared more sensitive to the treatment, as the growth inhibition for such cells was clearly higher than that observed for SH-SY5Y (Figure 15).

Example 12. Intracellular residence time of doxorubicin is increased in the presence of IKR.

HL-60/VCR cells were incubated with doxorubicin (DOX, 50 µg/ml) for up to 2 hours, in the presence or absence of Rs-ahx-IKR (5 µM). Free DOX can enter HL-60/VCR cells, but pGP activity causes its intracellular levels to drop over time. Accordingly, the intracellular levels, as determined by flow cytometry, of DOX decreased in absence of Rs-ahx-IKR. However, they dramatically increased when Rs-ahx-IKR was coadministered. This effect was evident also for liposomal formulations, which comprised either DOX, or both DOX and IKR. However, in that case the increased
residence time only became evident after 4 hours of incubation. This is presumably linked to the slower entry of liposomes into cells, and to the non-instantaneous release of DOX from the liposomal vesicles (Figure 16).
REFERENCE LIST


CLAIMS

1. A compound comprising a tripeptide represented by the sequence XAB, wherein X, A, and B are amino acids, wherein X can be any amino acid and A and B are basic amino acids, for use in the treatment, delay, prevention, cure, or stabilization of a disorder, preferably cancer, of a subject in need thereof, comprising administration to the subject of an effective dose of the compound, wherein the compound inhibits p-glycoprotein in its ability to export substances from a cell.

2. A compound for use according to claim 1, wherein X and/or B are terminal amino acids.

3. A compound for use according to any one of claims 1 or 2, wherein X, A, and B are natural amino acids, preferably L-amino acids.

4. A compound for use according to any of the preceding claims, wherein A and B are each independently selected from the group consisting of Arginine, Lysine, and Histidine, preferably selected from Arginine or Lysine.

5. A compound for use according to any of the preceding claims, wherein X is selected from the group consisting of Asparagine, Tryptophan, and any amino acid with a side chain that consists of only carbon and hydrogen atoms.

6. A compound for use according to any of the preceding claims, wherein X is selected from the group consisting of Alanine, Isoleucine, Asparagine, and Tryptophan.

7. A compound for use according to any of the preceding claims, wherein X is selected from the group consisting of Alanine, Isoleucine, Asparagine, and Tryptophan, and wherein A and B are each independently selected from the group consisting of Arginine, Lysine, and Histidine, preferably selected from Arginine or Lysine.
8. A compound for use according to any of the preceding claims, wherein the compound consists of a tripeptide represented by the sequence XAB, wherein XAB preferably is IKR or AKR, more preferably IKR.

9. A compound for use according to any of the preceding claims, wherein the compound further comprises a peptide, a drug and/or a label.

10. A compound as defined in any of the preceding claims.

11. A composition comprising a compound according to claim 10 and further comprising an excipient, preferably a pharmaceutically acceptable excipient, and optionally further comprising a further therapeutic compound.

12. A composition according to claim 11 for use in the treatment, delay, prevention, cure, or stabilization of a disorder, preferably cancer, of a subject in need thereof, comprising administration to the subject of an effective dose of the composition, wherein the composition inhibits p-glycoprotein in its ability to export substances from a cell.

13. Use of a compound according to claim 10 or a composition according to 11 in the treatment, delay, prevention, cure, or stabilization of a disorder, preferably cancer, of a subject in need thereof, comprising administration to the subject of an effective dose of a compound according to claim 10 or a composition according to claim 11, wherein the compound or composition inhibits p-glycoprotein in its ability to export substances from a cell.

14. An in vitro, in vivo or ex vivo method for inhibiting p-glycoprotein in its ability to export substances from a cell, said method comprising contacting the cell with a compound according to claim 10 or a composition according to claim 11.

15. A compound for use according to any one of claims 1-9, a composition for use according to claim 12, use according to claim 13, and a method according to claim 14, further comprising administration of an effective dose of another therapeutic compound or composition to the subject.
Fig. 2

A  PI positive cells (%)  100  80  60  40  20  0

B  Annexin V positive (%)  100  80  60  40  20  0

Counet: DNR+Pt

DNR+P-Te

DNR+R-9

DNR+D-IL-2

DNR+IL-2
Fig. 4
Fig. 7

Fluorescence ratio (ATP/AMP)

- hLF
- Truncated
- IKR
- R8-Ahx-IKR

Fig. 8

Cell viability (%)

- DNR
- Lip(DNR)
- DNR+Lipo(IKR)
- Lip(DNR)+Lipo(AKR)
- Lip(DNR)+Lipo(IKR)+Lipo(AKR)
- Lip(DNR+AKR)
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Date of the actual completion of the international search: 5 October 2016

Date of mailing of the international search report: 14/10/2016

Authorized officer: Pillings, Stephen
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