An Approach Towards More Selective Anticancer Agents

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A promising approach towards better targeted anticancer drug therapy takes advantage of enhanced expression of proteases associated with human malignancies. Especially plasminogen activator activity has been found to be substantially increased, leading to an enhanced activity of the serine protease plasmin. Bifunctional alkylating agents, such as *N*-(2-chloroethyl)-*N*-nitrosoureas, display broad spectrum anticancer activity, but also exhibit considerable systemic toxicity. We describe here the synthesis of new *N*-nitrosourea-based prodrugs designed to become activated by tumor-associated proteases, to provide for enhanced antitumor activity and reduced systemic toxicity. Tripeptides representing substrates for plasmin were linked by an amide bond to *N'*-(2-aminoethyl)-*N*-(2-chloroethyl)-*N*-nitrosourea and the corresponding *N'*-methyl derivative. Synthesis and plasmin-triggered decomposition of these new tripeptide conjugates is described. Cancer cells expressing high plasminogen activator activity are highly sensitive to the new prodrugs in the presence of plasminogen, but not in its absence.

Introduction

Malignant tumors represent one of the most common human diseases worldwide. Based on an estimation made in the United States, cancer will become the leading cause of death in the year 2000.[1]

Treatment of malignant diseases with anticancer drugs is an option of steadily increasing importance, especially for systemic malignancies or for metastatic cancers which have passed the state of surgical curability.[2] Unfortunately, the subset of human cancer types that are amenable to curative treatment today still is rather small. Although we witness tremendous progress in understanding the molecular events that lead to malignancy and we also know of many agents that effectively kill cancer cells, progress in development of drugs that can cure human cancer is slow.[3,4] The heterogeneity of malignant tumors with respect to their genetics, biology and biochemistry as well as primary or treatment-induced resistance to therapy mitigate against curative treatment.[5–7] Moreover, many anticancer drugs display only a low degree of selectivity, causing often severe or even life threatening toxic side effects, thus preventing the application of doses high enough to kill all cancer cells. Searching for antineoplastic agents with improved selectivity to malignant cells remains therefore a central task for drug development.[8]

An approach that offers some promise is to exploit enhanced expression of tumor-associated enzyme activities for local activation of latent prodrugs, converting the prodrug from the innocuous "mute" stage into the cytotoxic principle.

Tumor-Associated Proteases

Tumors, especially those that are invasive and metastasizing, often express significantly higher plasminogen activator activities than normal tissues. Plasminogen is a single chain glycoprotein, consisting of 791 amino acids with a molecular weight of about 93 kDa. Plasminogen is activated to the serine protease plasmin by a single proteolytic cleavage (Arg_{560}–Val_{561}).[9] This reaction is mainly catalyzed by two different plasminogen activators: urokinase-type (uPA) and tissue-type (tPA) activator that represent products of different genes. Whereas tPA has been recognized as a physiological fibrinolytic enzyme,[10,11] uPA was found to be a key enzyme in proteolytic reactions that are required for the spreading and invasiveness of cells, both in cancer and in tissue remodeling processes.[12–16] Plasmin consists of two polypeptide chains, connected via a disulfide bridge: a heavy A-chain (65 kDa), containing binding sites for fibrin, and a light B-chain (25 kDa) containing the active center.[9]

The primary structure of uPA has been determined by protein sequencing and from the cloned cDNA. uPA is the translated, active product of a 2.4 kb-long mRNA, transcribed from a 6.5 kb gene present on the long arm of chromosome 10. The primary translation product is prepro-uPA, consisting of 431 amino acids, which gives rise to the secreted pro-uPA by removal of a 20 residue long signal peptide. Pro-uPA is a single chain glycosy-

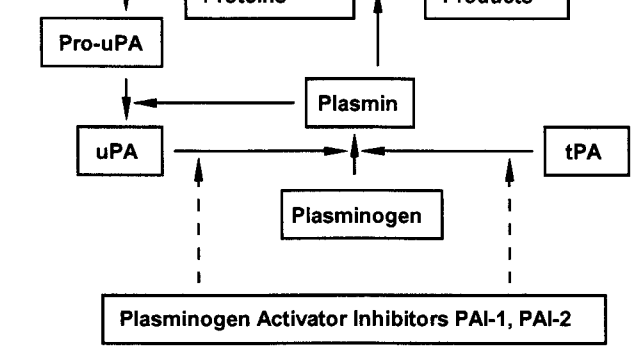


Figure 1. Schematic illustration of the formation of plasmin catalyzed by plasminogen activators and the proteolysis of plasmin substrates.
Abbreviations: uPA: urokinase-type plasminogen activator; tPA: tissue-type plasminogen activator; →Activation; ⇢ Inhibition

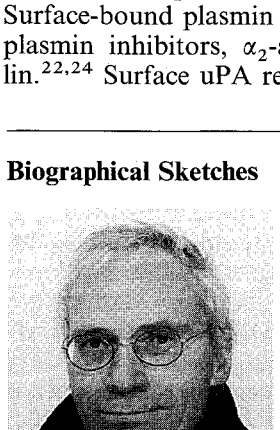
lated polypeptide with a molecular weight of about 48 kDa. Pro-uPA is converted to two-chain uPA by proteolytic cleavage after lysine 158. This reaction can be catalyzed again by plasmin. Two types of specific, fast-acting inhibitors of plasminogen activators (PAI-1 and PAI-2) have been identified in tissues, body fluids and cell cultures.[17-19] They are able to inhibit both uPA and tPA (Figure 1).

A unique characteristic of uPA is that it interacts with a specific high affinity receptor on the cell surface ($K_d = 0.1$ nM).[20-21] Receptor-bound uPA is catalytically active and remains on the surface of the cell without internalization.[20] Interaction with its substrate, plasminogen, produces plasmin which is still bound to the cell surface.[22-23]

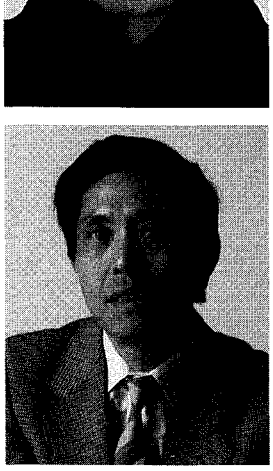
The affinity of plasminogen and plasmin for their receptors is in the range of $K_d = 0.1 - 1$ nM. The half-life of surface-bound plasmin is much shorter than that of uPA. Surface-bound plasmin is resistant to the physiological plasmin inhibitors, α_2-antiplasmin or α_2-macroglobulin.[22,24] Surface uPA receptors of many malignant cell lines are saturated with pro-uPA. It has been suggested that this can provide a selective advantage to migrating cells, in particular cancer cells.[24] It appears that the rate of synthesis of receptor and of uPA may play an important role in the invasive capacity of cancer cells.

An increased activity and expression of uPA has been found in a series of malignant tumor cell lines and human solid tumors, such as lung tumors,[25,26] prostate cancers,[27,28] breast cancers,[26,29,30] gastric and colorectal carcinomas,[30-36] ovarian carcinomas,[37] squamous basal cell carcinomas,[38,39] and melanomas,[40] but also in mammary carcinoma of rats,[41] and B 16 melanoma of mice.[42] A direct correlation has been found between levels of uPA activity and metastatic potential, and uPA activity of tumors has been tested for its potential usefulness as a prognostic marker.[43-46] Immunohistochemical studies on 96 human lung carcinomas revealed a positive correlation between the uPA activity and the size of the tumor. Patients with high uPA levels in tumor cell cytosol showed increased metastasis, as compared to patients with low uPA levels.[47] Similar results have been obtained in primary breast cancer. Breast cancer patients

Biographical Sketches



Gerhard Eisenbrand graduated from University of Freiburg, Breisgau, in Pharmacy and Food Chemistry. He prepared his doctorate in the Research Group of Preventive Medicine at the Max Planck Institute for Immunebiology, Freiburg, and received his Ph.D. in 1971. He joined the Institute of Toxicology and Chemotherapy at the German Cancer Research Centre, Heidelberg, working as a postdoc, later as senior scientist in the Division of Environmental Carcinogenesis headed by Prof. R. Preussmann. He achieved his habilitation at the University of Stuttgart in 1977. In 1982 he accepted the call to the Chair of Food Chemistry & Environmental Toxicology, Department of Chemistry, University of Kaiserslautern. His research interests comprise mechanisms of chemical carcinogenesis and biochemical toxicology, trace analysis of carcinogens in food and environment and biotransformation and interaction with biomolecules of carcinogens. He also has a prime interest in design and development of anticancer agents.



Weici Tang born in Jiangsu (China) in 1937; studied pharmaceutical chemistry at the University of Greifswald, Germany (1955-1960); postdoctoral stay in the Institute of Toxicology and Chemotherapy (Prof. Dr. D. Schmähl), German Cancer Research Centre as an Alexander von Humboldt Fellow (1979-1981) and as visiting Professor (1984-1986); scientific co-worker and senior researcher in the division of Food Chemistry and Environmental Toxicology (Prof. Dr. G. Eisenbrand), Department of Chemistry, the University of Kaiserslautern, Germany, since 1986; main scientific interests comprise anticancer drug development and natural products of plant origin.

Susanne Lauck-Birkel studied food chemistry at the University of Kaiserslautern obtaining her diploma (Staatsexamen) in 1989. She then worked a full practical year, spending six months each in both the food industry and a government institute for food analysis after which she obtained her second Staatsexamen earning her professional qualification as food chemist. In 1990, she began her doctorate work in cancer research in the working group of Prof. Dr. G. Eisenbrand at the University of Kaiserslautern where she specialized in synthesis and *in vitro* testing of plasmin activated anticancer prodrugs. Upon completion and defense of her doctoral thesis in 1994, she joined the Hoechst Schering AgrEvo GmbH. Dr. Susanne Lauck-Birkel is responsible for Toxicokinetics in the Ecochemistry Group of Environmental Sciences in AgrEvo, Frankfurt.

with high levels of uPA showed increased metastasis and decreased survival time.[48,49] Using a chromogenic substrate, an eightfold higher uPA activity was found in cultured human colon carcinoma cells, as compared to normal colon cells.[50] Human ovarian carcinoma cells expressed uPA in a pattern that was variable in intensity and distribution.[51] In addition, high levels of PAI-2 have been found to correlate with favorable prognosis in primary breast cancer.[52,53] A recombinant chimeric protein capable of binding the uPA receptor has been found inhibitory against tumor progression and metastasis.[54] Antibodies to plasminogen activator can also inhibit human tumor metastasis.[38,55]

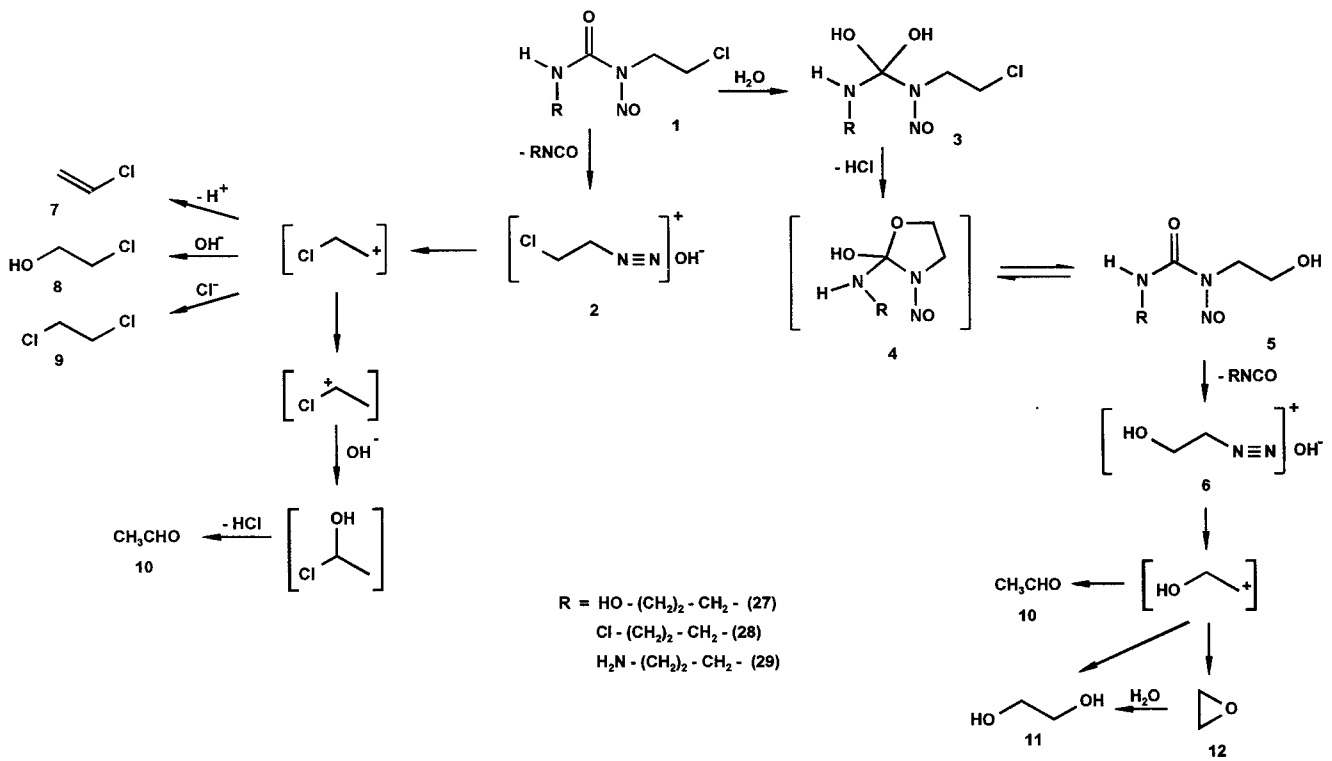
Antineoplastic *N*-(2-Chloroethyl)-*N*-nitrosoureas

N-(2-Chloroethyl)-*N*-nitrosoureas (CNUs **1**) are clinically used bifunctional alkylating agents that alkylate DNA and induce DNA–DNA crosslinks by forming alkyldiazonium ions or alkylcarbenium ions that alkylate biological macromolecules (Scheme 1).[56] One reaction pathway gives rise to the 2-chloroethyldiazonium ion **(2)**, which on further decay yields a spectrum of secondary products, such as vinyl chloride **(7)**, chloroethanol **(8)**, 1,2-dichloroethane **(9)** and acetaldehyde **(10)**. The second pathway leads through the tetrahedral intermediate **3** via the *N*-nitrosooxazolidine intermediate **4** to *N*-(2-hydroxyethyl)-*N*-nitrosourea **(5)**. The latter may decompose through the 2-hydroxyethyldiazonium ion **(6)** into **10**, ethylene glycol **(11)**, and ethylene oxide **(12)** (Scheme 1).[57-61]

In the presence of DNA, alkylation of DNA bases occurs. This results in the formation of adducts at the nitrogen ring atoms of purine and pyrimidine bases, such as *N7*-(2-hydroxyethyl)guanine **(13)**, *N7*-(2-chloroethyl)guanine **(14)** or *N3*-(2-hydroxyethyl)cytosine **(15)** (Figure 2). Adducts resulting from reaction at exocyclic oxygen atoms are exemplified by O^6-(2-hydroxyethyl)guanine **(16)**.[57,62] As a result of bifunctional alkylation resulting in an interstrand crosslink, 1-[deoxycytid-3-yl]-2-[deoxyguanos-1-yl]ethane **(20)** has been identified.[63-65] O^6-(2-Chloroethyl)guanosine **(17)** is thought to be the initial reaction product that is supposed to cyclize to the reactive intermediate *N1*,O^6-ethanodeoxyguanosine **(18)**. The latter reacts with the Watson–Crick paired cytosine **(19)** on the opposite strand at position N3 to form the interstrand crosslinked base pair **20**.[61,66] In contrast, di[guan-7-yl]-ethane **(21)** was found to result from intrastrand crosslinking.[67] Furthermore, ethano derivatives *N3*,N^4-ethanocytosine **(22)**,[68] *N1*,N^6-ethanoadenine **(23)**[69-71] and N^2,*N3*-ethanoguanine **(24)**[72] have also been islolated from DNA reacted with **1**.

The phosphodiester bond of the sugar phosphate backbone of DNA is another target for alkylating agents. Methyl and ethyl phosphotriesters are highly stable. In contrast, 2-hydroxyethyl phosphotriesters formed from hydroxyethylating intermediates (Scheme 1), rapidly decompose, causing DNA single strand breaks (Scheme 2).[73,74]

DNA strand breaking and interstrand crosslinking by bifunctional alkylating agents in general and by 2-chloroethylnitrosoureas in particular has been extensively studied in vitro and in vivo. Such DNA lesions are measured either by individually assaying the corresponding base adducts or, more conveniently, by measuring



Scheme 1. Generation of alkylating species and decomposition products from *N*-(2-chloroethyl)-*N*-nitrosoureas at near neutral conditions (R = alkyl)

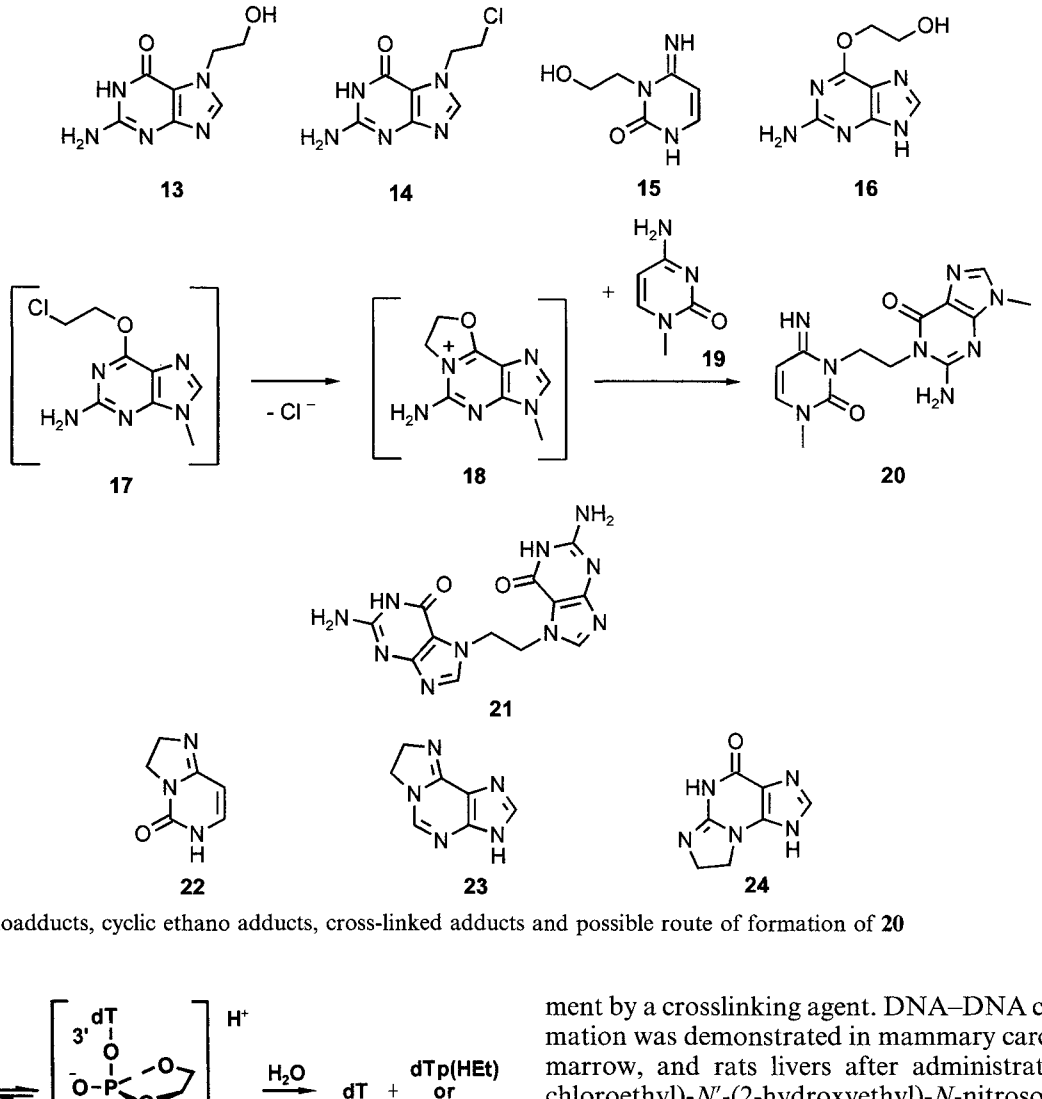
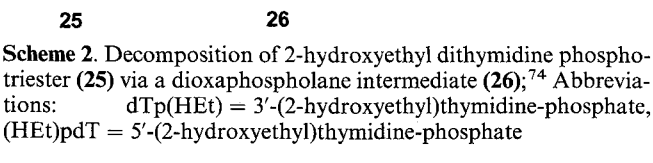


Figure 2. Monoadducts, cyclic ethano adducts, cross-linked adducts and possible route of formation of **20**



Scheme 2. Decomposition of 2-hydroxyethyl dithymidine phosphotriester **(25)** via a dioxaphospholane intermediate **(26)**;[74] Abbreviations: dTp(HEt) = 3′-(2-hydroxyethyl)thymidine-phosphate, (HEt)pdT = 5′-(2-hydroxyethyl)thymidine-phosphate

changes in physicochemical properties of the DNA macromolecule. The so-called alkaline filter elution technique, originally developed by Kohn et al.[75] has become a standard technique for measuring DNA damage. To determine strand breaks, DNA is denatured and passed through a membrane filter of defined pore size (0.2 μm) under alkaline conditions (pH 12). Whereas intact single stranded DNA cannot pass the filter, DNA damage results in strand breaks that allow fragments to elute through the filter. Since γ-irradiation strongly induces strand breaks, this can be used to calibrate the assay and express results in radiation equivalents. In contrast, DNA interstrand crosslinking causes retention on the filter, since DNA strands cannot segregate. Thus, DNA–DNA crosslinking can be quantified by comparing the elution of γ-irradiated DNA with and without additional treatment by a crosslinking agent. DNA–DNA cross-link formation was demonstrated in mammary carcinoma, bone marrow, and rats livers after administration of *N*-(2-chloroethyl)-*N*′-(2-hydroxyethyl)-*N*-nitrosourea (HECNU, **27**).[76]

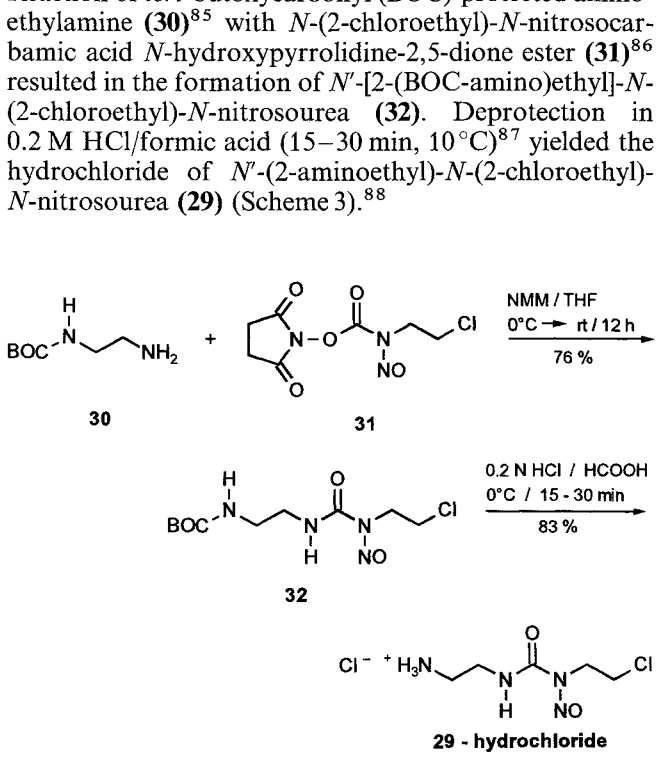
The decay of a nitrosourea yields a second highly reactive intermediate, RNCO (Scheme 1). The isocyanate is known to contribute substantially to toxic side effects. 2-Chloroethylisocyanate formed from *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea (BCNU, **28**) blocks many enzymes by carbamoylation. Inhibition of glutathione reductase in the lungs of cancer patients has been shown to be the primary cause for late and often fatal pulmonary fibrosis induced by **28** as a consequence of compromised antioxidative defense.[77–79] However, if a nucleophilic group is present in β-position of the *N*′-alkyl substituent, the isocyanate is quenched by internal reaction and toxicity is reduced. Thus, **27** has not been found to induce pulmonary toxicity. There is good evidence that nucleophilic attack of the β-hydroxyl group on the urea carbonyl of **27** initiates breakdown into the alkylating intermediate and 1,3-oxazolidin-2-one.[80]

On the basis of this observation it appeared promising to replace the β-hydroxyl group of **27** by an amino group and to attach to it a tripeptide that might be cleaved off by a tumor associated protease, such as plasmin. This approach should lead to a stable prodrug that should be locally activated by proteolytic enzymes associated with

tumor cells. Typical tripeptides behaving as substrates for plasmin are D-valyl-L-leucyl-L-lysine or D-alanyl-L-phenylalanyl-L-lysine, the *N*-terminal D-amino acid preventing unspecific cleavage by exopeptidases. Such plasmin substrates have already been explored earlier for synthesis of plasmin activated prodrugs and have shown promising activity in uPA expressing cell systems.[81-84] However, to the best of our knowledge, this approach has not been further investigated. We therefore have initiated studies to further develop this concept, aiming at protease-mediated liberation of bifunctional alkylating activity. Therefore, *N'*-(2-aminoethyl)-*N*-(2-chloroethyl)-*N*-nitrosourea **(29)** and the corresponding *N'*-methyl derivative were synthesized together with the respective tripeptide-linked prodrugs.

Synthesis of *N'*-(2-Aminoethyl)-*N*-(2-chloroethyl)-*N*-nitrosourea (29) and Its Tripeptide Conjugates

Reaction of *tert*-butoxycarbonyl (BOC)-protected aminoethylamine **(30)**[85] with *N*-(2-chloroethyl)-*N*-nitrosocarbamic acid *N*-hydroxypyrrolidine-2,5-dione ester **(31)**[86] resulted in the formation of *N'*-[2-(BOC-amino)ethyl]-*N*-(2-chloroethyl)-*N*-nitrosourea **(32)**. Deprotection in 0.2 M HCl/formic acid (15–30 min, 10 °C)[87] yielded the hydrochloride of *N'*-(2-aminoethyl)-*N*-(2-chloroethyl)-*N*-nitrosourea **(29)** (Scheme 3).[88]



Scheme 3. Synthesis of *N'*-(2-aminoethyl)-*N*-(2-chloroethyl)-*N*-nitrosourea hydrochloride

Tripeptides D-valyl-L-leucyl-L-lysine and D-alanyl-L-phenylalanyl-L-lysine were synthesized using general methods of peptide chemistry. The dipeptides (tripeptides) were made from *N*-BOC or *N*-benzyloxycarbonyl (Z)-protected amino acids (dipeptides) by condensation via an active ester with the corresponding amino acids or their methyl esters. In the case of lysine, N^{ω}-BOC-lysine or its methyl ester was used.

Compound **29** was released in situ from its hydrochloride using *N*-methylmorpholine (NMM), and coupled to D-(*N*-BOC-valyl)-L-leucyl-L-(N^{ω}-BOC)-lysine **(40)** using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), *N*-(2-chloroethyl)-*N'*-[2-[D-(*N*-BOC-valyl)-L-leucyl-L-(N^{ω}-BOC-lysyl)]aminoethyl]-*N*-nitrosourea **(46)** was obtained in 20 % yield only. Stepwise synthesis of *N*-(2-chloroethyl)-*N'*-[2-(tripeptidyl)aminoethyl]-*N*-nitrosoureas gave conjugates in higher yields. 2-(*N*-Z-amino)-ethylamine **(43)** has been found to be a suitable starting compound prepared from Z-aminopropionic acid amide via Hoffmann rearrangement by treatment with [bis(trifluoroacetoxy)iodo]benzene.[89] Condensation of **43** with *N*-BOC-protected tripeptides gave *N*-Z-*N'*-(BOC-tripeptidyl)ethanediamines **(44,45)** in high yields.

Hydrogenolytic elimination of the Z protecting group yielded BOC-protected tripeptidylaminoethylamine which gave BOC-protected tripeptide *N*-nitrosourea conjugates **(46,47)** by reaction with **31**. Elimination of the BOC group with HCl/formic acid[87] yielded tripeptide-linked *N*-(2-chloroethyl)-*N*-nitrosourea hydrochlorides **(48,49)**. The synthetic route to *N*-(2-chloroethyl)-*N'*-[2-(D-alanyl-L-phenylalanyl-L-lysylamino)ethyl]-*N*-nitrosourea dihydrochloride **(49)** is demonstrated in Scheme 4.

The concept for synthesis of *N*-(2-chloroethyl)-*N'*-[2-(tripeptidyl)aminoethyl]-*N*-nitrosoureas could not be used for the preparation of *N'*-methyl analogs because the latter are not stable under acidic conditions. The 9-fluorenylmethoxycarbonyl (FMOC) group was therefore used for protection of the amino functions, because it can be eliminated under mild basic conditions.[90-92]

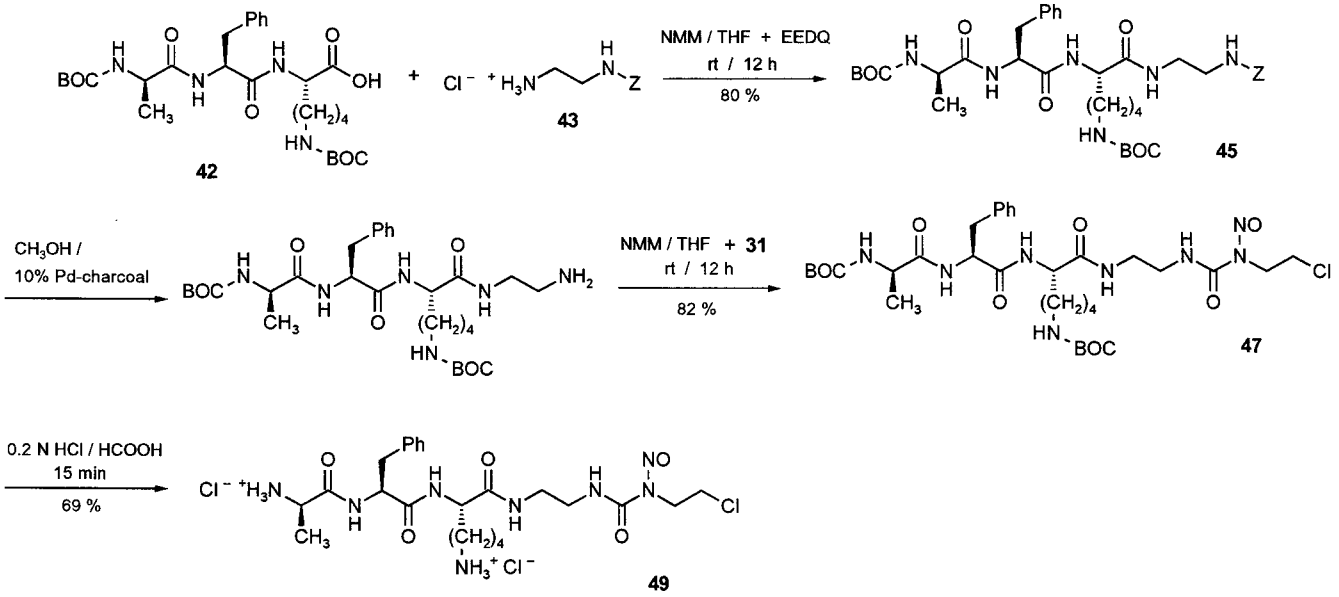
Two FMOC-protected tripeptides, D-(*N*-FMOC-valyl)-L-leucyl-L-(N^{ω}-FMOC-lysine) **(54)** and D-(*N*-FMOC-alanyl)-L-phenylalanyl-L-(N^{ω}-FMOC-lysine) **(56)** have been synthesized by coupling the corresponding FMOC-protected dipeptides **(51, 52)** with N^{ω}-FMOC-lysine **(55)** (Bachem). FMOC-protected dipeptides can be obtained by condensation of FMOC-protected amino acids with amino acid *tert*-butyl esters and subsequent cleavage of the ester bond by HBr in acetic acid.[93]

Synthesis of *N*-BOC-*N*-methylaminoethylamine **(36)** was achieved, starting from BOC-protected sarcosine **(33)** via BOC-protected sarcosylamide **(34)** to the nitrile **35** and its catalytic hydrogenation.[85]

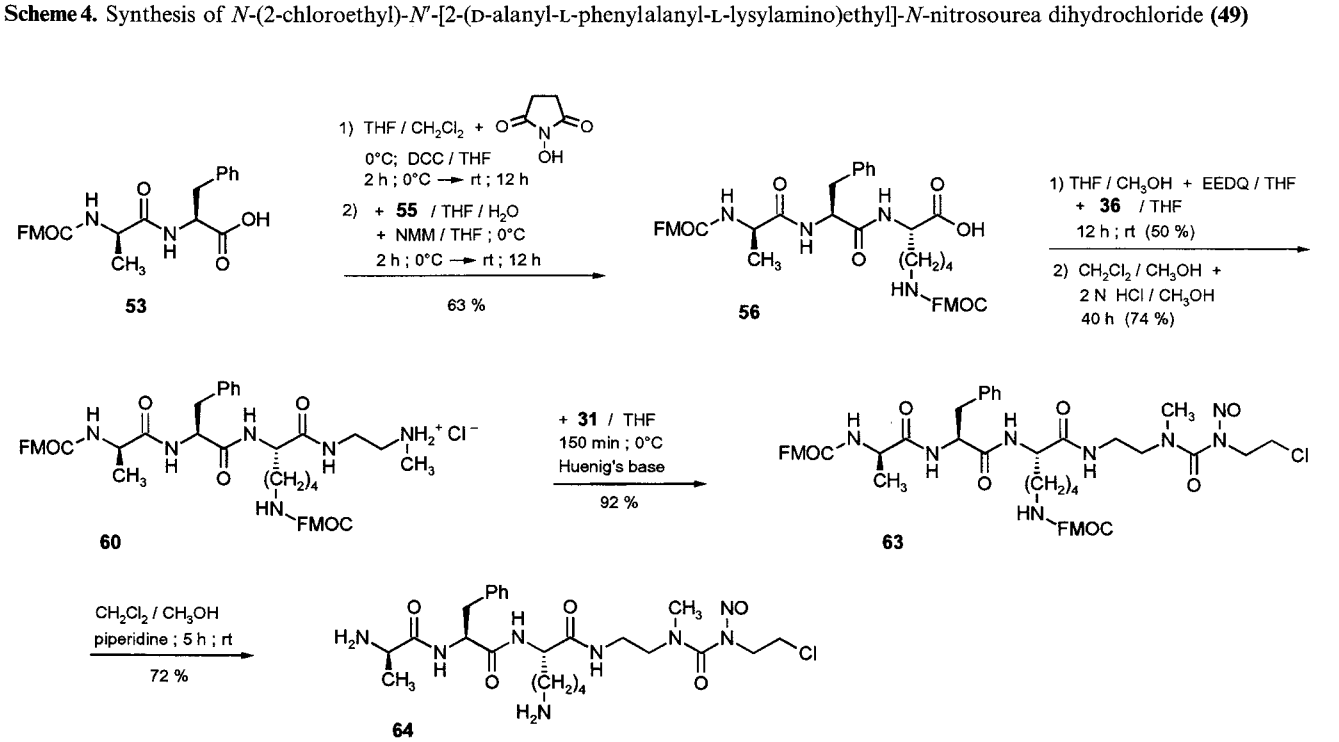
Reaction of FMOC-protected tripeptides with **36** yielded 1-(*N*-BOC-*N*-methylamino)-2-(FMOC-tripeptidylamino)ethanes **(57, 59)**. Elimination of BOC-protecting groups in methanolic HCl yielded 1-(*N*-methylamino)-2-(FMOC-tripeptidylamino)ethane hydrochlorides **(58, 60)**. Reaction of **58** or **60** with **31** gave *N*-(2-chloroethyl)-*N'*-methyl-*N'*-[2-(FMOC-tripeptidyl)aminoethyl]-*N*-nitrosoureas **(61,63)**. Elimination of FMOC groups with piperidine gave *N*-(2-chloroethyl)-*N'*-methyl-*N'*-[2-(D-valyl-L-leucyl-L-lysylamino)ethyl]-*N*-nitrosourea **(62)** from **61** or *N*-(2-chloroethyl)-*N'*-methyl-*N'*-[2-(D-alanyl-L-phenylalanyl-L-lysyl-amino)ethyl]-*N*-nitrosourea **(64)** from **63** (Scheme 5).[93]

Stability of *N'*-(2-Aminoethyl)-*N*-(2-chloroethyl)-*N*-nitrosourea (29) and the Tripeptide Conjugates

Conjugation of **29** with tripeptides enhanced its half life. Introduction of a methyl group at the *N'*-position resulted in further stabilization of the conjugates (Table 1).



Scheme 4. Synthesis of *N*-(2-chloroethyl)-*N'*-[2-(D-alanyl-L-phenylalanyl-L-lysylamino)ethyl]-*N*-nitrosourea dihydrochloride (**49**)



Scheme 5. Synthetic route of *N*-(2-chloroethyl)-*N'*-methyl-*N'*-[2-(D-alanyl-L-phenylalanyl-L-lysylamino)ethyl]-*N*-nitrosourea (**64**)[93]

Table 1. Half lives of *N'*-(2-aminoethyl)-*N*-(2-chloroethyl)-*N*-nitrosourea (**29**) hydrochloride and the tripeptide conjugates of *N'*-(2-aminoethyl)-*N*-(2-chloroethyl)-*N*-nitrosourea (**48**, **49**) and of *N'*-(2-aminoethyl)-*N*-(2-chloroethyl)-*N'*-methyl-*N*-nitrosourea (**62**, **64**) in Tris-buffer (pH 7.4) at 37 °C

Compound	k ($\times 10^{-3}$)	$t_{1/2}$ [min]
$H_2N(CH_2)_2NHCNC$ (**29**) · HCl	−20.2	34
D-Val-leu-lys-$NH(CH_2)_2NHCNC \cdot 2HCl$ (**48**)	−8.08	86
D-Ala-phe-lys-$NH(CH_2)_2NHCNC \cdot 2HCl$ (**49**)	−11.1	62
D-Val-leu-lys-$NH(CH_2)_2N(CH_3)CNC$ (**62**)	−2.17	319
D-Ala-phe-lys-$NH(CH_2)_2N(CH_3)CNC$ (**64**)	−2.04	340

CNC: *N*-(2-chloroethyl)-*N*-nitrosocarbamoyl group

Decomposition of the tripeptide-linked prodrugs **62** and **64** was significantly accelerated in the presence of plasmin (Table 2).

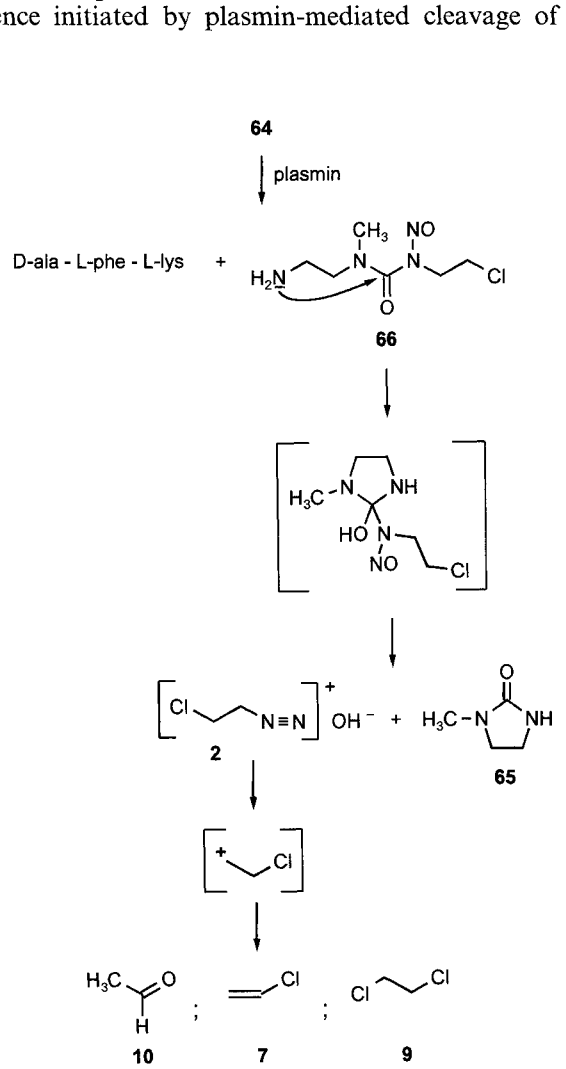
Decomposition and Activation of *N'*-(2-Aminoethyl)-*N*-(2-chloroethyl)-*N*-nitrosoureas (29) Hydrochloride and *N*-(2-Chloroethyl)-*N'*-methyl-*N'*-[2-(D-alanyl-L-phenylalanyl-L-lysyl-amino)ethyl]-*N*-nitrosourea (64)

Imidazolidin-2-one was obtained nearly quantitatively by incubation of the hydrochloride of **29** in phosphate buffer (pH 7.4) at room temperature for 48 h.[88] In the presence of plasmin, incubation of **64** in 0.1 M Tris/HCl (pH 7.4) yielded 1-methylimidazolidin-2-one (**65**). Fur-

Table 2. Half lives of *N*-(2-chloroethyl)-*N'*-methyl-*N'*-[2-(D-valyl-L-leucyl-L-lysylamino)ethyl]-*N*-nitrosourea (**62**) and *N*-(2-chloroethyl)-*N'*-methyl-*N'*-[2-(D-alanyl-L-phenylalanyl-L-lysylamino)ethyl]-*N*-nitrosourea (**64**) in Tris-buffer (pH 7.4) with and without plasmin (0.32 U/mL) and in human plasma at 37 °C

Compound	k ($\times 10^{-3}$)	$t_{1/2}$ [min]
D-Val-leu-lys-NH$(CH_2)_2$N(CH_3)CNC(**62**)		
0.1 M Tris/HCl	−2.17	319
0.1 M Tris/HCl + plasmin	−3.74	186
human plasma	−2.14	324
D-Ala-phe-lys-NH$(CH_2)_2$N(CH_3)CNC(**64**)		
0.1 M Tris/HCl	−2.04	340
0.1 M Tris/HCl + plasmin	−4.23	164
human plasma	−1.86	372

thermore, **10,7** and **9** were identified by gas chromatography/mass spectrometry as decomposition products generated via a 2-chloroethyldiazonium intermediate **(2)** (Scheme 6).[94] The generation of **65** from **64** in the presence of plasmin can be rationalized by a reaction sequence initiated by plasmin-mediated cleavage of the bond between the tripeptide and **66**.[93,94] Generated **66** decomposes rapidly by nucleophilic attack of the β-amino group on the carbonyl group, the tetrahedral intermediate breaking down into **65** and **2**. In the absence of plasmin, formation of **65** was not observed.



Scheme 6. Plasmin-mediated activation of *N*-(2-chloroethyl)-*N'*-methyl-*N'*-[2-(D-alanyl-L-phenylalanyl-L-lysylamino)ethyl]-*N*-nitrosourea **(64)** and the formation of 1-methylimidazolidin-2-one **(65)**

Inhibition of Tumor Cell Growth in Vitro by Plasmin-Sensitive Prodrugs

To test the validity of the concept a series of human tumors maintained as xenografts in immune deficient nude mice were screened for PA activity both in solid tumor tissue and in cell culture. In solid tumor tissue PA activity was determined after homogenization in Tris buffer (pH 7.5), containing 0.1 % of Tween 80. Incubation of the cell extract or conditioned medium with human plasminogen (Fluka) for 1 h resulted in the release of plasmin. Plasmin activity was determined using 7-(D-valyl-L-leucyl-L-lysylamino)-4-methylcoumarin (Bachem) as fluorogenic substrate. The released 7-amino-4-methylcoumarin was measured fluorometrically at 380 nm for excitation and at 460 nm for emission.[95] Receptor-bound PA was determined by incubation of the cells with plasminogen in 0.1 M Tris buffer (pH 7.5) at 37 °C for 1 h, proceeding further as described. Urokinase (Sigma) was used as reference enzyme.

Out of several human tumor xenografts tested, a large cell lung carcinoma xenograft LXFL 529, originating from a 34 year old patient (115 days survival after operation) and passaged in nude mice since 1984, was found to express high PA activity (5.40 ± 0.70 IU/mg protein). The corresponding cultured cells, LXFL 529L, secreted high levels of PA into serum-free conditioned medium (2.45 ± 0.64 IU/mg protein) and showed high membrane associated PA activity (2.11 ± 0.42 IU/mg protein). The murine melanoma cell line B 16 secreted a PA activity of 0.35 ± 0.09 IU/mg protein into serum-free medium and showed a membrane-associated PA activity of 0.53 ± 0.14 IU/mg protein in cultured cells.

Compounds **62** and **64** were tested for their growth inhibitory activity in vitro against human large cell lung cancer xenograft LXFL 529L and B 16 melanoma cells, using the sulforhodamine B assay. This assay is based on quantitation of cellular protein which is directly proportional to the number of vital cells.[96] Cells were plated in 24-well culture plates (2×10^4 cells per well) in complete medium and incubated at 37 °C in a humidified 5 % CO_2 atmosphere for 48 h. The medium was removed and cells were incubated in serum- and phenol red-free RPMI 1640 medium for 24 h with prodrugs, with or without addition of plasminogen. Compound **27** served as reference substance with proven clinical activity in a spectrum of tumor malignancies. After removal of incubation medium, cells were grown for 48 h (B 16 cells 24 h) in complete medium and were then fixed with trichloroacetic acid. The fixed cells were stained with sulforhodamine B. After standing for 30 min at room temperature, the supernatant was decanted, the dye in the cells extracted with Tris base (pH 10.5) and determined photometrically at 564 nm.

In the presence of plasminogen, prodrug **64** was highly active in the human xenograft LXFL 529L and in murine

B 16 melanoma cells, comparable or even superior to the reference substance. The effectiveness is expressed as T/C value (%), defined as percent of surviving cells in the test group, compared to untreated control (test/control × 100). The cytotoxicity of **64** was concentration dependent. In the absence of added plasminogen, **64** was much less effective. This finding strongly suggests that plasmin-mediated prodrug activation contributes to a major extent to the growth inhibitory activity of **64**. In LXFL cells, **64** in the presence of plasminogen displays the same antitumor activity as **27**. Since in the absence of plasminogen **64** is much less effective, it is conceivable that a major proportion of the prodrug is not activated due to the lack of plasmin activity. It is also of interest that B 16 cells are more resistant to **27** and **64** without plasminogen but that in the presence of plasminogen a remarkable increase in efficacy becomes apparent at 100 μmol. Compound **27** at this concentration still is relatively inefficient, very similar to **64** without plasminogen. It appears that B 16 cells are less resistant to the cytotoxic agent liberated from **64** by plasmin-mediated cleavage than to **27** (Figure 3).

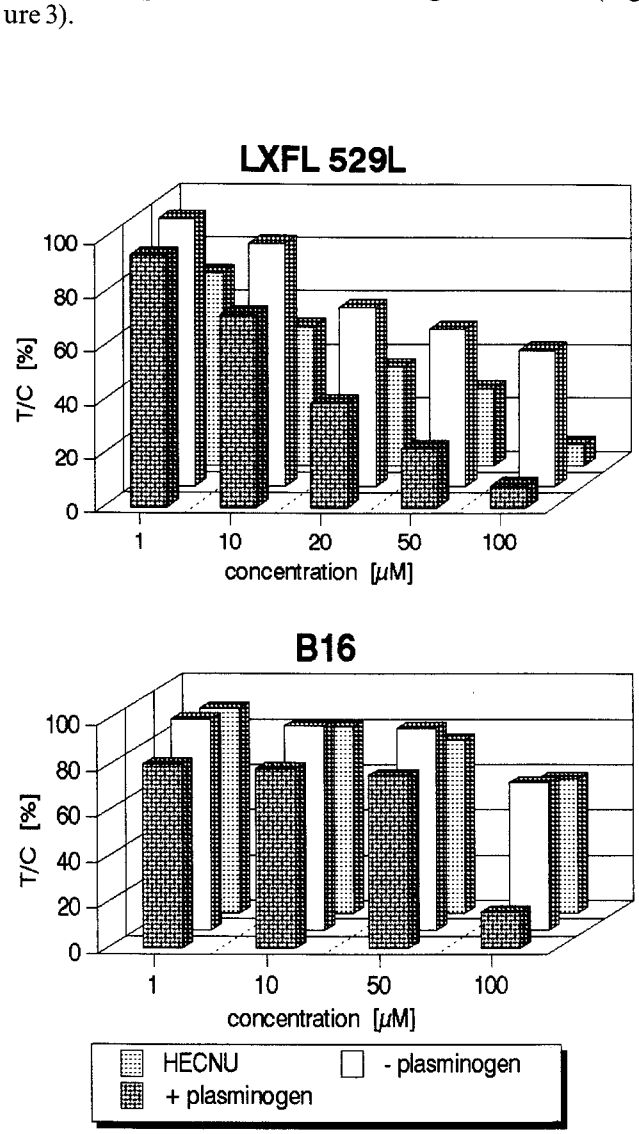


Figure 3. Growth inhibition of LXFL 529L and B 16 cells by prodrug **64** with and without plasminogen in comparison to a clinically effective reference compound **27**

Closing Remarks

A series of malignant tumor cell lines and many human solid tumors secrete substantially increased amounts of plasminogen activators, compared to normal tissues. As a consequence, enhanced plasmin activity is present in close vicinity to such tumor tissues.

Plasmin activated *N*-(2-chloroethyl)-*N*-nitrosourea prodrugs appear to be promising new antineoplastic agents that should preferably target human tumors high in plasminogen activator activity. Data obtained in the present study clearly show that tumor cells expressing plasminogen activator become significantly more sensitive to such prodrugs in the presence of plasminogen.

It remains to be established whether the observed increase in cytotoxic activity in plasminogen activator expressing tumor cells is also accompanied by a decrease in systemic toxicity. Appropriate experiments in tumor-bearing animals are the next step needed to establish such a gain in therapeutic breadth. Obviously the concept deserves further exploration. Research into optimizing carrier peptides and cytotoxic groups of such peptide conjugates is in progress.

Melting points (mp) were measured on a Büchi 510 apparatus and are not corrected. Elemental analysis were determined on a Perkin-Elmer 2400 CHN elemental analyser. IR spectra were recorded on Perkin-Elmer IR-Spectrophotometer 394. ^1H and ^{13}C NMR spectra were recorded on Varian EM 360, Varian EM 390, Bruker AM 200, Bruker AM 400 or Bruker AM 500. Chemical shifts are given in ppm relative to TMS as an internal standard.

Decomposition of 64 and Product Analysis by Head Space Gas Chromatography and Gas Chromatography/Mass Spectrometry (GC/MS):

Compound **64** (2.7 μmol), dissolved in 0.1 M Tris/HCl buffer (10 mL), containing 0.1 % Tween 80 was incubated at 37 °C in 40 mL septum-sealed head space vials in the presence and absence of plasmin (3 mL, 13 U/mL). Controls contained identical volumes of Tris/HCl, containing 0.1 % Tween 80 with and without the corresponding amount of plasmin. The head space volume was flushed with helium (15 mL/min) at specified time intervals (1, 3 , 5, 7 h) and was cryofocused (150 mL) in a liquid N_2 cooled glass lined tubing trap (40 cm, 0.8 mm id.). Trapped volatiles were transferred to the analytical GC column by heating the trap with a hot air gun (300 °C). GC conditions: 25 m column, 0.53 mm, Paraplot S, Chrompack; helium 10 mL/min; program: 50 °C (1 min), 12 °C/min to 175 °C, then isothermal. Mass spectrometer: Finnigan TSQ 70; Electron impact 200 μA, 70 eV; flame ionisation detector: hydrogen 30 mL/min, air 300 mL/min helium make-up 40 mL/min.

***N*-[2-(*tert*-Butoxycarbonylamino)ethyl]-*N*-(2-chloroethyl)-*N*-nitrosourea (32):**

A solution of **31** (11.5 mmol) in THF (20 mL) was added at 0 °C into a solution of **30** (10 mmol) and *N*-methylmorpholine (NMM) (10 mmol) in THF (20 mL) and stirred overnight at r.t. After evaporation the residue was dissolved in EtOAc (20 ml) and washed successively with 10% citric acid, (10 ml) $NaHCO_3$ (10 ml) and brine (10 ml) and dried (Na_2SO_4). After removing EtOAc, crude **32** was purified by chromatography on silica gel (CH_3OH–$CHCl_3$, 1:10); yield: 2.5 g (76 %).

^1H NMR (90 MHz, $CDCl_3$): δ = 7.70 (t, 1 H), 5.25 (t, 1 H), 4.20 (t, 2 H), 3.70–3.40 (m, 6 H), 1.40 (s, 9 H).

IR (KBr): υ = 3320, 2950, 1675, 1520–1470, 1355 cm^{-1}.

Anal.: Calcd for $C_{10}H_{19}ClN_4O_4$: C, 40.8; H, 6.5; N, 19.0. Found C, 40.6; H 6.3; N, 18.5.

***N'*-(2-Aminoethyl)-*N*-(2-chloroethyl)-*N*-nitrosourea (29) Hydrochloride:**
Compound **32** (10 mmol) was treated with 0.2 M HCl in formic acid (55 mL) at 10 °C for 15–30 min until gas development ceased. After evaporation of formic acid, the crude hydrochloride was dissolved in CH_3OH (5 mL), precipitated by addition of Et_2O, and purified by repeated re-precipitation from CH_3OH and Et_2O; yield: 2.0 g (85 %).

^{1}H NMR (90 MHz, DMSO-d_6): δ = 8.95 (t, 1H), 4.15 (t, 2H), 3.75–3.50 (m, 4H), 3.20–3.00 (m, 2H).

$^{13}C\{^1H\}$ NMR (500 MHz, MeOD): δ = 156.0 (s), 41.4 (t), 40.9 (t), 40.1 (t), 39.2 (t).

IR (KBr): υ = 3300, 1710, 1510, 1470, 650 cm^{-1}.

Anal.: Calcd for $C_5H_{12}Cl_2N_4O_2$: C, 26.0; H, 5.2; N, 24.3 Found C, 26.3; H 5.2; N, 24.2.

***N*-(*tert*-Butoxycarbonyl)sarcosine (33):**
A solution of sarcosine (18 g, 0.2 mol) in 1M NaOH (200 mL) and dioxane (350 mL) was cooled to 0 °C and di-*tert*-butyl carbonate (48.5 g, 0.22 mol) in dioxane (50 mL) added dropwise with stirring. The pH-value was maintained at 9–10 (NaOH). After stirring overnight the mixture was concentrated, cooled in ice water, acidified with citric acid to pH 2–3, and extracted with EtOAc (3 × 100 mL). The combined organic extract was washed with brine (2 × 30 mL), dried (Na_2SO_4), and concentrated to give colorless crystals, yield: 36.3 g (95 %).

^{1}H NMR (60 MHz, $CDCl_3$): δ = 3.95 (m, 2H), 2.90 (s, 3H), 1.45 (s, 9H).

Anal.: Calcd for $C_8H_{15}NO_4$: C, 50.8; H, 8.0; N, 7.4. Found C, 50.7; H 8.1; N, 7.3.

***N*-(*tert*-Butoxycarbonyl)sarcosylamide (34):**
Chloroformic acid ethyl ester (8.4 mL, 88 mmol) in THF (20 mL) was added dropwise into a solution of **33** (16.65 g, 88 mmol) and triethylamine (12.4 mL, 88 mmol) in THF (250 mL). The mixture was stirred overnight after addition of 20 % aq NH_3 (18.8 mL, 220 mmol). After evaporation the residue was extracted with EtOAc (3 × 50 mL) to give colorless crystals, yield: 11.4 g (69 %).

^{1}H NMR (60 MHz, $CDCl_3$): δ = 6.20 (br s, 2H), 3.85 (s, 2H), 2.90 (s, 3H), 1.45 (s, 9H).

Anal.: Calcd for $C_8H_{16}N_2O_3$: C, 51.1; H, 8.6; N, 14.9. Found C, 51.1; H 8.5; N, 14.7.

***N*-(*tert*-Butoxycarbonyl)sarcosinenitrile (35):**
A solution of **34** (7.2 g, 38 mmol) and NMM (9.2 mL, 84 mmol) in THF (30 mL) was mixed with trifluoroacetic anhydride (6 mL, 42 mmol) under ice cooling. After stirring for 3 h at r.t., the mixture was diluted with H_2O (30 mL) and evaporated. The residue was partitioned between H_2O and Et_2O. The organic phase was washed successively with 10 % citric acid (10 mL), 0.1 M NaOH (10 mL), and brine (10 mL), dried (Na_2SO_4), and concentrated. The crude product was purified by vacuum distillation to give **35**, bp 59–60 °C/0.2 Torr, yield: 5.2 g (80 %).

^{1}H NMR (90 MHz, $CDCl_3$): δ = 4.15 (s, 2H), 2.85 (s, 3H), 1.45 (s, 9H).

2-Amino-1-[*N*-(*tert*-butoxylcarbonyl)-*N*-methylamino]ethane (36):
A solution of **35** (13.3 g, 76 mmol) was hydrogenized in NH_3–sat. EtOH (300 mL) over Raney Ni (12 h; 30 bar; 50 °C). Filtration and evaporation of the mixture gave the crude product. Vacuum distillation gave **36**, bp 77–79 °C/0.4 Torr, yield: 11.3 g (86 %).

^{1}H NMR (60 MHz, $CDCl_3$): δ = 3.25 (t, 2H), 2.85 (s, 3H), 2.75 (t, 2H), 1.45 (s, 9H), 1.10 (s, 2H).

D-(*N*-*tert*-Butoxycarbonylvalyl)-L-leucine Methyl Ester (37):
A solution of D-(*N*-BOC)-valine (5 g, 23 mmol), leucine methyl ester hydrochloride (4.2 g, 23 mmol), and l-hydroxybenzotriazole (3.2 g, 23 mmol) in DMF (35 mL) was treated with triethylamine (3.2 mL) at 0 °C. A solution of dicyclohexylcarbodiimide (DCC) (5.3 g, 25 mmol) in CH_2Cl_2 (55 mL) was added dropwise, and the mixture was stirred for 4 h at 0 °C and then for 24 h at r.t. The precipitated dicyclohexylurea was filtered off and the filtrate was concentrated in vacuo. The crude product was dissolved in EtOAc (50 mL), washed successively with 10 % citric acid (10 mL), sat. $NaHCO_3$ (10 mL), and brine (10 mL) and dried (Na_2SO_4). After evaporation of EtOAc, **37** was crystallized from hexane as colorless crystals, mp 89–90 °C, yield: 7 g, (88 %).

^{1}H NMR (90 MHz, $CDCl_3$): δ = 6.55 (d, 1H), 5.05 (d, 1H), 4.70–4.40 (m, 1H), 3.95 (q, 1H), 3.70 (s, 3H), 2.10–1.90 (m, 2H), 1.65 (q, 2H), 1.45 (s, 9H), 0.95 (m, 12H).

Anal.: Calcd for $C_{17}H_{32}N_2O_5$: C, 61.7; H, 7.47; N, 8.0. Found C, 61.5; H, 7.34; N, 7.4.

D-(*N*-*tert*-Butoxycarbonylvalyl)-L-leucine (38):
A solution of **37** (7 g, 20 mmol) in acetone/DMF (120 mL + 20 mL) was treated with 1 M NaOH (10 mL) under stirring for 1 h at r.t. After removing the solvents the residue was dissolved in water (200 mL) and washed with Et_2O. The product was extracted from the aqueous solution with EtOAc (3 × 50 mL) after acidification with citric acid to pH 2–3. The combined organic phase was washed (H_2O) (2 × 30 mL), dried (Na_2SO_4), evaporated in vacuo to give **38** as colorless crystals (hexane), mp 143–144 °C, yield: 6.3 g (95 %).

^{1}H NMR (90 MHz, $CDCl_3$): δ = 9.40 (s, 1H), 6.90 (d, 1H), 5.50 (d, 1H), 4.80–4.40 (m, 1H), 4.30–4.00 (m, 1H), 2.30–1.95 (m, 2H), 1.65 (q, 2H), 1.45 (s, 9H), 1.00–0.70 (m, 12H).

Anal.: Calcd for $C_{16}H_{30}N_2O_5$: C, 58.2; H, 9.2; N, 8.5. Found C, 57.9; H, 9.3; N, 8.3.

D-(*N*-*tert*-Butoxycarbonylvalyl)-L-leucyl-L-(N^{ω}-(*tert*-Butoxycarbonyl)lysine Methyl Ester (39):
A solution of **38** (3.3 g, 10 mmol), N^{ω}-BOC-lysine methyl ester hydrochloride (3 g, 10 mmol), *N*-hydroxypyrrolidine-2,5-dione (1.15 g), and NMM (1.1 mL, 10 mmol) in DMF (35 mL) was treated with Et_3N (1.4 mL) at 0 °C. A solution of DCC (2.3 g, 11 mmol) in CH_2Cl_2 (55 mL) was added dropwise and the mixture was stirred for 4 h at 0 °C and then for 24h at r.t. The precipitated dicyclohexylurea was removed and the solution was concentrated in vacuo. The residue was dissolved in EtOAc (50 mL), washed successively with 10 % citric acid (10 mL), sat. $NaHCO_3$ (10 mL), and brine (10 mL) and dried (Na_2SO_4). After evaporation of EtOAc **39** was crystallized as colorless crystals (hexane), mp 147–148 °C, yield: 4.4 g (76 %).

^{1}H NMR (90 MHz, $CDCl_3$): δ = 7.60–7.20 (m, 2H), 5.80–5.60 (m, 1H), 5.40–5.10 (m, 1H), 4.70–4.30 (m, 2H), 4.15–3.95 (m, 1H), 3.70 (s, 3H), 3.25–2.95 (m, 2H), 2.30–1.20 (m, 28H), 1.00–0.80 (m, 12H).

Anal.: Calcd for $C_{28}H_{52}N_4O_8$: C, 58.7; H, 9.2; N, 9.8. Found C, 58.4; H, 8.9; N, 9.5.

D-(*N*-*tert*-Butoxycarbonylvalyl)-L-leucyl-L-(N^{ω}-*tert*-Butoxycarbonyl)lysine (40):
Compound **40** was prepared by the same procedure as described for **38** using **39** as starting material, colorless crystals, yield: 3 g (88 %).

^{1}H NMR (90 MHz, $CDCl_3$): δ = 9.90 (s, 1H), 4.70–4.30 (m, 2H), 4.20–3.90 (m, 1H), 3.30–2.90 (m, 2H), 2.40–1.35 (m, 28H), 1.10–0.90 (m, 12H).

$^{13}C\{^1H\}$ NMR (500 MHz, DMSO-d_6): δ = 173.3 (s), 172.1 (s), 171.3 (s), 155.5 (s), 155.0 (s), 78.0 (s), 77.3 (s), 60.0 (d), 51.9 (d), 50.4 (d), 40.5 (t), 30.5 (t), 30.2 (d), 29.0 (t), 28.2 (q), 28.1 (q), 24.0 (q), 23.2 (q), 22.8 (t), 22.7 (t), 19.1 (q), 18.2 (q).

Anal.: Calcd for $C_{27}H_{50}N_4O_8$: C, 58.1; H, 9.0; N, 10.0. Found C, 58.2; H 8.8; N, 9.8.

D-(*N*-*tert*-Butoxycarbonylalanyl)-L-phenylalanine Methyl Ester (41):
Compound **41** was prepared by the same procedure as described for **37** using D-(*N*-BOC)-alanine and L-phenylalanine methyl ester as starting materials, colorless crystals, yield: 4.9 g (61 %).

^{1}H NMR (90 MHz, $CDCl_3$): δ = 7.25 (s, 5H), 4.60–3.70 (m, 2H), 3.70 (s, 3H), 3.25–2.60 (m, 2H), 1.40 (s, 9H), 1.00 (d, 3H).

Anal.: Calcd for $C_{18}H_{26}N_2O_5$: C, 61.7; H, 7.5; N, 8.0. Found C, 61.2; H 8.0; N, 8.2.

D-(*N*-*tert*-Butoxycarbonylalanyl)-L-phenylalanyl-L-(N^{ω}-*tert*-butoxycarbonyl)lysine (42):
Compound **42** was prepared by the same procedure as described for **39** using D-(*N*-BOC-alanyl)-L-phenylalanine, released in situ from **41** as described for **38**, and L-(N^{ω}-BOC)-lysine as starting materials, colorless crystals, yield: 3.0 g (54%).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.10 (d, 1H), 8.00 (d, 1H), 7.20 (s, 5H), 6.80 (d, 1H), 6.75 (t, 1H), 4.60–4.50 (m, 1H), 4.20–4.10 (m, 1H), 4.00–3.90 (m, 1H), 3.00–2.75 (m, 4H), 1.75–1.60 (m, 2H), 1.40–1.20 (m, 22H), 0.90 (d, 3H).

^{13}C{^{1}H} NMR (500 MHz, DMSO-d_6): δ = 173.4 (s), 172.4 (s), 171.1 (s), 155.5 (s), 155.0 (s), 137.7 (s), 129.3 (d), 127.8 (d), 126.1 (d), 78.0 (s), 77.3 (s), 53.2 (d), 52.0 (d), 49.6 (d), 37.4 (t), 30.7 (t), 29.1 (t), 28.2 (q), 28.1 (q), 22.8 (t), 22.7 (t), 18.0 (q).

Anal.: Calcd for $C_{28}H_{44}N_4O_8$: C, 59.6; H, 7.9; N, 9.9. Found C, 59.6; H 7.8; N, 9.4.

2-(*N*-Benzyloxycarbonylamino)ethylamine Hydrochloride (43):
A solution of *N*-Z-aminopropionyl amide (20 mmol) and pyridine (40 mmol) in a mixture of MeCN and H_2O (each 60 mL) was mixed with [bis(trifluoroacetoxy)iodo]benzene (20 mmol) and stirred overnight at r.t. The mixture was then poured into H_2O (400 mL), acidified with HCl to pH 2, washed with Et_2O (5 × 75 mL). The precipitated solid mass was recrystallized from EtOH or EtOH–Et_2O, mp 172–173 °C, yield: 3.6 g (78%).

^{1}H NMR (90 MHz, CD_3OD): δ = 7.65 (s, 5H), 5.10 (s, 2H), 3.45 (t, 2H), 3.10 (t, 2H).

Anal.: Calcd for $C_{10}H_{15}ClN_2O_2$: C, 52.1; H, 6.6; N, 12.1 Found C, 51.9; H 6.5; N, 11.9.

N-Benzyloxycarbonyl-*N'*-(*tert*-Butoxycarbonyltripeptidyl)alkanediamines, General Procedure:
A solution of **43** (5 mmol) and NMM (5 mmol) in THF (30 mL) was added with BOC-protected tripeptide (5 mmol) and EEDQ (6 mmol). The mixture was stirred overnight at r.t. After addition of EtOAc (100 mL), the solution was washed successively with 10% citric acid (20 mL), sat. Na_2CO_3 (20 mL), and brine (20 mL), dried (Na_2SO_4), and concentrated in vacuo. The crude products were purified by repeated reprecipitation from CH_3OH and Et_2O.

1-(N-Benzyloxycarbonylamino)-2-[D-(N-tert-butoxycarbonylvalyl)-L-leucyl-L-(N^{ω}-tert-butoxycarbonyl)lysylamino]ethane **(44):**
The general procedure was followed, colorless crystals, mp 154–155 °C, yield: 2.9 g (78%).

^{1}H NMR (90 MHz, DMSO-d_6): δ = 8.20–7.60 (m, 3H), 7.35 (s, 5H), 6.90–6.50 (m, 3H), 5.05 (s, 2H), 4.45–4.30 (m, 2H), 4.20–3.85 (m, 2H), 3.75 (t, 1H), 3.20–2.75 (m, 8H), 2.00–1.20 (m, 24H), 1.00–0.75 (m, 12H).

Anal.: Calcd for $C_{37}H_{62}N_6O_9$: C, 60.5; H, 8.5; N, 11.4 Found C, 60.6; H 8.5; N, 11.2.

1-(N-Benzyloxycarbonylamino)-2-[D-(N-tert-butoxycarbonylalanyl)-L-phenylalanyl-L-(N^{ω}-tert-butoxycarbonyl)lysylamino]ethane **(45):**
The general procedure was followed, colorless crystals, mp 135–136 °C, yield: 3.0 g (80%).

^{1}H NMR (90 MHz, DMSO-d_6): δ = 8.20–7.60 (m, 3H), 7.35 (s, 5H), 7.25 (s, 5H), 6.90–6.50 (m, 2H), 5.00 (s, 2H), 4.50–4.30 (m, 1H), 4.30–3.75 (m, 2H), 3.20–2.70 (m, 8H), 2.00–1.00 (m, 24H) 0.95 (d, 3H).

IR (KBr): υ = 3300, 2980, 2930, 1710–1630, 1550–1490, 1390, 1370, 1250, 1170, 705 cm^{-1}.

Anal.: Calcd for $C_{38}H_{56}N_6O_9$: C, 61.6; H, 7.6; N, 11.3. Found C, 61.1; H, 7.5; N, 11.3

***N'*-[2-(*tert*-Butoxycarbonyltripeptidylamino)ethyl]-*N*-(2-chloroethyl)-*N*-nitrosoureas; General Procedure:**
The corresponding *N*-Z-*N'*-(BOC-tripeptidyl)ethanediamines (2 mmol) were hydrogenized in CH_3OH over 10% Pd/charcoal. After filtration and evaporation the residue was dissolved in THF (20 mL) and added to a solution of **31** (0.25 mmol) and NMM (0.25 mmol) in THF (20 mL). The mixture was stirred overnight at r.t., evaporated, dissolved in CH_2Cl_2 (20 mL), washed successively with 10% citric acid (10 mL), sat. Na_2CO_3 (10 mL), and brine (10 mL), dried (Na_2SO_4), and concentrated in vacuo. Crude products from CH_2Cl_2 were purified by chromatography on silica gel and reprecipitation from suitable solvents.

N-(2-Chloroethyl)-N'-{2-[D-(N-tert-butoxycarbonylvalyl)-L-leucyl-L-(N^{ω}-tert-butoxycarbonyl)lysylamino]ethyl}-N-nitrosourea **(46):**
The general procedure was followed, yellowish crystals, yield: 1.1 g (77%). ^{1}H NMR (90 MHz, $CDCl_3$): δ = 8.75 (t, 1H), 8.20–7.80 (m, 2H), 7.60–6.90 (m, 2H), 5.40–5.10 (m, 1H), 4.55–4.05 (m, 5H), 3.95–2.95 (m, 8H), 2.30–1.20 (m, 28H), 1.20–0.85 (m, 12H).

Anal.: Calcd for $C_{32}H_{59}ClN_8O_9$: C, 54.9; H, 8.5; N, 16.0. Found C, 55.0; H, 8.5; N, 15.9.

N-(2-Chloroethyl)-N'-{2-[D-(N-tert-butoxycarbonylalanyl)-L-phenylalanyl-L-(N^{ω}-tert-butoxycarbonyl)lysylamino]ethyl}-N-nitrosourea **(47):**
The general procedure was followed, yellowish crystals, yield: 1.2 (82%).

^{1}H NMR (90 MHz, DMSO-d_6): δ = 8.75 (t, 1H), 8.05–7.65 (m, 3H), 7.25 (s, 5H), 6.95–6.55 (m, 2H), 4.60–4.30 (m, 1H), 4.30–3.75 (m, 4H), 3.60 (t, 2H), 3.50–3.10 (m, 4H), 3.10–2.70 (m, 4H), 1.85–1.10 (m, 24H), 0.95 (d, 3H).

^{13}C{^{1}H} NMR (200 MHz, CD_3OD): δ = 176.9 (s), 174.7 (s), 173.7 (s), 158.4 (s), 157.9 (s), 155.3 (s), 138.5 (s), 130.3 (d), 129.5 (d), 127.9 (d), 80.6 (s), 79.7 (s), 65.2 (d), 55.3 (d), 51.7 (d), 41.2 (t), 40.1 (t), 40.0 (t), 37.7 (t), 32.0 (t), 30.3 (t), 28.8 (q), 24.3 (t), 17.6 (q).

IR (KBr): υ = 3290, 3080, 2980, 2940, 1720–1630, 1570–1480, 1450, 1390, 1370, 1250, 1170, 755, 705 cm^{-1}.

Anal.: Calcd for $C_{33}H_{53}ClN_8O_9$: C, 53.5; H, 7.2; N, 15.1 Found C, 53.0; H 7.0; N, 14.7.

N-(2-Chloroethyl)-*N'*-(2-tripeptidylaminoethyl)-*N*-nitrosourea Hydrochlorides; General Procedure:
The corresponding *N*-(2-chloroethyl)-*N'*-[2-(BOC-tripeptidylamino)ethyl]-*N*-nitrosoureas were treated with 1.1 equivalent 0.2 M HCl in formic acid for each BOC group for 15 min. Formic acid was evaporated and the residue treated with EtOAc. The crude products were purified by reprecipitation from CH_3OH and Et_2O.

N-(2-Chloroethyl)-N'-[2-(D-valyl-L-leucyl-L-lysylamino)ethyl]-N-nitrosourea Dihydrochloride **(48)**:
The general procedure was followed, yellowish crystals, yield: 85%.

^{1}H NMR (200 MHz, DMSO-d_6): δ = 8.75 (t, 1H), 8.15 (d, 1H), 8.10–8.00 (m, 2H), 4.35–4.27 (m, 1H), 4.18–4.11 (m, 1H), 4.09 (t, 2H), 3.75–3.68 (m, 1H), 3.60 (t, 2H), 3.40–3.25 (m, 4H), 2.80–2.70 (m, 2H), 2.15–2.07 (m, 2H), 1.75–1.35 (m, 8H), 0.95–0.83 (m, 12H).

^{13}C{^{1}H} NMR (500 MHz, DMSO-d_6): δ = 171.8 (s), 171.6 (s), 168.8 (s), 152.7 (s), 57.5 (d), 52.9 (d), 51.4 (d), 40.4 (t), 38.3 (t), 38.0 (t), 30.8 (t), 29.8 (d), 26.4 (t), 24.0 (d), 23.1 (q), 22.5 (t), 20.8 (q), 18.3 (q), 17.6 (q).

Anal.: Calcd for $C_{22}H_{45}Cl_3N_8O_5$: C, 43.5; H, 7.5; N, 18.4. Found C, 43.3; H 7.3; N, 17.9.

N-(2-Chloroethyl)-N'-[2-(D-alanyl-L-phenylalanyl-L-lysylamino)-ethyl]-N-nitrosourea Dihydrochloride **(49):**
The general procedure was followed, yellowish crystals, yield: 69%.

^{1}H NMR (90 MHz, DMSO-d_6): δ = 8.85–8.55 (m, 2H), 8.50–8.05 (m, 2H), 7.30 (s, 5H), 6.0–5.0 (br), 4.60–4.30 (m, 1H), 4.30–3.75 (m, 4H), 3.60 (t, 2H), 3.60–2.70 (m, 8H), 1.90–1.00 (m, 6H), 0.95 (d, 3H).

IR (KBr): υ = 3420–3120, 3060, 2980, 2930, 1670–1620, 1540–1450, 1250, 1170, 745, 700 cm^{-1}.

Anal.: Calcd for $C_{23}H_{39}Cl_3N_8O_5$: C, 45.0; H, 6.4; N, 18.3. Found C, 45.6; H 6.8; N, 17.7.

D-(*N*-9-Fluorenylmethoxycarbonylvalyl)-L-leucine *tert*-Butyl Ester (50):
A solution of D-(*N*-FMOC)-valine (5.4 g, 16 mmol) and *N*-hydroxypyrrolidine-2,5-dione (2.1 g, 18 mmol) in THF (100 mL) was added dropwise at 0 °C to DCC (3.7 g, 18 mmol) in THF (100 mL).

The mixture was stirred for 2 h at 0 °C, and then mixed with L-leucine *tert*-butyl ester, obtained from L-leucine *tert*-butyl ester hydrochloride (5.1 g, 23 mmol) and 1 M NaOH (23 mL), in THF (100 mL) under ice cooling, and stirred overnight at r.t. After evaporation the residue was dissolved in EtOAc (100 mL), washed successively with 10% citric acid (30 mL), sat. Na_2CO_3 (30 mL) and H_2O (30 mL), and dried (Mg_2SO_4). Concentration of the EtOAc gave **50** as colorless crystals (EtOAc), yield: 6.9 g (85%).

^{1}H NMR (90 MHz, $CDCl_3$): δ = 7.80–7.20 (m, 8H), 6.30 (br d, 1H), 5.40 (br d, 1H), 4.70–4.00 (m, 5H), 2.30–2.00 (m, 2H), 1.80–1.55 (m, 2H), 1.40 (s, 9H) 1.10–0.90 (m, 12H).

Anal.: Calcd for $C_{30}H_{40}N_2O_5$: C, 70.8; H, 7.9; N, 5.5. Found C, 69.5; H 8.0; N, 5.9.

D-(*N*-9-Fluorenylmethoxycarbonylvalyl)-L-leucine (51):
Compound **50** (6.8 g, 13.5 mmol) in HOAc (120 mL) was treated with 2 M HBr in HOAc (13.5 mL, 27 mmol). After completion of reaction, the mixture was poured into ice water and **51** precipitated as a white solid, yield: 5.8 g (95%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76–7.28 (m, 8H), 6.95 (d, 1H), 5.86 (d, 1H), 4.65–4.19 (m, 5H), 2.10–1.46 (m, 4H), 1.00–0.94 (m, 12H).

Anal.: Calcd for $C_{26}H_{32}N_2O_5$: C, 69.0; H, 7.1; N, 6.2. Found C, 68.3; H 7.1; N, 6.1.

D-(*N*-9-Fluorenylmethoxycarbonylalanyl)-L-phenylalanine *tert*-Butyl Ester (52):
Compound **52** was prepared by the same procedure as described for **50** using D-(N-FMOC)-alanine and L-phenylalanine *tert*-butyl ester as starting materials, colorless crystals (EtOAc), yield: 6.9 g (84%).

^{1}H NMR (90 MHz, $CDCl_3$): δ = 7.80–7.00 (m, 13H), 6.45 (d, 1H), 5.40 (d, 1H), 4.75 (q, 1H), 4.45–4.00 (m, 4H), 3.20 (d, 2H), 1.40 (s, 9H), 1.30 (d, 3H).

Anal.: Calcd for $C_{31}H_{34}N_2O_5$: C, 72.3; H, 6.7; N,5.4. Found C, 72.2; H 6.8; N, 5.6.

D-(*N*-9-Fluorenylmethoxycarbonylalanyl)-L-phenylalanine (53):
Compound **53** was prepared by the same procedure as described for **51** using **52** as starting material, white solid, yield: 5.4 g (87%).

^{1}H NMR (90 MHz, $CDCl_3$): δ = 7.90–6.95 (m, 13H), 6.70 (d, 1H), 5.70 (d, 1H), 4.90 (q, 1H), 4.50–3.90 (m, 4H), 3.15 (d, 2H), 1.20 (d, 3H).

D-(*N*-9-Fluorenylmethoxycarbonylvalyl)-L-leucyl-L-(N^{ω}-9-Fluorenylmethoxycarbonyl)lysine (54):
A solution of **51** (5.3 g, 11.8 mmol) in THF/CH_2Cl_2 (100 mL + 30 mL) mixed with *N*-hydroxypyrrolidine-2,5-dione (1.5 g, 13 mmol) was cooled to 0 °C, and DCC (2.7 g, 13 mmol) in THF (80 mL) was added dropwise. The mixture was stirred for 2 h under ice cooling and then overnight at r.t. L-(N^{ω}-FMOC)-lysine **(55)** (5.2 g, 13.5 mmol) in THF/H_2O (200 mL + 100 mL) was added. After cooling to 0 °C, NMM (1.6 mL, 14.2 mmol) in THF (50 mL) was added slowly. The reaction was completed by stirring for 2 h under ice cooling and then overnight at r.t. After evaporation, the residue was partitioned between EtOAc and 10% aq citric acid. The organic phase was washed with H_2O, dried ($MgSO_4$), and concentrated. Treatment with Et_2O gave crystalline **54**, yield: 8.0 g, (85%).

^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.10 (d, 1H), 8.00 (d, 1H), 7.95–7.10 (m, 17H), 5.55 (d, 1H), 4.40–4.10 (m, 7H), 3.90 (t, 1H), 3.60 (t, 1H), 2.95 (br t 2H), 2.00–1.00 (m, 10H), 0.95–0.70 (m, 12H).

^{13}C{^{1}H} NMR (400 MHz, DMSO-d_6): δ = 173.5 (s), 172.1 (s), 171.3 (s), 156.4 (s), 156.2 (s), 144.2 (s), 143.8 (s), 140.8 (s), 127.7 (d), 127.6 (d), 127.1 (d), 125.4 (d), 125.3 (d), 125.2 (d), 120.1 (d), 65.9 (t), 65.3 (t), 60.7 (d), 52.0 (d), 50.5 (d), 46.9 (d), 46.7 (d), 40.6 (t), 33.3 (t), 30.7 (t), 30.2 (d), 29.1 (t), 24.5 (d), 24.2 (q), 23.2 (q), 22.9 (t), 19.2 (q), 18.5 (q).

D-(*N*-9-Fluorenylmethoxycarbonylalanyl)-L-phenylalanyl-L-(N^{ω}-9-Fluorenylmethoxycarbonyllysine (56):
Compound **56** was prepared by the same procedure as described for **54** using **53** and **55** as starting materials, white solid, yield: 5.6 g (63%).

^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.81 (d, 1H), 8.10 (d, 1H), 7.88–7.13 (m, 22H), 5.60 (d, 1H), 4.60 (q, 1H), 4.30–4.00 (m, 7H), 3.35 (m, 1H), 3.10–2.90 (m, 2H), 3.00 (br t, 2H), 1.70–1.00 (m, 6H), 0.98 (d, 3H).

^{13}C{^{1}H} NMR (400 MHz, DMSO-d_6): δ = 175.2 (s), 173.3 (s), 172.3 (s), 156.1 (s), 155.6 (s), 143.9 (s), 140.7 (s), 137.6 (s), 129.2 (d), 127.8 (d), 127.5 (d), 127.0 (d), 126.1 (d), 125.4 (d), 125.3 (d), 125.1 (d), 120.0 (d), 65.7 (t), 65.2 (t), 53.2 (d), 52.1 (d), 50.1 (d), 46.8 (d), 46.6 (d), 43.2 (t), 33.3 (t), 30.7 (t), 28.9 (t), 22.8 (t), 18.0 (q).

Anal.: Calcd for $C_{48}H_{48}N_4O_8$: C, 71.3; H, 6.0; N, 6.9. Found C, 70.3; H 6.5; N, 6.8.

1-(*N*-tert-Butoxycarbonyl-*N*-methyl)amino-2-[D-(*N*-9-fluorenylmethoxycarbonylvalyl)-L-leucyl-L-(N^{ω}-9-fluorenylmethoxycarbonyllysyl)amino]ethane (57):
Compound **57** was prepared by the same procedure as described for **54**, through the *N*-hydroxypyrrolidine-2,5-dione ester (3 g, 3.7 mmol). A solution of **36** (970 mg, 5.6 mmol) in THF (20 mL) was added into the solution containing active ester. The reaction was completed after stirring for 2 h at 0 °C and overnight at r.t. After evaporation the residue was dissolved in EtOAc, washed with 10% citric acid and H_2O, and dried ($MgSO_4$). Concentration of the organic solution gave **57** as colorless crystals, yield: 2.8 g (78%).

^{1}H NMR (90 MHz, DMSO-d_6): δ = 8.20 (br d, 1H), 8.05–7.00 (m, 19H), 5.50 (br d, 1H), 4.40–3.40 (m, 9H), 3.15–3.00 (m, 6H), 2.65 (s, 3H), 1.95–1.10 (m, 10H), 1.40 (s, 9H), 1.00–0.65 (m, 12H).

Anal.: Calcd for $C_{55}H_{70}N_6O_9$: C, 68.9; H, 7.4; N, 8.7. Found C, 68.3; H 7.7; N, 8.8

1-Methylamino-2-(D-(*N*-9-fluorenylmethoxycarbonylvalyl)-L-leucyl-L-(N^{ω}-9-fluorenylmethoxycarbonyl)lysylamino]ethane Hydrochloride (58):
Compound **57** (2 g, 2.1 mmol) was treated in CH_3OH (200 mL) with 2 M methanolic HCl (10 mL). The mixture was stirred for about 20 h and evaporated. Recrystallization of the crude product from CH_3OH/Et_2O gave **58** as colorless crystals, yield: 1.5 g (77%).

^{1}H NMR (400 MHz, DMSO-d_6): δ = 9.03 (br s, 2H), 8.58 (br d, 1H), 8.10 (br s, 1H), 7.96–7.34 (m, 19H), 4.30–4.14 (m, 7H), 3.92 (m, 2H), 3.40 (q, 2H), 3.10–2.97 (m, 4H), 2.90 (s, 3H), 2.02–1.28 (m, 10H), 0.95–0.89 (m, 12H).

Anal.: Calcd for $C_{50}H_{63}ClN_6O_7$: C, 67.1; H, 7.1; N, 9.4. Found C, 66.3; H 6.9; N, 9.2.

1-(*N*-*tert*-Butoxycarbonyl-*N*-methyl)amino-2-[D-(*N*-9-fluorenylmethoxycarbonylalanyl)-L-phenylalanyl-L-(N^{ω}-9-fluorenylmethoxycarbonyllysyl)amino]ethane (59):
Compound **56** (5 g, 6.2 mmol) in THF/CH_3OH (600 mL + 120 mL) was treated with EEDQ (3.1 g, 12.4 mmol) in THF (80 mL) and mixed dropwise with a solution of **36** (1.4 g, 8.1 mmol) in THF (60 mL). The mixture was stirred overnight and evaporated. Chromatography of the crude product on silica gel (CH_2Cl_2/CH_3OH, 12:1) gave **59** as colorless crystals, yield: 3 g (50%).

^{1}H NMR (90 MHz, $CDCl_3$): δ = 7.80–6.90 (m, 25H), 5.20 (br d, 1H), 4.80–3.80 (m, 9H), 3.60–3.20 (m, 4H), 3.10–2.90 (m, 4H), 2.80 (s, 3H), 2.00–1.50 (m, 6H), 1.40 (s, 9H), 1.10 (d, 3H).

Anal.: Calcd for $C_{56}H_{64}N_6O_9$: C, 69.7; H, 6.7; N, 8.7. Found C, 69.5; H 6.8; N, 9.4.

1-Methylamiono-2-(D-(*N*-9-fluorenylmethoxycarbonylalanyl)-L-phenylalanyl-L-(N^{ω}-9-fluorenylmethoxycarbonyllysyl)amino]ethane Hydrochloride (60):
Compound **59** (3 g, 3.1 mmol) in CH_2Cl_2/CH_3OH (120 mL + 10 mL) was stirred with 2 M methanolic HCl (30 mL) for 40 h. After evaporation recrystallization of the crude product from CH_3OH/Et_2O gave colorless crystals, yield: 2.1 g (74%).

^{1}H NMR (90 MHz, $CDCl_3/CD_3OD$, 1:1): δ = 8.60 (br s, 2H), 7.80 (br s, 1H), 7.70–7.00 (m, 24H), 4.70–3.90 (m, 9H), 3.80–3.50 (m, 2H), 3.25–2.90 (m, 6H), 2.65 (s, 3H), 2.10–1.10 (m, 6H), 0.95–0.75 (d, 3H).

Anal.: Calcd for $C_{51}H_{56}N_6O_7$: C, 67.9; H, 6.4; N, 9.3. Found C, 67.4; H 6.3; N, 8.4.

N-(2-Chloroethyl)-N'-methyl-N'-{2-[D-(N-9-fluorenylmethoxycarbonylvalyl)-L-leucyl-L-(N^{ω}-9-fluorenylmethoxycarbonyllysyl)amino]-ethyl}-N-nitrosourea (61):

A solution of **58** (1.5 g, 1.7 mmol) in THF/CH_3OH (each 100 mL) was mixed with **31** (860 mg, 3.4 mmol). The mixture was cooled to 0 °C and then treated with Hünig's base (0.6 mL, 3.4 mmol) in THF (60 mL). After stirring for 2 h at 0 °C, the mixture was concentrated to about 20 mL. Addition of Et_2O gave **61** as pale yellow crystals, yield: 1.5 g (91%).

^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$, 5:1): δ = 7.77–7.28 (m, 20H), 5.93 (br d, 1H), 4.45–4.30 (m, 2H), 4.35 (d, 4H), 4.28–4.21 (m, 1H), 4.19–4.17 (m, 1H), 4.09 (t, 2H), 3.76–3.73 (m, 1H), 3.56 (t, 2H), 3.45 (q, 4H), 3.27–3.21 (m, 2H), 3.12 (s, 3H), 2.01–1.05 (m, 10H), 1.02–0.90 (m, 12H).

$^{13}C\{^1H\}$ NMR (400 MHz, $CDCl_3/CD_3OD$, 5:1): δ = 174.5 (s), 173.1 (s), 172.6 (s), 157.4 (s), 157.1 (s), 155.7 (s), 143.8 (s), 143.6 (s), 143.4 (s), 141.2 (s), 127.7 (d), 127.6 (d), 127.0 (d), 126.9 (d), 124.9 (d), 119.9 (d), 67.1 (t), 66.5 (t), 61.5 (d), 53.2 (d), 52.8 (d), 47.1 (d), 46.9 (d), 42.5 (t), 40.4 (t), 39.6 (t), 39.5 (t), 36.9 (t), 30.4 (t), 29.8 (d), 29.0 (t), 25.4 (t), 24.8 (d), 23.1 (q), 23.0 (t), 20.6 (q), 19.1 (q), 18.9 (q), 18.8 (q).

Anal.: Calcd for $C_{53}H_{65}ClN_8O_9$: C, 64.1; H, 6.6; N, 11.3. Found C, 63.8; H 6.6; N, 11.0.

N-(2-Chloroethyl)-N'-methyl-N'-[2-(D-valyl-L-leucyl-L-lysylamino)-ethyl]-N-nitrosourea (62):

Compound **61** (1.5 g, 1.5 mmol) in CH_2Cl_2 and CH_3OH (20 mL + 8.5 mL) was stirred with piperidine (1.5 mL, 15 mmol) for 5 h at r.t. Et_2O was added until the solution became turbid. Precipitated **62** was washed with CH_2Cl_2 (2 mL)/Et_2O (1:2) and dried in vacuo, pale yellow solid, yield: 540 mg (66%).

^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$, 5:1): δ = 7.65 (d, 1H), 7.60 (d, 1H), 7.25 (d, 1H), 7.20 (t, 2H), 6.12 (t, 2H), 4.60 (m, 1H), 4.18 (t, 1H), 4.13 (m, 1H), 3.98 (t, 2H), 3.46 (t, 2H), 3.44–3.38 (m, 2H), 3.11–3.08 (m, 1H), 3.03 (s, 3H), 2.97 (m, 2H), 2.51 (t, 2H), 1.88–1.11 (m, 10H), 0.82–0.73 (m, 12H).

$^{13}C\{^1H\}$ NMR (400 MHz, $CDCl_3/CD_3OD$, 5:1) δ = 176.0 (s), 173.3 (s), 172.9 (s), 156.2 (s), 60.6 (d), 53.5 (d), 52.4 (d), 42.9 (t), 41.1 (t), 40.7 (t), 40.1 (t), 37.4 (t), 32.1 (t), 31.7 (t), 31.6 (d), 25.1 (d), 24.3 (t), 23.1 (t), 22.9 (q), 21.5 (q), 19.5 (q), 17.1 (q), 16.6 (q).

N-(2-Chloroethyl)-N'-methyl-N'-{2-[D-(N-9-fluorenylmethoxycarbonylalanyl)-L-phenylalanyl-L-(N^{ω}-9-fluorenylmethoxycarbonyllysyl)amino]ethyl}-N-nitrosourea (63):

A solution of **60** (2 g, 2.2 mmol) in THF and CH_3OH (100 mL + 50 mL) was neutralized to pH 7 with Hünig's base and mixed with **31** (1.1 g, 4.4 mmol) in THF (50 mL). The mixture was cooled to 0 °C, treated with Hünig's base (0.8 mL, 4.4 mmol) in THF (80 mL), and stirred for 150 min under ice cooling. After evaporation and addition of Et_2O **63** was crystallized, pale yellow crystals, yield: 2 g (92%).

^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$, 10:1): δ = 7.80–7.15 (m, 25H), 6.20 (m, 1H), 4.50 (q, 1H), 4.44–4.25 (m, 7H), 4.09 (t, 2H), 3.56 (t, 2H), 3.25 (m, 1H), 3.17–3.05 (m, 6H), 2.95 (m, 2H), 2.74 (s, 3H), 1.90–1.25 (m, 6H), 1.15 (d, 3H).

$^{13}C\{^1H\}$ NMR (400 MHz, $CDCl_3/CD_3OD$, 10:1): δ = 174.3 (s), 172.2 (s), 171.4 (s), 156.8 (s), 155.6 (s), 143.7 (s), 143.5 (s), 141.1 (s), 136.1 (s), 128.8 (d), 128.4 (d), 127.6 (d), 127.5 (d), 126.8 (d), 124.8 (d), 124.4 (d), 119.8 (d), 67.8 (t), 66.3 (t), 54.7 (d), 53.2 (t), 50.5 (d), 50.1 (d), 47.0 (d), 46.7 (d), 42.4 (t), 40.2 (t), 39.6 (t), 36.5 (t), 36.3 (t), 30.5 (t), 28.9 (t), 25.3 (t), 17.6 (q), 17.1 (q).

Anal.: Calcd for $C_{54}H_{59}ClN_8O_9$: C, 64.9; H, 5.9; N, 11.2. Found C, 64.3; H 5.6; N, 10.0

N-(2-Chloroethyl)-N'-methyl-N'-[2-(D-alanyl-L-phenylalanyl-L-lysylamino)ethyl]-N-nitrosourea (64):

Compound **64** was prepared by the same procedure as described for **62** using **63** as starting material, pale yellow solid, yield: 60 mg (72%).

^{1}H NMR (400 MHz, CD_3OD): δ = 7.78 (d, 1H), 7.72 (d, 1H), 7.38–7.20 (m, 8H), 6.12 (t, 2H), 4.60 (q, 1H), 4.28 (m, 1H), 4.08 (t, 2H), 3.60 (t, 2H), 3.49–3.41 (m, 2H), 3.21–3.18 (m, 1H), 3.14 (s, 3H), 3.05 (m, 2H), 2.95–2.84 (m, 4H), 1.74–1.65 (m, 6H), 1.07 (d, 3H).

$^{13}C\{^1H\}$ NMR (400 MHz, CD_3OD): δ = 178.1 (s), 174.1 (s), 173.7 (s), 157.3 (s), 138.4 (s), 130.3 (d), 129.5 (d), 127.9 (d), 56.1 (d), 54.6 (d), 51.5 (d), 47.9 (t), 45.9 (t), 45.1 (t), 43.7 (t), 41.0 (t), 40.8 (t), 40.5 (t), 38.5 (t), 32.3 (t), 29.2 (t), 24.3 (t), 21.6 (q), 21.2 (q).

Anal.: Calcd for $C_{24}H_{39}ClN_8O_5$: C, 52.0; H, 7.1; N, 20.2. Found C, 51.7; H 6.9; N, 19.5.

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