Advances in Brief

Extracellularly Tumor-activated Prodrugs for the Selective Chemotherapy of Cancer: Application to Doxorubicin and Preliminary in Vitro and in Vivo Studies

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Abstract

Oligopeptidic derivatives of anthracyclines unable to penetrate cells were prepared and screened for their stability in human blood and their reactivation by peptidases secreted by cancer cells. N-β-alanyl-L-leucyl-L-leucyl-doxorubicin was selected as a new candidate prodrug. The NH₂-terminal β-alanine allows a very good blood stability. A two-step activation by peptidases found in conditioned media of cancer cells ultimately yields N-i-leucyl-doxorubicin. In vitro, when MCF-7/6 cancer cells are exposed to the prodrug, they accumulate about 14 times more doxorubicin than MRC-5 normal fibroblasts, whereas when exposed to doxorubicin the uptake is slightly higher in fibroblasts than in MCF-7/6 cells. This increased specificity of the prodrug over doxorubicin was confirmed in cytotoxicity assays using the same cell types. In vivo, the prodrug proved about nine times less toxic than doxorubicin in the normal mouse and also much more efficient in two different experimental chemotherapy models of human breast tumors.

Introduction

Chemotherapy remains the major systemic treatment of malignant diseases. The effectiveness of chemotherapy is however greatly limited by the severe side effects of the cytotoxic agents used, as well as by the development of resistance. Both of these limitations explain the low cure rates achieved in most instances (1). It is clear that increasing the selectivity of the available cytotoxic agents by delivering them specifically to malignant cells would reduce the toxicity of chemotherapy. This would allow the use of much higher doses of the drugs and/or more frequent treatments. As a result, increased tumor exposure to the cytotoxic agent would increase treatment efficacy. Furthermore, increased tumor concentrations of the cytotoxic could, at least in certain cases, overcome resistance to treatment. One approach to achieve such a goal consists in developing prodrugs of anticancer agents that are activated only in the vicinity of or within tumor masses. A number of different strategies have been used for many years and are still being used to develop such prodrugs (reviewed in Ref. 2), but as of today however, not a single compound has been approved for clinical use. One of the approaches is based on the activation of prodrugs by tumor-associated enzymes, particularly peptidases, a number of which, such as plasmin, are known to participate and collaborate in tumor invasion and metastasis (3–6). The most promising results published thus far were obtained with two peptidic derivatives of Dox³ that target selectively prostate cancer cells through cleavage by prostate-specific antigen (7–9). On the basis of our previous experience with L-Dox (10–14), we tried to develop a new compound that would meet the criteria we consider essential for the development of a successful prodrug. The ideal prodrug (a) should be stable in blood and body fluids; (b) should be unable to enter cells as such; and (c) should be activated by enzymes specifically released by solid tumor cells. This concept of extracellularly tumor-activated prodrugs (ETAP) would of course ensure a very low toxicity. Rather than to go after a known peptidase specific of a given tumor type, we preferred to use a more empirical approach. Various peptide conjugates of our model drug, Dox, were screened for their stability in whole blood and their activation by enzymes released by cancer cell lines. A tetrapeptidic derivative, N-β-alanyl-L-leucyl-L-leucyl-L-leucyl-Dox, was identified as a candidate ETAP prodrug. Because this compound overcomes two major limitations of L-Dox (i.e., instability in blood and ability to freely diffuse inside cells), it was nicknamed SLD.

Materials and Methods

Drugs, Amino Acids, Peptides, and Conjugates. Dox was obtained from Meiji Seika Kaisha Ltd. (Tokyo, Japan). Amino acids were obtained from Novabiochem (Laußel, Switzerland) and N-Fmoc-β-alanyl-L-leucyl-L-leucyl-L-leucine was custom synthesized by UCB-Bioproducts (Braine-l’Alleud, Belgium). Amino acid and peptide conjugates of Dox were synthesized by coupling the carboxyl group of Fmoc-protected amino acids or peptides to the free amino group of the anthracycline. O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (Aldrich, Milwaukee, WI) was used as the coupling agent. Fmoc-protecting groups were removed using piperidine (Fluka, Buchs, Switzerland) as described previously (15), except that the reaction was quenched with a 10% (w/v; pH 3) citrate buffer. Structure of the prodrugs was confirmed by mass spectrometry, elemental, and nuclear magnetic resonance analyses.

Cell Lines. The MCF-7/6 human breast cancer cell line was received from Prof. M. M. Mareel (University of Ghent, Belgium). The MRC-5 human normal fibroblast strain was a kind gift from Dr. Luc Fabry (SmithKline Beecham, Rixensart, Belgium). Both cell types were routinely cultured in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% (v/v) FCS (Life Technologies, Inc.). Conditioned media were prepared by incubating subconfluent cultures for 24 h in a serum-free medium made of a 1:1 mixture of DMEM and Ham’s F12 supplemented with 200 μg/ml BSA (receptor grade; Serva, Heidelberg, Germany), 1 μg/ml insulin (Novo, Bagsvaerd, Denmark), and 1 μg/ml transferrin (Sigma, St. Louis, MO). After recovery, the conditioned media were cooled down to 4°C, buffered, centrifuged to remove cells and debris, concentrated 20-fold by ultrafiltration, and used immediately.

³ The abbreviations used are: Dox, doxorubicin; L-Dox, N,I-leucyl-Dox; Fmoc, 9-fluorenylmethoxycarbonyl; ETAP, extracellularly tumor-activated prodrug; HPLC, high-performance liquid chromatography; RTV, relative tumor volume; SLD, Super-Leu-Dox; T:C ratio, treated to control ratio.
Blood Stability Studies. Blood from healthy human donors was collected in citrated tubes and used immediately. The Dox conjugates were incubated at 37°C in whole blood at a final concentration of 17.24 μM. At selected time points, three 25-μl aliquots were removed, and conjugates and their metabolites were extracted. The samples (in a final volume of 500 μl) were added to tubes containing 1.8 ml of a 4:1 mixture of chloroform and methanol (Labscan, Dublin, Ireland). One hundred microliters of a freshly prepared solution of the internal standard (N-prolyl-daunorubicin, 345 μμM) were added to allow quantification, followed by 600 μl of a 0.5 M borate buffer (pH 9.8). The tubes were vigorously shaken, centrifuged, and the organic layer was collected. After solvent evaporation and dissolution of the residue in 500 μl of a 70:30 mixture of 0.1% (w/v) ammonium formate (pH 4.0) and acetonitrile (Labscan), all samples were filtered (0.22 μm). HPLC analysis was performed with Super-ODS reverse phase columns (4.6 × 100 mm; TosohHaas) under isocratic conditions [30% acetonitrile (v/v), 0.1% trifluoroacetic acid (v/v) in water] with a flow rate of 1.5 ml/min for 6.5 min. Fluorescence detection (λex = 480 nm; λem = 560 nm) was used, and the drugs and metabolites were identified according to their respective relative retention times as determined from a set of standards.

Activation by Conditioned Media. Conjugates were incubated at 37°C in MCF-7/6 cell conditioned media, and the generation of metabolites as a function of time was analyzed as in the case of blood stability studies.

Uptake Studies. Subconfluent cultures of MCF-7/6 and MRC-5 cells were incubated at 37°C in the presence of 17.24 μM of Dox, L-Dox, or SLD for up to 24 h. At selected time points, three flasks were removed in each group. The cells were immediately washed three times with ice-cold PBS (2.8 mM NaH2PO4, 7.2 mM Na2HPO4, 150 mM NaCl, pH 7.4). They were recovered in 1 ml of cold PBS using a cell scraper, and after a 30-s ultrasonication at 100 W, drugs and metabolites were extracted and quantified as described above.

Cytotoxicity Assays. Twenty-four hours after seeding, MCF-7/6 and MRC-5 cells were exposed to increasing concentrations (0.17 nM to 1.2 mM) of standards.

Activity Studies. MCF-7/6 estrogen-responsive human breast tumor samples were filtered (0.22 μm). HPLC analysis was performed with Super-ODS reverse phase columns (4.6 × 100 mm; TosohHaas) under isocratic conditions [30% acetonitrile (v/v), 0.1% trifluoroacetic acid (v/v) in water] with a flow rate of 1.5 ml/min for 6.5 min. Fluorescence detection (λex = 480 nm; λem = 560 nm) was used, and the drugs and metabolites were identified according to their respective relative retention times as determined from a set of standards.

Results and Discussion

Selection of a Candidate Prodrug. One limitation of L-Dox was its ability to enter any cell type, normal as well as tumoral. Its increased safety and potency was very likely the result of a higher accumulation of the free drug in tumor cells as compared to normal cells because of higher intracellular leucine aminopeptidase activities in the former (16). This suggested that a better prodrug could be generated if L-Dox could be prevented from entering normal cells. Of course, these prodrugs would have to be stable in blood as well as in body fluids and normal tissues, but should be activated into L-Dox in the vicinity of tumor cells. To achieve this, we synthesized a number of peptide (three to five residues) conjugates of Dox, keeping a dipeptidyl derivative Ala-Leu-Dox. L-Dox progressively appears later on, which might suggest a two-step extracellular activation of the candidate prodrug. Interestingly, the parent drug Dox is never detected in conditioned media, even when these experiments are carried out for longer periods of time.

In Vitro Specificity for Tumor Cells. SLD was then compared to Dox and L-Dox in uptake studies performed with MCF-7/6 breast cancer cells and with MRC-5 normal fibroblasts. All drugs were used at equimolar concentrations. Over 24 h, Dox accumulates slightly more in fibroblasts as compared to MCF-7/6 cancer cells (Fig. 2). Between 6 and 24 h, no increase in the intracellular concentration is.
AN EXTRACELLULARLY TUMOR-ACTIVATED PRODRUG OF DOXORUBICIN

The improved specificity of SLD for tumor cells was confirmed in \textit{in vitro} cytotoxicity assays. Fig. 3 compares the ratio of IC\textsubscript{50} values of Dox, L-Dox, and SLD in MRC-5 normal fibroblasts versus MCF-7/6 breast cancer cells. Interestingly, and as expected, this ratio is about three times higher in the case of SLD as compared to L-Dox and more than four times higher as compared to the parent drug Dox.

\textit{In Vivo Toxicity.} SLD was then compared to L-Dox and Dox in \textit{in vivo} toxicity assays. The LD\textsubscript{50} values were estimated 28 days after the initiation of i.p. treatment of OF-1 male mice with increasing dose levels of the three compounds. All drugs were administered once a day for 5 consecutive days. \textit{In vivo} also, SLD is much less toxic than Dox or even L-Dox. With the dosing protocol used here, SLD is close to nine times less toxic than Dox with cumulative LD\textsubscript{50} values of 181 and 20.5 \(\mu\text{mol/kg}\), respectively. It is also about four times less toxic than L-Dox. These results are in good agreement with the \textit{in vitro} results and the prodrug nature of SLD. Although this remains to be demonstrated formally, based on the \textit{in vitro} results, the reduced

![Graph](image-url)

Fig. 2. MCF-7/6 human breast cancer cells and MRC-5 normal fibroblasts were cultured for up to 24 h in the presence of 17.24 \(\mu\text{mol}\) Dox, L-Dox, or \(\text{N'}\text{-alanyl-l-leucyl-l-alanyl-l-leucyl-Dox}\) (SLD). At selected time points, three flasks from each group were removed from the incubator, cells were washed and homogenized prior to extraction, and HPLC analysis of drugs and metabolites was performed as described in “Materials and Methods.” Results from a typical experiment. Data points represent mean amount of drug or metabolite detected per mg cell protein \(\pm SD\) \((n = 3)\). \(\bullet\), \(\text{N'-alanyl-l-leucyl-l-leucyl-Dox}\); \(\square\), \(\text{N'-l-leucyl-l-leucyl-Dox}\); \(\triangle\), L-Dox; and \(\mathbf{m}\), Dox.

![Graph](image-url)

Fig. 3. Differential cytotoxicity of Dox, L-Dox, or \(\text{N'}\text{-alanyl-l-leucyl-l-alanyl-l-leucyl-Dox}\) (SLD) for MCF-7/6 cancer cells as compared to MRC-5 fibroblasts. The IC\textsubscript{50} ratio of the different drugs for MRC-5 fibroblasts versus MCF-7/6 cells is presented.

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Fig. 4. Experimental chemotherapy with \(\text{N'-alanyl-l-leucyl-l-alanyl-l-leucyl-Dox}\) (SLD) as compared to Dox in two different models of human breast cancer. Athymic mice bearing established MCF-7/6 and MAXF-1162 tumors (at least 100 mm\(^3\)) were treated with five consecutive daily i.p. injections of either Dox or \(\text{N'-alanyl-l-leucyl-l-alanyl-l-leucyl-Dox}\). The evolution of median RTVs in the different treatment groups is presented. \(\bullet\), controls; \(\bigcirc\), 1.7 \(\mu\text{mol/kg}\) Dox; \(\times\), 2.1 \(\mu\text{mol/kg}\) Dox; \(\square\), 2.6 \(\mu\text{mol/kg}\) Dox; \(\bigcirc\), 34.5 \(\mu\text{mol/kg}\) SLD; and \(\mathbf{m}\), 51.7 \(\mu\text{mol/kg}\) SLD. * two mice of five died during the study in these groups.

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toxicity is presumed to be attributable to the inability of the prodrug to enter cells combined to a relatively good systemic stability. These results indicate that significantly higher doses of SLD can be administered safely to treat tumors.

**In Vivo Activity of the Prodrug against Human Breast Tumors.** Fig. 4 shows the results of the experimental chemotherapy of two human breast tumor types implanted s.c. in the flanks of athymic mice. MCF-7/6 and MAXF-1162 tumors were used, and i.p. treatment was not initiated before tumor volume reached at least 100 mm³. Tumor growth curves in the different treatment groups are presented. Mice bearing MCF-7/6 tumors were treated once daily for 5 consecutive days with 2.1 μmol/kg Dox or with 51.7 μmol/kg SLD. This latter dose level is higher than the LD₅₀ value as previously determined in normal OF-1 male mice, but in the tumor-bearing, athymic mice, it did not prove toxic. No mortality was observed and the animals lost a maximum of 12% only of their body weight by day 9 after initiation of treatment. Body weight then progressively increased up to the original value by day 50. Whereas Dox treatment clearly does not slow down MCF-7/6 tumor growth, SLD does. The effect is particularly marked after day 30 and a minimal T:C ratio of about 45% is reached on day 50. This five consecutive i.p. injection dosing protocol was also used for the treatment of MAXF-1162 tumors. No mortality was observed in the dose group treated with 34.5 μmol/kg SLD/injection, but two animals of five died in both Dox groups (1.7 and 2.6 μmol/kg). In this model also, SLD proved clearly more effective than the parent drug Dox (Fig. 4). SLD completely blocked tumor growth and allowed a minimum T:C ratio of tumor volumes of about 14% (reached on day 28). Dox showed more toxicity and allowed only a minimum T:C ratio of 55% in the 2.6-μmol/kg dose group, also reached on day 28.

**Conclusion**

Based on the very simple ETAP concept and a straightforward screening methodology, we developed a new class of peptidic prodrugs of anticancer agents. The model compound, N-β-alanyl-L-leucyl-L-leucyl-Dox or SLD, is relatively insensitive to blood peptidases, is poorly absorbed by cells as such, and is cleaved into Ala-Leu-Dox and then into L-Dox by unknown enzymes secreted by cancer cells. L-Dox is a well-known prodrug of Dox that freely diffuses inside cells where it is activated into the fully active drug. Finally, the prodrug nature of SLD was confirmed *in vivo* by its reduced toxicity and enhanced activity in two experimental models. Further efficacy and mechanistic studies will determine whether this new compound deserves clinical development. Studies are also under way to identify and characterize the enzyme(s) responsible for SLD activation. One potential advantage of our compound over other interesting prodrugs of this type (8, 9) is that it is expected to be active on different tumor types. Furthermore, because of the extracellular activation, a bystander effect can also be anticipated.

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**References**


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