Improved Therapeutic Efficacy of Doxorubicin through Conjugation with a Novel Peptide Drug Delivery Technology (Vectocell)

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Improvement in the therapeutic index of doxorubicin, a cytotoxic molecule, has been sought through its chemical conjugation to short (15−23 amino acid) peptide sequences called Vectocell peptides. Vectocell peptides are highly charged drug delivery peptides and display a number of characteristics that make them attractive candidates to minimize many of the limitations observed for a broad range of cytotoxic molecules. The studies reported here characterized the in vitro and in vivo efficacy of a range of Vectocell peptides conjugated to doxorubicin through different linkers. These studies show that the in vivo therapeutic index of doxorubicin can be improved by conjugation with a specific Vectocell peptide (DPV1047) through an ester linker to C14 of doxorubicin, in both colon and breast tumor models. This conjugate was also shown to have significant in vivo antitumoral activity in a model resistant to doxorubicin, suggesting that this conjugate is able to circumvent the multidrug resistance (MDR) phenotype. These experiments therefore provide support for the use of the Vectocell technology with other cytotoxic agents.

Introduction

The objective of this research was to assess the in vivo therapeutic benefit of novel peptidic−doxorubicin conjugates in both drug-sensitive and drug-resistant cancer models. Vectocell peptides (or DPV for Diatos Peptide Vectors) are short (15−23 amino acid) peptide sequences that are able to transport molecules across the cell membrane via endocytosis and have been developed as tools for intracellular delivery of a broad range of therapeutic molecules.[1−3] These peptides originate from human heparin binding proteins and/or anti-DNA antibodies and, once conjugated to a therapeutic molecule, can selectively deliver the molecule to either the cytoplasm or the nuclei of mammalian cells.[4,5]

The conjugation of Vectocell peptides to cytotoxic molecules can modify the in vivo distribution of the therapeutic molecules, improving pharmacokinetic properties and/or reducing systemic toxicity by driving tissue and intracellular delivery.[2,5] In addition, conjugation of Vectocell peptides to small molecules/drugs may also provide a means of inhibiting the extracellular export of the therapeutic agents by proteins involved in multidrug resistance (MDR).[6]

Multidrug resistance can seriously limit cancer chemotherapy treatment, for example, through the overexpression of membrane transporters that mediate unidirectional energy-dependent drug efflux, thus reducing intracellular drug levels. These membrane transporters are normally expressed in high levels within cells involved in detoxification, such as the liver, kidney, and colon.[7,8] Tumors arising from these cells are often resistant to chemotherapy treatment from their onset, while other tumors can acquire resistance through the induction of MDR transport proteins during treatment.[8−10] Many inhibitors of MDR transporters have been identified. However, these inhibitors also interfere with the clearance of the cytotoxic drug, resulting in elevated plasma concentrations of the cytotoxic agent and associated toxicity.[11] An alternative approach is to circumvent rather than to directly inhibit MDR mechanisms, by developing anticancer therapies that are not substrates for extracellular export.

Doxorubicin, an anthracycline antibiotic, remains among the most widely used cytotoxic agents for the treatment of a broad spectrum of cancers, including breast, stomach, non-Hodgkin's lymphoma, and bladder cancer.[12,13] As with many cytotoxic drugs, doxorubicin has severe short- and long-term side effects, in this case mostly associated with bone marrow and myocardial cell toxicity.[12,14] Cardiotoxicity limits the cumulative dosage of doxorubicin to 500−600 mg/m^2, which may be a dose at which tumor is still responding to treatment but for which no further doxorubicin treatment can be given.[15,16] Another drawback of doxorubicin is the emergence of drug resistance that results in the reduction of the intracellular concentration of doxorubicin.[17]

The present study aimed to generate novel peptidic−doxorubicin conjugates by use of three Vectocell peptides that differ in terms of their charge, size, and intracellular delivery characteristics and to assess their ability to enhance the therapeutic potential of doxorubicin and to prevent the appearance of MDR. Different conjugation sites and linkers of different stabilities were used to generate Vectocell−doxorubicin conjugates in order to evaluate their effect on efficacy. Chemical routes were developed to allow the conjugation of doxorubicin to Vectocell peptides through ester, thioether, and amide chemical linkers. The ester and thioether involved carbon 14 of doxorubicin, and the amide carbon 3′. In vitro and in vivo characterization has defined the optimal conjugate−linker combination that significantly increases efficacy above unconjugated doxorubicin in both doxorubicin-sensitive and -resistant models. The data presented therefore provide in vivo proof of concept for the use of Vectocell peptides to improve the therapeutic index of doxorubicin, and potentially many other cytotoxic or small molecule anticancer drugs.

Results

Chemistry. The synthesis of three different types of doxorubicin conjugates covalently attached to Vectocell peptides has

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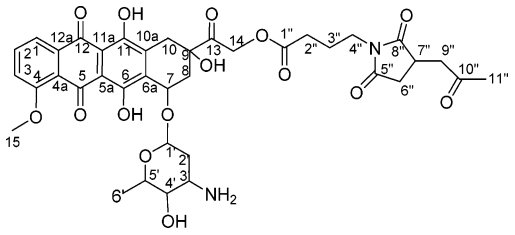


Figure 1. Acetone adduct byproduct of the 14-halodaunorubicin esterification.

been undertaken. Ester or thioether bonds have been used to attach the Vectocell peptide at position 14 (C_{14}, Figure 1), which should not inhibit the interaction of doxorubicin with DNA. The 3′ amino group (Figure 1) was used to conjugate the Vectocell peptide via an amide bond, which has the potential of inhibiting interaction of the amine group with DNA.[12,13]

One Vectocell−doxorubicin conjugation route has been suggested by the work of Horton et al.,[18] who described the synthesis of doxorubicin and 14-*O*-acetyldoxorubicin from daunorubicin via 14-bromodaunorubicin. This chemical route (Scheme 1) was shown to allow the generation of the desired doxorubicin−peptide conjugate, through either an ester bond (cleavable) or a thioether bond (noncleavable).

The first step of this synthesis (Scheme 1) was the halogenation of daunorubicin hydrochloride (**1**) on its 14 position[19] via the 14-bromo-13-dimethylketal-daunorubicin (**2**) intermediate, which was directly hydrolyzed (without purification) to 14-halodaunorubicin (**3**) (mixture of 14-bromo- and 14-chlorodaunorubicin) rather than the expected 14-bromodaunorubicin (Scheme 2) due to the use of sodium chloride during the extraction step (see Experimental Section). Halogen exchange can be prevented by using saturated sodium bromide instead of sodium chloride during this step. Analysis of the acetal intermediate showed the presence of 14-chlorodaunorubicin (9% of peak area), presumably arising because the daunorubicin starting material was a hydrochloride salt. It was possible to form exclusively 14-bromodaunorubicin by avoiding the presence of chloride ions.

14-Halodaunorubicin (**3**) (Scheme 2) was then acylated by use of sodium maleimidobutyrate (**5**) [previously obtained by treatment with sodium hydrogen carbonate of the parent acid (**4**)] to generate 14-doxorubicinyl maleimidobutyrate (**6**). One major impurity was formed and identified, by mass spectrometry, as an acetone adduct of 14-doxorubicinyl maleimidobutyrate. The byproduct obtained is not reactive in the following step and can be removed by extraction.

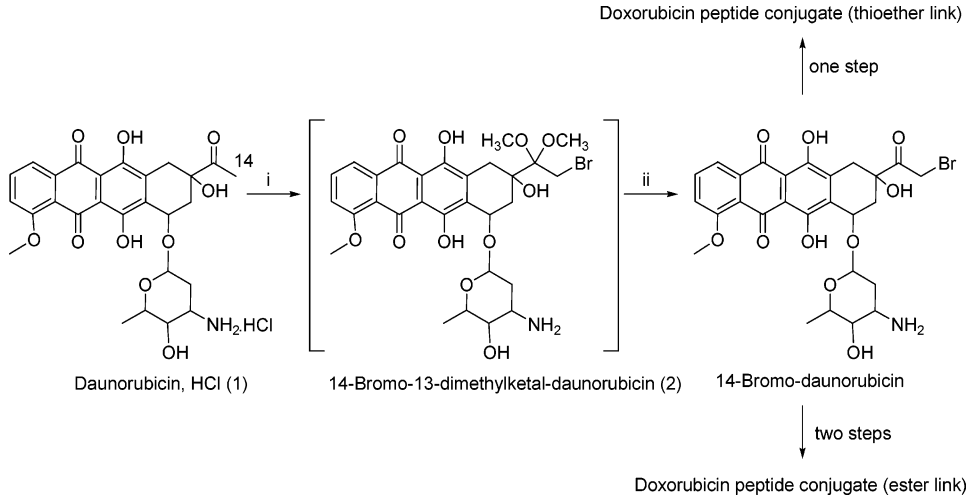
The next step consists of the coupling of 14-doxorubicinyl maleimidobutyrate (**6**) via its maleimido moiety with the thiol group of the peptide (**7a**−**c**) (see Experimental Section and Scheme 2), resulting in the desired peptide−doxorubicin ester conjugate (**8a**−**c**). A second route was investigated that consists of reacting halogenated daunorubicin (**3**) directly with the thiol moiety of the peptide (**7a**−**c**) as described in Scheme 2, thereby obtaining a more stable thioether conjugate (**9b**−**c**).

Doxorubicin peptide conjugates were also prepared from doxorubicin, in which the peptide is coupled on the 3′ amino group of the daunosamine sugar of the anthracycline (**10**) (Scheme 3). After conjugation of maleimidobutyric acid (**4**) to the amino group of doxorubicin (**10**), the activated anthacycline (**11**) was conjugated with the peptide (**7a,b**) through the thiol group as described above. Thus, peptide−doxorubicin amide conjugates (**12a,b**) were obtained.

Selection of the Optimal Conjugate. The Vectocell peptides (**7a**, **7b**, and **7c**; DPV1047, DPV10, and DPV7b, respectively) were conjugated to doxorubicin (**10**) via the three types of chemical bond described above, and the in vitro cytotoxicity of the synthesized compounds was evaluated in two doxorubicin-sensitive (HCT116, MCF7/6) tumor cell lines (Table 1). The Vectocell peptides alone showed no cytotoxic activity (data not shown[2]). The Vectocell−doxorubicin conjugates **8a**−**c** showed no loss of cytotoxicity compared to free doxorubicin. Compounds **9b** and **9c** showed a loss of activity in the HCT116 model but not in the MCF-7/6 cell model, compared to free doxorubicin (**10**). In contrast, the compounds **12a** and **12b** showed a significant loss of cytotoxicity in both tumor cell lines (loss of 1−2 logs in IC_{50}). The in vitro data, therefore, suggest that the ester (**8a**−**c**) and thioether (**9b** and **9c**) bonds are optimal for Vectocell−doxorubicin conjugate activity and that conjugation to the 3′ position via an amide bond (**12a** and **12b**) partially inactivates doxorubicin.

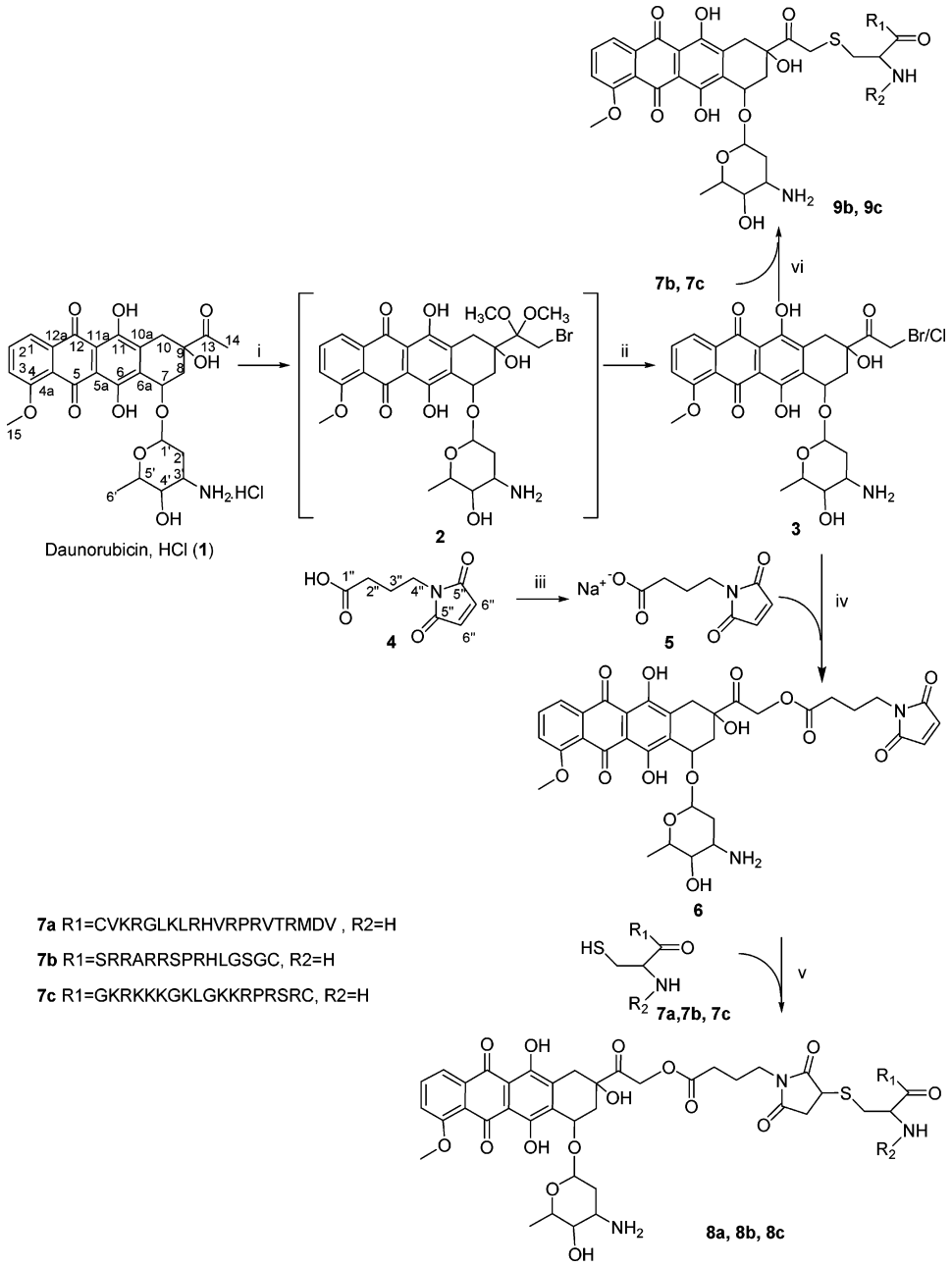
The in vitro cytotoxicity of the conjugates was also tested in a doxorubicin-resistant cell model (MCF7-Adr, Table 1). These experiments showed that it is possible to overcome the doxorubicin-resistant phenotype by conjugation of doxorubicin to Vectocell peptides. Vectocell peptides DPV1047 (**8a** and **12a**) and DPV10 (**8b** and **9b**) are able to inhibit the doxorubicin-

Scheme 1. Synthesis of Doxorubicin−Peptide Conjugates Starting from Daunorubicin[a]



[a] Reagents and conditions: (i) Trimethyl orthoformate, methanol, dioxane, Br_2, and then propylene oxide and diisopropyl ether; (ii) 48% HBr, acetone, and then NaCl.

Scheme 2. Synthesis of Doxorubicin−Peptide Conjugates in C_{14} Position[a]



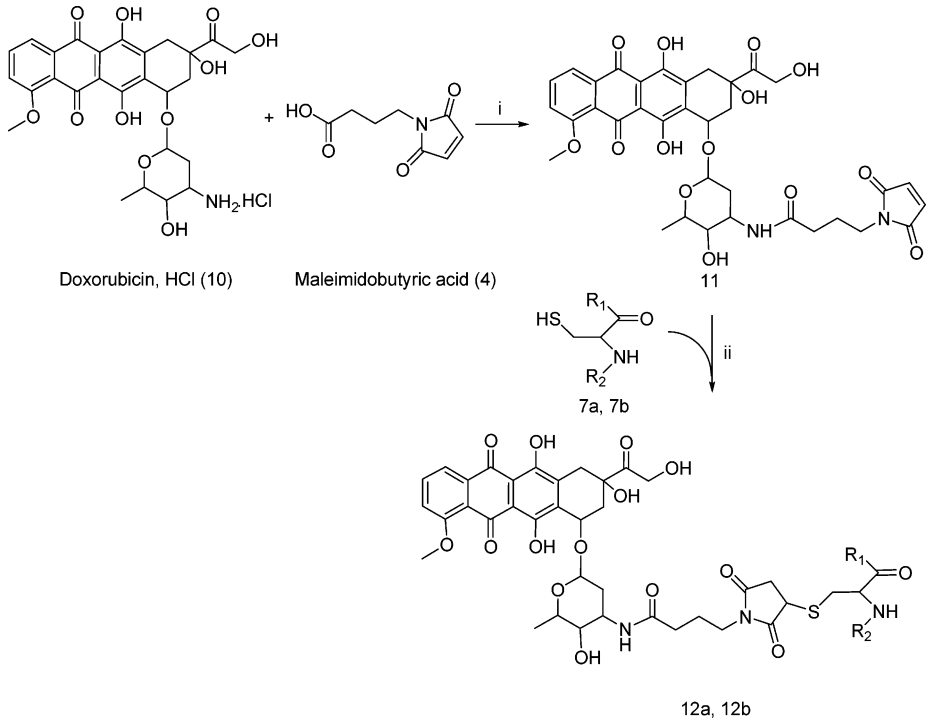
[a] Reagents and conditions: (i) Trimethyl orthoformate, methanol, dioxane, Br_2, and then propylene oxide and diisopropyl ether; (ii) 48% HBr, acetone, and then NaCl; (iii) $NaHCO_3$, H_2O; (iv) acetone, refluxed; (v) H_2O and then CH_2Cl_2, DMF; (vi) methanol, K_2CO_3, and then Tris-HCl buffer pH 9, $CHCl_3$, wherein R_1 = -OH or -NH-peptide; R_2 = -H or -CO-peptide.

resistant phenotype of MCF7-Adr cells. However, the compounds **8a** and **9b** exhibited the greatest cytotoxic activity in the doxorubicin-resistant cell model, although **9b** showed a loss of cytotoxic activity in the HCT116 in vitro model (Table 1). The in vitro data therefore suggested that the optimal conjugate for both doxorubicin-sensitive and -resistant models was **8a**.

The in vivo antitumoral efficacy of the different conjugates was compared in the human HCT116 colon carcinoma model (doxorubicin-sensitive) administered by intravenous injection (iv) (Table 1). The conjugates **8a**−**c** all showed activity; the most active was **8a**. Compound **12a** showed no activity and confirmed the in vitro data. In a second experiment, conjugates **9c**, **9b**, and **8a** were compared. Compound **9c** was completely inactive and **9b** was only partially active, which correlated with the reduction of in vitro cytotoxicity of both **9c** and **9b** compared to **8a** observed in the HCT116 model. The in vivo evaluation of the Vectocell−doxorubicin conjugates confirmed that **8a** (15 μmol/kg) is the optimal conjugate with a T/C value of 29.5% (Table 1), whereas T/C obtained with the other conjugates ranged from 34.1% (**8c**) to 86.2% (**9c**). Moreover, in this experiment, the conjugate **8a** (15 μmol/kg) showed better efficacy than doxorubicin (**10**, 6.5 μmol/kg: doxorubucin's maximal tolerated dose, MTD), with a T/C of 49.6%. It should be noted that it is possible to administer **8a** at twice the MTD of doxorubicin (**10**), demonstrating that **8a** is less toxic and more active than **10**. For this reason **8a** was therefore selected for further preclinical evaluation in both doxorubicin-sensitive and -resistant models.

Extensive Evaluation of 8a. Experiments were undertaken to evaluate whether conjugation of the Vectocell peptide to doxorubicin was required to enhance activity. Compounds **10**

Scheme 3. Synthesis of Doxorubicin−Peptide Conjugates with an Amide Link[a]



[a] Reagents and conditions: (i) DIPEA, HATU, DMF; (ii) H_2O and then CH_2Cl_2, DMF; wherein R_1 = -OH or -NH-peptide; R_2 = -H or -CO-peptide.

Table 1. In Vitro and in Vivo Selection of Vectocell−Doxorubicin Conjugates[a]

compd	composition	HCT116 [S] (μM)	MCF-7/6 [S] (μM)	MCF-7/Adr [R] (μM)	in vivo efficacy (HCT116) T/C (%)/dose (μmol/kg)
10	doxorubicin	3	18	> 1000	49.6/6.5
8a	DPV1047-E−Dox	6	29	133	29.5/15
8b	DPV10-E−Dox	2	9	532	39/15
8c	DPV7b-E−Dox	5	10	> 1000	34.1/15
9b	DPV10-TE−Dox	153	37	121	55.8/10
9c	DPV7b-TE−Dox	30	17	862	86.2/10
12a	DPV1047-A−Dox	nd	108	500	79.7/15
12b	DPV10-A−Dox	200	114	625	nd

[a] In vitro data: Mean IC_{50} values (micromolar) from three or more independent experiments are given. [S] Doxorubicin-sensitive cell line; [R] doxorubicin-resistant cell line. In vivo HCT116 experiment: NMRi nude mice were intradermally grafted with HCT116 tumor cells. Mice were treated when tumors were established by iv injections (three injections per week for 3 weeks) with the different compounds. Conjugates were administered at their maximal administrable dose (MAD); doxorubicin was administered at its maximal tolerate dose (MTD). Tumor volume was evaluated and T/C was calculated.

Table 2. Confirmation That Chemical Conjugation of DPV1047 to Doxorubicin is Required to Increase the in Vitro Cytotoxicity of Doxorubicin[a]

compd	HCT116 [S] (μM)	MCF-7/Adr [R] (μM)
doxorubicin (**10**)	3	>1000
DPV1047 (**7a**)	>1000	>1000
doxorubicin (**10**) + DPV1047 (**7a**) (nonconjugated)	4	>1000
DPV1047-E−Dox (**8a**, conjugated)	7	260

[a] Mean IC_{50} values (micromolar) from three or more independent experiments are given. [S] Doxorubicin-sensitive cell line; [R] doxorubicin-resistant cell line.

(doxorubicin) and **7a** (DPV1047) were coincubated (without chemical conjugation) with both doxorubicin-sensitive and -resistant cell lines (Table 2). Administration of the Vectocell peptide (**7a**) alone has no antitumoral activity in vitro. In the doxorubicin-resistant cell line, coadministration of **10** and **7a** showed no antitumoral activity, confirming that conjugation of the Vectocell peptide to doxorubicin is required to elicit the cytotoxic effect in this doxorubicin-resistant cell line [as demonstrated in Table 2 by the activity of **8a** (DPV1047-E−Dox) on MCF7-Adr cells].

The in vivo antitumor activity of **8a** was then evaluated in the MDA-MB-231 human breast adenocarcinoma model (Figure 2A). In this study, DPV1047-E−doxorubicin conjugate (**8a**) was shown to have a significant dose-dependent efficacy, with a optimal T/C of 27.6% at 8 μmol/kg ($p < 0.001$) and a optimal T/C of 16.4% at 10 μmol/kg. Moreover, at the higher dose **8a** treatment induced three transient and one complete regression. At an equitoxic dose [as shown by the same maximum body weight loss (Figure 2B)], **8a** was also found to be more active than doxorubicin (**10**), with optimal T/C values of 43.4% for doxorubicin (**10**) at 6 μmol/kg and 16.4% for **8a** at 10 μmol/kg ($p < 0.001$, Figure 2A). The improved efficacy was still significant at 8 μmol/kg **8a**, (T/C = 27.6%, $p < 0.001$) compared to doxorubicin (**10**) at 6 μmol/kg.

The DPV1047-E−doxorubicin conjugate (**8a**) was then compared to doxorubicin (**10**) in the HCT15 human colorectal adenocarcinoma model (Figure 3A). This cell line has been shown to constitutively express P-glycoproteins (Pgps) and was thus chosen for its partial resistance to doxorubicin.[20] The in vivo expression of Pgp in this tumor model was confirmed by immunohistochemistry and showed a high level of immunoreactivity in the membrane of the tumor cells (not shown). In

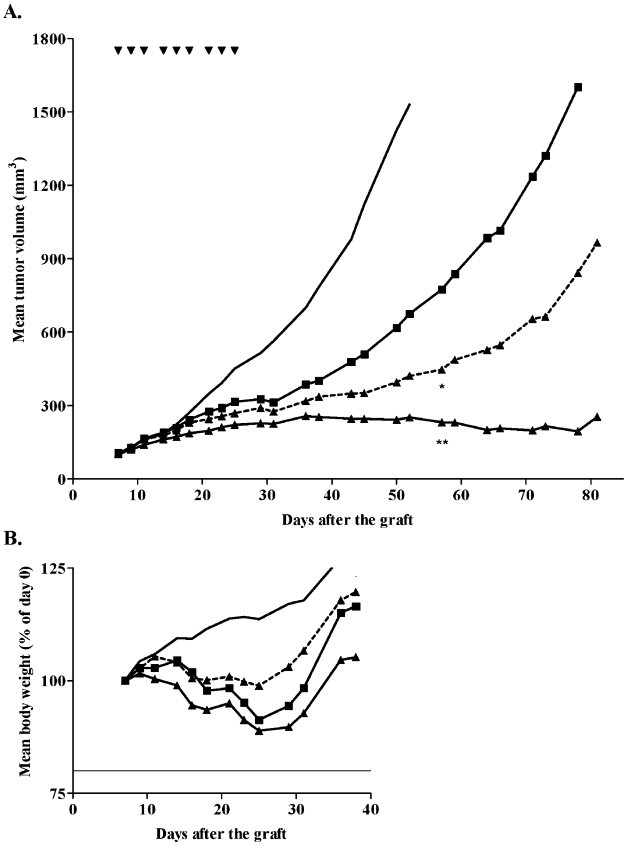


Figure 2. Antitumoral efficacy (A) and toxicity (B) of DPV1047-E−Dox (**8a**) and doxorubicin (**10**) in MDA-MB-231 human breast cancer model. Compounds were administered three times a week for 3 weeks (see arrowheads, ▼). (—) Vehicle-treated animals; (—■—) **10** (doxorubicin), 6 μmol/kg; (--▲--) **8a** (DPV1047-E−Dox), 8 μmol/kg; (—▲—) **8a** (DPV1047-E−Dox), 10 μmol/kg. *p < 0.05, **p < 0.01 (in comparison with doxorubicin).

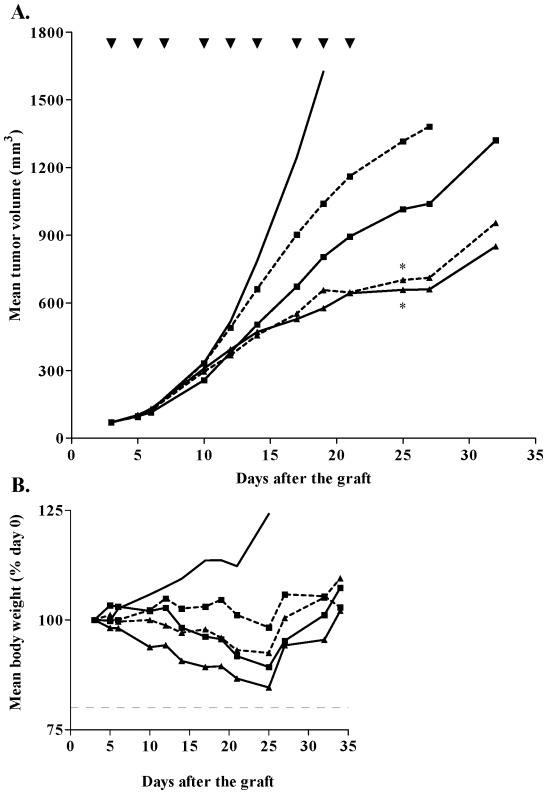


Figure 3. Antitumoral efficacy (A) and toxicity (B) of DPV1047-E−Dox (**8a**) and doxorubicin (**10**) in the doxorubicin-resistant HCT15 human colon cancer model. Compounds were administered three times a week for 3 weeks (see arrowheads, ▼). (—) Vehicle-treated animals; (--■--) **10** (doxorubicin), 4.8 μmol/kg; (—■—) **10** (doxorubicin), 6 μmol/kg; (--▲--) **8a** (DPV1047-E−Dox), 8 μmol/kg; (—▲—) **8a** (DPV1047-E−Dox), 10 μmol/kg. *p < 0.05 (in comparison with doxorubicin).

this partially resistant, Pgp-expressing, tumor model (Figure 3) the DPV1047-E−doxorubicin conjugate (**8a**) displayed a significantly (p < 0.05) better antitumoral efficacy than free doxorubicin (**10**). The body weight losses induced by both drugs at these doses were similar, indicating equitoxicity (Figure 3B). In this experiment, the dose-related efficacy was evaluated by administering a dose 20% inferior to the MTD of both drugs. Reducing the optimal dose of doxorubicin (**10**) or **8a** by 20% significantly reduced the efficacy of doxorubicin (**10**, optimal T/C of 50% vs 38.6%), whereas the antitumoral activity of the DPV1047-E−doxorubicin conjugate (**8a**) was maintained (optimal T/C of 26.6% vs 24.7%, Figure 3A).

In Vitro Inhibition of Multidrug Resistance. To further explore the ability of the DPV1047-E−doxorubicin conjugate (**8a**) to circumvent MDR, its cytotoxicity was correlated with the expression of the MDR protein Pgp (P-glycoprotein) in three doxorubicin-resistant cell lines (Table 3). In these resistant cell lines, DPV1047-E−Dox (**8a**) always showed better antiproliferative activity than doxorubicin. In MCF7-Adr and MES-SA-dx5 cells, which express high levels of Pgp, the enhanced efficacy of DPV1047-E−Dox (**8a**) was highly significant compared to that of doxorubicin alone (p < 0.001).

To verify that DPV1047-E−Dox is able to circumvent the action of Pgp drug pumps, experiments were performed with an inhibitor of Pgp function, verapamil (Vp).[8,21] Vp was added to cultured cells prior to the cytotoxic compound and was able to increase the sensitivity of the resistant cells to doxorubicin (**10**) between 11- and 33-fold (Table 4). In the case of Vp followed by DPV1047-E−Dox (**8a**) treatment, only a moderate increase in sensitivity was observed, with only a 3.6−5-fold increase in activity. The difference between doxorubicin (**10**) and DPV1047-E−Dox (**8a**) cytotoxicity following Vp treatment suggests that DPV1047-E−Dox (**8a**) is a poor substrate for Pgp-mediated cell extrusion, which could result in an increase in the intracellular concentration of the therapeutic molecule.

Table 3. DPV1047-E−Dox (**8a**) and Doxorubicin (**10**) Cytotoxicity in Three Doxorubicin-Resistant Human Cell Lines[a]

	HCT15	MES-SA/dx5	MCF-7/Adr
Pgp expression[b]	5	22	28
doxorubicin (μM)	283	600	>1000
DPV1047-E−Dox (**8a**) (μM)	50	81***	260***

[a] Mean IC_{50} values (micromolar) from three or more independent experiments are given. ***p < 0.001 in comparison with doxorubicin. [b] Mean fluorescence intensity by flow cytometry.

Discussion

The search for better cancer therapies relies on either identifying new therapeutic molecules with new mechanisms of action or improving the therapeutic index of chemotherapeutic agents that are already available. The goal of this study was to evaluate the capacity of Vectocell peptides to improve the therapeutic index of doxorubicin (**10**), a drug that still remains one of the most prescribed drugs for cancer treatment.[12] Vectocell peptides are polycationic cell-penetrating peptides (15−23 amino acid residues) originating from human proteins. Vectocell peptides have been extensively studied by de Coupade et al.[2] and were shown to be internalized by different mechanisms, delivered to different cellular compartments, and have

Table 4. Effect of Pgp Expression on Cytotoxic Activity of DPV1047-E−Dox (**8a**) and Doxorubicin (**10**)[a]

		HCT116 [S] (μM)	HCT15 [R] (μM)	MCF-7/Adr [R] (μM)	MES-SA/dx5 [R] (μM)
doxorubicin (**10**)	alone	4	228	> 1000	600
	+ 20 μM Vp	2	20	30	21
DPV1047-E−Dox (**8a**)	alone	5	50	260	79
	+ 20 μM Vp	4	11	52	22

[a] Mean IC_{50} values (micromolar) from three or more independent experiments are given. [S] Doxorubicin-sensitive cell line; [R] doxorubicin-resistant cell line. Vp, verapamil.

different intracellular delivery levels. The internalization of Vectocell peptides occurs following their interaction with cell surface glycosaminoglycans (GAGs, polyanionic molecules present on the surfaces of all adherent animal cells that interact with many families of growth factors and growth factor receptors and influence cell growth and differentiation) via energy-dependent caveolar endocytosis, as has been described for other cell-penetrating peptides.[2] One particular peptide, DPV1047 (**7a**), that partially originates from an anti-DNA antibody is internalized in a caveolar-independent manner. In the present study, Vectocell peptides that show different internalization characteristics have been linked to doxorubicin to assess in vitro and in vivo efficacy.

A number of doxorubicin−peptidic conjugates have been reported in the literature,[22−24] and all have been shown to require peptide cleavage in order to reactivate doxorubicin. It was, however, hypothesized that the use of a positively charged Vectocell peptide could stabilize the doxorubicin interaction with negatively charged DNA, allowing improved activity, suggesting that a stable chemical linker may enhance activity. This hypothesis was evaluated by the conjugation of Vectocell peptides to different positions on doxorubicin by labile or nonlabile chemical bonds.

The selection of an optimal chemical bond and conjugation position was found to be important for increased activity. In contrast with the initial expectation, the use of chemically stable bonds for conjugation of the peptide at position 14 (thioether) or at position 3′ (amide) of doxorubicin (**10**) resulted in significant or complete loss of activity in vivo, presumably because the Vectocell peptides inhibit the interaction of doxorubicin (**10**) with DNA. A relatively labile chemical bond, allowing doxorubicin release, is therefore required, and for this, the C_{14} position and an ester bond are ideal. In fact, the ester bond was shown to be partially labile in plasma in vivo, with both the ester conjugate [DPV1047-E−Dox (**8a**)] and the metabolite doxorubicin being present in the blood 30 min after DPV1047-E−Dox (**8a**) injection (the ratio of **8a**/doxorubicin at 30 min post-**8a** injection is approximately 0.1, data not shown). This confirmed that the ester link is labile but that it is sufficiently stable to allow DPV-driven intracellular delivery and improve in vivo efficacy compared to free doxorubicin.

As expected, the choice of Vectocell peptide also affected the in vitro and in vivo efficacy of the conjugate. DPV1047 and DPV10 ester conjugates (**8a** and **8b**, respectively) had an improved cytotoxic activity on resistant cell lines compared to free doxorubicin, which was not the case for the DPV7b ester conjugate (**8c**). In the in vivo models that are sensitive to doxorubicin (**10**), the use of **8a** (DPV1047-E−Dox), **8b**, and **9c** shows a clear benefit over the use of doxorubicin (**10**) alone. This benefit was not, however, observed in the in vitro cytotoxicity data (Table 1), which suggests that **8a** (DPV1047-E−Dox), **8b**, and **9c** alter the in vivo biodistribution and/or pharmacokinetic properties of doxorubicin (**10**), thereby reducing the exposure of normal tissues to doxorubicin and increasing its concentration in the tumor (**10**). This appears to be the case as DPV1047-E−Dox (**8a**) reduced the systemic toxicity of doxorubicin (**10**) and at equitoxic doses it exhibits significantly improved antitumoral efficacy compared to doxorubicin (**10**) alone in three different in vivo tumor models.

The present study suggests that conjugation of doxorubicin to a Vectocell peptide allows circumvention of the MDR phenotype. The mechanism of Vectocell−doxorubicin internalization may deliver doxorubicin to intracellular compartments that are less accessible to Pgps. During the preparation of this paper, Liang and Yang[25] have confirmed this observation using a TAT (CGGGYGYGRKKRRQRRR)−doxorubicin conjugate. The TAT−doxorubicin conjugate published by Liang and Yang corresponds most closely to compounds **12a,b**, conjugates that were active but not the optimal molecule selected in our studies.

The ability of DPV1047-E−Dox (**8a**) to increase the efficacy and reduce the systemic toxicity of doxorubicin (**10**) suggests not only that DPV1047-E−Dox (**8a**) will be useful in indications for which doxorubicin (**10**) is currently used, but also that DPV1047-E−Dox (**8a**) may be applied to indications that are currently refractory to doxorubicin treatment. The mechanism of DPV1047-E−Dox (**8a**) intracellular delivery, although not fully characterized, appears to be different from that of other Vectocell or cell-penetrating peptides such as TAT.[2] This may be, in part, due to the lower charge-to-mass ratio observed for DPV1047 compared to other cell-penetrating peptides, which in turn results in an improved therapeutic index.

In conclusion, our results demonstrate that this novel doxorubicin conjugate, DPV1047-E−Dox (**8a**, subsequently to be named DTS-101), shows increased antitumoral efficacy against doxorubicin-sensitive and -resistant tumor models. In vitro and in vivo data indicate that doxorubicin (**10**) delivered in the form of DPV1047-E−Dox (**8a**) has a significantly higher activity than doxorubicin (**10**) and suggests that DPV1047-E−Dox delivers doxorubicin to an intracellular compartment that is less accessible to the action of membrane Pgps. Our results also suggest that the Vectocell technology could be used to increase the therapeutic index of other cytotoxic and small molecule anticancer drugs.

Experimental Section

Chemistry Reagents and Conditions. Daunorubicin hydrochloride and doxorubicin hydrochloride were obtained from Meiji Seika Kaisha Ltd., Japan (these compounds and their derivatives should be handled carefully due to their carcinogenic properties). The peptide DPV1047 (**7a**, CVKRGLKLRHVRPRVTRMDV) was produced by Neosystem, France, and the peptides DPV7b (**7c**, GKRKKKGKLGKKRPRSRC) and DPV10 (**7b**, SRRARRSPRHLGSGC) were produced by Bachem, Switzerland.

Maleimidobutyric acid, *n*-butanol, absolute dioxane, and sodium bicarbonate were obtained from Fluka, Switzerland. Acetone, bromine, hydrobromic acid 48% in water, diisopropyl ether, methanol, potassium carbonate, trifluoroacetic acid (TFA), trimethyl orthoformate, diisopropylethylamine (DIPEA), and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) were obtained from Aldrich, France. Acetic acid and tris(hydroxymethyl)aminomethane (Tris) were from Acros, Belgium. Acetonitrile and dichloromethane were from SDS, France. Chloroform and *n*-hexane were from Labscan, Ireland. Dimethylforma-

mide (DMF) was from Merck, United States. Propylene oxide was from Janssen, United States, and sodium chloride was from Vel, Belgium. HPLC analyses of all synthesized compounds were performed at ambient temperature on a Agilent 1100 series system equipped with a diode array and fluorescence detectors and using Luna C18(2), 3 μm, 4.6 × 100 mm (Phenomenex) column with 0.1% TFA in water as solvent A, 0.1% TFA in acetonitrile as solvent B, and a flow of 1.2 mL/min. Gradient elution conditions were 5−60% B in 10 min, 60−90% B in 1 min, and 90% B for 3 min. The purity of the generated compounds was assessed as the percentage surface area of the peaks at 214 nm. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker FRX 250 and 400 MHz spectrometer with tetramethylsilane as an internal standard. Chemical shifts (δ) are reported in parts per million (ppm) and J values are quoted in hertz (Hz). ^{1}H assignments were supported by (^{1}H−^{1}H) COSY spectra. The splitting systems are as follows: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad, br s = broad singlet, br d = broad doublet, br t = broad triplet, dd = doublet doublet, ddd = doublet doublet doublet.

LC-MS analyses were performed with electrospray ionization. Electrospray mass spectra (direct injections) were performed with an LCQ DECA Ion trap MS^n system (Thermo-Finnigan, Belgium) in the ESI positive mode.

14-Halodaunorubicin (3) via 14-Bromo-13-dimethylketal-daunorubicin (2). Daunorubicin hydrochloride (**1**) (16.92 g; 30.0 mmol) was dissolved in a mixture of anhydrous 1,4-dioxane (207 mL) and anhydrous methanol (207 mL). Trimethyl orthoformate (15.15 mL; 138.5 mmol) was added and the mixture was stirred at room temperature for 5 min. The solution was cooled to 11 °C and bromine (2.64 mL; 51.5 mmol) was added over 2 min. The mixture was stirred at 12 ±2 °C for 2 h. Propylene oxide (5.46 mL; 78.0 mmol) was added at 4 °C and the solution was stirred at 2 ± 2 °C for 75 min. Diisopropyl ether (1.74 L) was added to precipitate the 14-bromo-13-dimethylketal-daunorubicin intermediate (**2**). The red solid was collected by filtration through Whatman no. 42 filter paper and washed with diisopropyl ether (540 mL).

The solid was dissolved in a mixture of acetone (690 mL) and 0.25 M hydrobromic acid (600 mL). The resulting red solution was stirred at room temperature for 66 h. HPLC showed that no acetal remained. The reaction was diluted with water (750 mL) and extracted with chloroform (3 × 750 mL). Saturated brine (saturated sodium chloride in water) (150 mL) was added to the aqueous solution and the product was extracted into *n*-butanol (2 × 1.5 L; 2 × 0.75 L) with the aqueous layer becoming essentially colorless. The 1-butanol extract was concentrated under vacuum at 35 °C (water bath temperature) to a volume of ca. 300 mL. *n*-Hexane (2 L) was added to precipitate the product, which was collected by filtration. The resulting red solid was vacuum-dried at room temperature. LC-MS analysis indicated that the product was a mixture of the salts of 14-bromodaunorubicin and 14-chlorodaunorubicin (**3**) (ca. 1:1). Thus, 15.46 g of product (**3**) was isolated [71% yield from daunorubicin hydrochloride (**1**), HPLC purity: 96%]. ^{1}H NMR (250 MHz, CD_3OD) 1.29 (d, 3H, H6′, J = 6.5 Hz); 1.88 (dd, 1H, H2′, J = 3.5, 12.7 Hz); 2.04 (ddd, 1H, H2′, J = 3.5, 12.7, 12.6 Hz); 2.19 (dd, 1H, H8, J_{gem} = 4.7, 14.6 Hz); 2.39 (br d, 1H, H8, J_{gem} = 14.6 Hz); 2.78 (d, 1H, H10, J_{gem} = 18.6 Hz); 3.05 (d, 1H, H10, J_{gem} = 18.6 Hz); 3.57 (m, 1H, H3′); 3.71 (br s, 1H, H4′); 4.01 (s, 3H, H15); 4.29 (br d, 1H, H5′, J = 6.5 Hz); 4.54 (d, 1H, H14, J_{gem} = 18.0 Hz); 4.67 (d, 1H, H14, J_{gem} = 18.0 Hz); 5.01 (s, 1H, H7); 5.47 (d, 1H, H1′, J = 3.5 Hz); 7.52 (d, 1H, H3, J = 7.7 Hz); 7.77 (br t, 2H, H1 and H2, J = 7.7 Hz). *m*/*z* (ESI): 562, 564 $[M + H]^+$ for 14-chlorodaunorubicin, 606, 608 $[M + H]^+$ for 14-bromodaunorubicin.

Sodium 4-Maleimidobutyrate (5). Sodium hydrogen carbonate (8.391 g; 0.10 mol) was dissolved in water in a 1-L volumetric flask to produce a 0.1 M solution. A portion of this solution (435 mL; 43.5 mmol) was added slowly via a dropping funnel to a stirred suspension of 4-maleimidobutyric acid (**4**) (8.014 g; 43.75 mmol) in water (80 mL). The resulting solution was stirred for 20 min and the water was evaporated in vacuo at 30 °C (water bath temperature) before final drying on a freeze-drying unit. The product (**5**) was obtained as an off-white solid (9.06 g; 100% yield). ^{1}H NMR (250 MHz, D_2O) δ 1.73 (q, 2H, H3″, J = 7.0 Hz); 2.10 (t, 2H, H2″, J = 7.4 Hz); 3.43 (t, 2H, H4″, J = 6.8 Hz); 6.73 (s, 2H, H6″). *m*/*z* (ESI): 182 $[M - H]^-$.

14-Doxorubicinyl Maleimidobutyrate Ester (6). 14-Bromodaunorubicin/14-chlorodaunorubicin HBr salt, approximately 1/1 (**3**) (8.4 g; 12.6 mmol), and sodium 4-maleimidobutyrate (**5**) (13.5 g; 65.8 mmol) in acetone (1.2 L) were stirred and heated under reflux and under argon, for 3 h. When cool, the mixture was filtered through Whatman no. 40 filter paper, the solid was well washed with acetone (total ca. 200 mL), and the filtrate was evaporated at reduced pressure (water bath at 30 °C). The red residue (10.3 g) was dissolved in water containing 0.1% TFA (340 mL) and chromatographed on reverse-phase silica (YMC ODS-A, 500 g). The column was eluted with a stepwise gradient of acetonitrile (10%, 20%, 25%, 30%, and 50%) in water containing 0.1% TFA and the fractions were checked by HPLC. The product fractions were combined and the solvent was removed, initially in a rotary evaporator (bath at 30−32 °C), and then on a freeze-drying unit. The product (**6**) was obtained as a red solid (7.2 g; 69% yield) containing one major impurity, which was isolated. This byproduct was formed consecutively to the esterification reaction and identified by mass spectrometry as an acetone adduct of doxorubicin 14-maleimidobutyrate. Compound **6**: ^{1}H NMR (250 MHz, CD_3OD) 1.31 (d, 3H, H6′, J = 6.4 Hz); 1.93 (m, 4H, H2′ and H3″); 2.12 (br d, 1H, H8); 2.47 (m, 3H, H8 and H2″); 2.98 (d, 1H, H10, J_{gem} = 18.5 Hz); 3.14 (d, 1H, H10, J_{gem} = 18.5 Hz); 3.61 (m, 4H, H3′, H4′, and H4″); 4.05 (s, 3H, H15); 4.33 (q, 1H, H5′, J = 6.4 Hz); 5.07 (m, 1H, H14, J_{gem} = 17.7 Hz); 5.10 (s, 1H, H7); 5.31 (d, 1H, H14, J_{gem} = 17.7 Hz); 5.46 (br s, 1H, H1′); 6.81 (s, 2H, H6″); 7.60 (d, 1H, H3, J = 8.2 Hz); 7.87 (t, 1H, H2, J = 8.2 Hz); 8.01 (d, 1H, H1, J = 7.5 Hz). *m*/*z* (ESI): 709 $[M + H]^+$. Acetone adduct: ^{1}H NMR (250 MHz, D_2O) 1.25 (d, 3H, H6′, J = 5.2 Hz); 1.94 (m, 4H, H2′ and H3″); 2.17 (s, 3H, H11″); 2.56 (m, 4H, H8 and H2″); 2.87 (m, 3H, H10 and H9″); 3.12 (m, 4H, H10, H6″, and H7″); 3.61 (m, 4H, H3′, H4′, and H4″); 3.82 (s, 3H, H15); 4.24 (br s, 1H, H5′); 5.24 (m, 3H, H14 and H7); 5.43 (br s, 1H, H1′); 7.30 (m, 2H, H3 and H2); 7.57 (m, 1H, H2). *m*/*z* (ESI): 767 $[M + H]^+$.

Peptide−Doxorubicin Ester Conjugate (8a−c). General procedure: A solution of doxorubicin-14-maleimidobutyrate (**6**) (4.59 g; 3.91 mmol) in oxygen-free water (160 mL) was added at room temperature under argon, in a glovebag, to a solution of peptide (**7a**) (11.878 g, gross; 66% peptide content, 7.92 g, net; 3.27 mmol) in oxygen-free water (160 mL). An additional 80 mL of water was used to rinse the flask that contained 14-doxorubicinyl maleimidobutyrate (**6**). The resulting solution was stirred at room temperature under argon for 48 h, and then extracted 30 times with 10% DMF in dichloromethane (100 mL), and three times with dichloromethane alone (100 mL). The aqueous layer was lyophilized to give 12.5 g of crude peptide conjugate as a red solid.

The solid was purified by means of preparative HPLC separation with a Micromass ZMD instrument and a Luna C18(2), 10 μm, 250 × 21.2 mm semipreparative column (Phenomenex ref 00G-4253-P0) with 0.1% TFA in water as solvent A and 0.1% TFA in acetonitrile as solvent B (gradient, 5−30% B in 10 min, 3 min at 30% B, 30−90% B in 1 min, 6 min at 90% B; flow, 20 mL/min; loading, 200 mg/run in 1 mL). For each injection, the desired product was detected and collected by means of both the UV response and the Fraction-Lynx mass detector system, set at *m*/*z* = 783 for the $[M + 4H]^{4+}$ ion of the desired conjugate. The relevant fractions for each injection were combined and the resulting solution was lyophilized to give doxorubicin−peptide conjugate (**8a**) TFA salt as a red solid. A total of 5.35 g of material (52% yield) was obtained from the 12.5 g of crude material. LC-MS for conjugate: *m*/*z* 783 $(M + 4H)^{4+}$, 1044 $(M + 3H)^{3+}$, 96% purity (surface area, HPLC). TFA analysis = 26.9% (theory = 26.7% for 10 molar equiv CF_3COOH). Compound **8b**: 86% yield; *m*/*z* (ESI) 1202 $(M + 2H)^{2+}$, 2403 $(M + H)^+$; 97.6% purity (surface area, HPLC). Compound **8c**: 84% yield; *m*/*z* (ESI) 705 $(M + 4H)^{4+}$, 940 $(M + 3H)^{3+}$, 1410 $(M + 2H)^{2+}$; 99.2% purity (surface area, HPLC).

Peptide−Doxorubicin Thioether Conjugate (9b,c). General procedure: 14-Halodaunorubicin (**3**) (0.350 mmol) was dissolved in dry methanol (12 mL) in a round-bottom flask and peptide (**7b,c**) (0.85 equiv with peptide content taken into account) was added, followed by K_2CO_3 (1.3 equiv) (pH must reach 10; if not, potassium carbonate was added). The reaction mixture was stirred for 30−90 min (depending on the peptide) under argon and protected from light. Workup was initiated by the addition of 0.5 M Tris-HCl buffer pH 9 ($^1/_{10}$ of methanol volume) and extractions with chloroform (6 × 1 volume) until the organic layer became colorless. The aqueous layer was then loaded on a YMC ODS-A solid-phase extraction resin (5 g/100 mg of crude compound) preconditioned with methanol and water in a glass frit. After washes with 0.1% TFA in water, the conjugate was recovered by elution with methanol. Methanol was evaporated, the residue was dissolved in water, and the resulting solution was lyophilized to yield the crude thioether conjugate. After preparative HPLC purification, pure conjugates **9b,c** were isolated. Compound **9b**: 78% yield; *m/z* (ESI) 1111 $(M + 2H)^{2+}$, 2221 $(M + H)^+$; 98.6% purity (surface area, HPLC). Compound **9c**: 84% yield; *m/z* (ESI) 1318 $(M + 2H)^{2+}$, 2636 $(M + H)^+$; 95.1% purity (surface area, HPLC).

***N*-Maleimidobutyrate Doxorubicin (11).** Doxorubicin hydrochloride (**10**) (0.05 g, 0.086 mmol, 1 equiv) and 4-maleimidobutyric acid (**4**) (0.015 g, 0.086 mmol, 1 equiv) were dissolved in DMF (3.5 mL). Diisopropylethylamine (DIPEA) (0.03 mL, 0.172 mmol, 2 equiv) was added and the solution was stirred for 15 min at room temperature. Then, HATU (0.036 g, 1.1 equiv) previously dissolved in DMF (1 mL) was added and the mixture was stirred for an additional hour at room temperature. The solution was evaporated at reduced pressure (water bath at 30 °C). The red residue was dissolved in water and purified on reverse-phase silica (YMC). The column was eluted with a stepwise gradient of acetonitrile (10%, 20%, 25%, 30%, and 50%) in water and the fractions were checked by HPLC. The product fractions were combined and the solvent was removed, initially in a rotary evaporator bath (at 30−32 °C), and then on a freeze-drying unit. The product (**11**) was obtained as a red solid (0.044 g; 71% yield). ^{1}H NMR (400 MHz, DMSO-d_6) 1.31 (d, 3H, H6′, $J = 6.4$ Hz); 1.41 (dd, 1H, H4′, $J = 4.4$, 12 Hz); 1.63 (m, 2H, H3″); 1.83 (dt, 1H, H3′, $J = 4$, 12.8 Hz); 2.01 (t, 2H, H2″, $J = 6$ Hz); 2.08 (dd, 1H, H2′, $J = 5.6$, 14 Hz); 2.18 (m, 1H, H2′); 2.64 (br d, 1H, H8, $J = 15.8$ Hz); 2.73 (m, 1H, H8); 2.95 (m, 2H, H4″); 3.42 (m, 1H, H10); 3.92 (m, 1H, H10); 3.94 (s, 3H, H15); 4.15 (m, 1H, H5′); 4.57 (m, 2H, H14); 4.71 (m, 2H, H7); 5.20 (m, 1H, H1′); 6.94 (s, 2H, H6″); 7.51 (d, 1H, H3, $J = 8$ Hz); 7.59 (m, 1H, NH); 7.86 (m, 2H, H1 and H2). *m/z* (ESI): 709 $(M + H)^+$; 86.1% purity (surface area, HPLC).

Peptide−Doxorubicin Amide Conjugate (12a,b). General procedure: A solution of *N*-maleimidobutyrate doxorubicin (**11**) (0.025 g, 0.035 mmol, 1 equiv) in DMF (0.7 mL) was added at room temperature under argon to a solution of peptide (**7a,b**) (0.7 equiv with peptide content taken into account) in oxygen-free water (7 mL). The resulting solution was stirred at room temperature under argon for 2 h and then extracted three times with dichloromethane. The aqueous layer was lyophilized to give the crude peptide conjugate as a red solid. The solid was purified by means of preparative HPLC separation. The relevant fractions (checked by HPLC) were combined and the resulting solution was lyophilized to give doxorubicin−peptide conjugate (**12a,b**) TFA salt as a red solid. Compound **12a**: 34% yield; *m/z* (ESI) 1565 $(M + 2H)^{2+}$; 95.9% purity (surface area, HPLC). Compound **12b**: 97% yield; *m/z* (ESI) 802 $(M + 3H)^{3+}$, 1202 $(M + 2H)^{2+}$, 2405 $(M + H)^+$; 93.7% purity (surface area, HPLC).

All the tested compounds are produced as TFA salts. Previous experiments performed with DPV−maleimide conjugates, which were synthesized as TFA salts, in vitro and in vivo[2] showed that the use of TFA for synthesis is not associated with toxicity up to 1 mM concentration.

In Vitro Pharmacology. The following human carcinoma cell lines were utilized: HCT116 (colorectal cancer, ATCC no. CCL-247), HCT15 (colorectal cancer constitutively expressing Pgp,[26] ATCC no. CCL-225), MCF7/6 (breast cancer, derived from MCF7 after antimycoplasma treatment that led to a change in its behavior;[27] gift of Professor M. M. Mareel, University of Ghent, Belgium), MCF7/Adr (breast cancer derived from MCF7 parental cell line by selection with doxorubicin, cultured in the presence of doxorubicin; gift from Professor J. Robert, Institut Bergonié, Bordeaux, France), MES-SA/dx5 (human uterine sarcoma, ATCC no. CRL1977), MDA-MB-231 (human mammary gland adenocarcinoma, ATCC no. HTB-26). For cytotoxicity studies, viability assays were performed with a WST-1 cytotoxicity assay (Roche, Mannheim, Germany). Briefly, cells were seeded in 96-well plates and incubated with increasing concentrations of drugs for 1 h. Short-term exposure to the conjugates was used to prevent any differences associated with linker stability [release of free doxorubicin (**10**)] interfering with the results. Drugs were then washed out and cells were postincubated with fresh medium for 48 h. The WST-1 test was performed and IC_{50} values (drug concentration that inhibits 50% of cell viability) were estimated from sigmoidal regressions with Graph Pad Prism 3.02 software.

For the in vitro studies with Verapamil (Vp), cells were preincubated overnight in the presence of 20 μM Vp and then incubated with same concentration of Vp for the duration of the experiment. The viability assay was performed as above.

Mice and Tumor Models. Female NMRI nu/nu mice, 6 weeks of age, were used for in vivo experiments. Mice were monitored for 1 week in the animal unit prior to experimentation. They received proper care and maintenance in accordance with institutional guidelines. On day 0, 1×10^7 HCT116 or HCT15 cells suspended in 100 μL of Dulbecco's modified Eagle medium (DMEM) (Invitrogen, France) or 3×10^6 MDA-MB-231 cells suspended in 100 μL of matrigel/DMEM (1/2 v/v) (BD Biosciences, France) were injected in the ventral skin of the mice.

Immunostaining to detect Pgp expression was performed on 10 μm thick tumor sections with a rabbit anti-human mdr1 antibody (Dako, France), according to the manufacturer's recommendations, and a secondary goat anti-rabbit antibody (Dako, France) and a DAB substrate (Dako, France).

Chemotherapy treatment began 3 days (HCT116 and HCT15) or 7 days (MDA-MB-231) after tumor implantation, when tumors reached a minimal size of 70 mm^3. Doxorubicin (**10**) and DPV1047-E−Dox (**8a**) were dissolved in NaCl 0.9%/H_2O (9/1 v/v) and filtered through a 0.2 μm filter, to obtain a concentration corresponding to the required dose to be administered in a volume of 10 μL/g of body weight. Drug concentrations were controlled with a spectrophotometer (U-2000, Hitachi). Drugs were administered following a (Q2D3) × 3W schedule, that is, three injections every 2 days during 3 weeks (nine injections in total). The cumulative doses of each compound were 54 μmol/kg for doxorubicin (**10**) and 72 or 90 μmol/kg for DPV1047-E−Dox (**8a**).

Evaluation of Antitumoral Activity. Tumor volumes were calculated by the following formula: length (millimeters) × width^2 (square millimeters)/2. Results are expressed as mean T/C, that is, mean tumor volume of treated versus control mice. Statistical analyses are performed with GraphPad Prism program. A one-way ANOVA was assessed, followed by a Newman−Keuls test, to compare the antitumor activity of the Vectocell−doxorubicin conjugate [DPV1047-E−Dox (**8a**)] to that of doxorubicin. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

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