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Carminomycin, 14-Hydroxycarminomycin and Its Novel Carbohydrate Derivatives Potently Kill Human Tumor Cells and Their Multidrug Resistant Variants†

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The new hydrophilic derivatives of 14-hydroxycarminomycin were obtained using 13-dimethyl ketal of 14-bromocarminomycin (**6**) as the starting compound. The reductive alkylation of **6** with melibiose or D-galactose followed by hydrolysis of the corresponding intermediate bromoketals **9** and **11** produced 3′-*N*-[-α-D-(galactopyranosyl-(1→6)-*O*-D-1-desoxyglucit-1-yl]-14-hydroxycarminomycin (**10**) and 3′-*N*-(1-desoxy-D-galactit-1-yl)-14-hydroxycarminomycin (**12**), respectively. These novel derivatives **10** and **12** were less toxic than carminomycin or 14-hydroxycarminomycin for leukemia (K562) and breast carcinoma (MCF-7) cells. Importantly, carminomycin, 14-hydroxycarminomycin and compounds **10** and **12** were similarly active for wild type cells and their multidrug resistant (MDR) sublines, K562i/S9 and MCF-7Dox.

Anthracycline antibiotics are effective agents widely used in cancer chemotherapy. However, the therapeutic activity of many drugs of this class is low if tumor cells express multidrug resistance (MDR) mediated by a decreased intracellular drug accumulation. This phenomenon is mediated by ATP binding cassette transporters that efflux their substrates out of the cell[1]. In the growing list of these transporters, P-glycoprotein (Pgp, ABCB1), a 140~170 kDa plasma membrane associated pump, remains a major molecular determinant of MDR. Pgp-mediated transport of a variety of metabolites and xenobiotics confers resistance to many chemotherapeutics including anthracycline antibiotics daunorubicin (**1**) and doxorubicin (**2**) (Fig. 1)[1]. Pgp overexpression is a negative preventive factor in leukemia and breast cancer, malignancies where anthracyclines are important components of therapeutic regimes[2~4]. Escalation of doses of anthracyclines (to accumulate more drug in the tumor) is frequently limited by serious side effects such as blood/bone marrow and heart toxicity due to cummulation effect[5]. The efficacy of anthracyclines against Pgp-positive cancers (*i.e.*, MDR reversal) could be potentiated by the combination of these agents with inhibitors of Pgp-mediated transport or by the administration of antitumor compounds not effluxed by Pgp. The latter opportunity seems straightforward, because the use of Pgp blockers may be hampered by their toxicity and overall limited efficacy[5].

Earlier we have demonstrated that antibiotic carminomycin (**3**)[6], and its derivatives such as 14-hydroxycarminomycin (**4**)[7] (Fig. 1), 14-*O*-hemiadipate and 14-*O*-hemipimelate of 14-hydroxycarminomycin, as well as its *N*-trifluoracetyl congeners[8] were more potent than **2** for tumor cells selected for survival in continuous presence of **2**. The average resistance indexes (*i.e.*, the ratio of concentrations that caused a 50% growth inhibition of resistance versus respective wild type cells) for MCF-7 human breast carcinoma cell line and its doxorubicin-selected subline were as high as 168 for **2** and 64 for **1**,

† This work is dedicated to the memory of Dr. LEONARD S. POVAROV.
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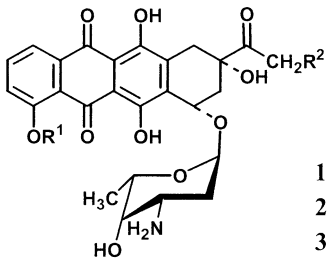
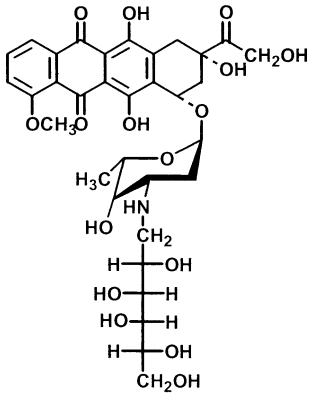
Fig. 1. The structures of anthracycline antibiotics.



1 R^1=CH_3, R^2=H Daunorubicin
2 R^1=CH_3, R^2=OH Doxorubicin
3 R^1=H, R^2=H Carminomycin
4 R^1=H, R^2=OH 14-Hydroxycarminomycin

Fig. 2. 3′*N*-(1-Desoxy-D-galact-1-yl)doxorubicin.



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whereas for **3**, **4** and *N*-trifluoroacetyl carminomycin the respective resistance indexes were only 10, 13 and 2[8]. Whereas carminomycin (**3**) has been successfully used in former USSR for the treatment of patients with uterus sarcomas[9], 14-hydroxycarminomycin was not proposed for the usage in medicinal practice first of all due to its high toxicity[7]. The aim of the present research was to improve the therapeutic indexes of carminomycin derivatives with the hope to retain their property to be active against tumors resistant to doxorubicin.

Previously we have synthesized *N*-substituted doxorubicin derivatives alkylated with mono- or disaccharides (such as **5**, Fig. 2), which were highly soluble in water, active against P388 leukemia, and were better tolerated by animals than parental doxorubicin[10].

We report here the synthesis of two new hydrophilic derivatives of 14-hydroxycarminomycin **10** and **12**, bearing polyole moieties at 3′*N*-position of daunosamine. These derivatives potently kill wild type human breast carcinoma and leukemia cells. Importantly, our novel compounds were similarly highly efficient against the isogenic sublines that expressed Pgp as a result of gene transfer or prolonged drug selection for an MDR phenotype.

Materials and Methods

General Experimental Procedures

Carminomycin hydrochloride was produced at the pilot plant of the Gause Institute of New Antibiotics, Moscow. 14-Hydroxycarminomycin (**4**)[7] and 3′*N*-(1-deoxy-D-galact-1-yl)doxorubicin (**5**)[10] were obtained as described earlier. All reagents and solvents were purchased from Aldrich, Fluka and Merck. Progress reaction products, column eluates, and all final samples were analyzed by TLC and HPLC. TLC was performed on Merck $G60F_{254}$ precoated plates in the following systems: $CHCl_3$ - MeOH - HCOOH, 7 : 1 : 0.1 (A); $CHCl_3$ - MeOH - H_2O - HCOOH, 13 : 6 : 1 : 0.1 (B). Reaction products were purified by column chromatography on Merck silanized silica gel 60 (0.063～0.200 mm). Melting points were determined on Buchi SMP-20 and are incorrected. HPLC analyses were performed on a Shimadzu HPLC LC 50 instrument equipped with a Diasorb C-16 column (4.0×250 mm, 7 μk, BioChem Mack, Russia) and variable wavelength UV detector set at 254 nm with an injection volume 10 μl. Elutions were carried out at a flow rate of 110 μl/minute by the 0.01 M H_3PO_4 - MeCN mixture at 20°C using gradient of MeCN from 25% to 80%. The sample concentration was 0.05～0.2 mg/ml. ^{1}H NMR spectra were recorded on a Varian VXR-400 spectrometer at 400 MHz using DQCOSY method. Mass spectra were determined by Electrospray

Ionization (ESI) on a Finnigan MAT 900S spectrometer (Germany, Bremen). The data for the predominant monoisotope peak are presented. All solutions were dried over sodium sulfate and evaporated at reduced pressure on a Buchi rotary evaporator at the temperature below 35°C.

13-Dimethylketal of 14-bromocarminomycin (**6**)

Carminomycin hydrochloride (**3**) (450 mg, 0.79 mmol) was dissolved in a mixture of MeOH (4 ml), dioxane (2 ml) and ethyl orthoformate (2 ml), then Br_2 (0.035 ml) was added. The reaction mixture was stirred for 2 hours at 20°C and afterwards dry K_2CO_3 (90 mg, 2.38 mmol) was added. Inorganic residue was filtered off quickly and the filtrate was evaporated. The resulting crude 13-dimethylketal of 14-bromocarminomycin (**6**) (~550 mg) was immediately used in the next stage without purification. Rf 0.40 (system A).

3′*N*-[-α-D-(Galactopyranosyl-(1→6)-*O*-D-1-desoxyglucit-1-yl]-14-hydroxycarminomycin (**10**)

Crude **6** (~0.550 mg) was dissolved in MeOH (20 ml). The solution of melibiose (**7**) (860 mg, 2.38 mmol) in H_2O (10 ml) was added, and the reaction mixture was kept at 40°C for 4 hours. Then $NaBH_3CN$ (100 mg, 1.59 mmol) was added, and the mixture was stirred overnight at 37°C. The resulting conjugate 3′*N*-[-α-D-(galactopyranosyl-(1→6)-*O*-D-1-desoxyglucit-1-yl]-13-dimethylketal of 14-bromocarminomycin (**9**) had Rf (system B) 0.33 and Rt 17.823 minutes. Water (50 ml) was added to the reaction mixture, the resulting residue was filtered off and dissolved in 0.25 N HBr (50 ml), and the aqueous solution was extracted with $CHCl_3$ (5×50 ml). The organic layers were combined and extracted with aqueous 0.25 N HBr (2×50 ml). The dark red residue formed between the layers was dissolved in 40 ml of 0.25 N HBr - MeOH (1 : 1) mixture and combined with the 0.25 N HBr extracts. The pooled extracts were incubated for 20 hours at 37°C (to hydrolyze 13-$(OMe)_2$-ketal groups), after that the solution of HCOONa in water (0.75 g/5 ml) was added to the reaction mixture (up to pH ~4.2). The mixture was kept at 37°C for 44 hours, diluted with H_2O to 500 ml, combined with the sorbent XAD-2 swelled in water (~100 ml) and stirred at r.t. for 6 hours until the red color of the solution disappeared. The sorbent was filtered off and washed with water (500 ml). The resulting compound **10** was eluted from the resin with the mixture *n*-BuOH - Me_2CO - H_2O (1 : 1 : 1), evaporated to a small volume of aqueous solution and purified by column chromatography on silanized silica gel, elution was carried out with 0.06% AcOH in H_2O. The resulting fractions containing **10** were combined and evaporated to a small volume. Addition of *i*-PrOH (45 ml) gave a precipitate which was filtered off, washed with Et_2O and dried in vacuum to yield **10** as amorphous dark red powder (54 mg; 8%). Rf 0.27 (system B), HPLC Rt 9.42 minutes, m.p. 169~171°C (decomp.). HR-ESI-MS: calculated for $C_{38}H_{49}NO_{21}$ MW 855.278, found 856.279 $(M+H)^+$.

3′-*N*-(1-Desoxy-D-galactit-1-yl)-14-hydroxycarminomycin (**12**)

Compound **12** was obtained as **10**, starting from **3** (350 mg, 0.62 mmol) and D-galactose (**8**) (560 mg, 3.09 mmol). Yield 20 mg (5%) as amorphous dark red powder. Rf (system B) 0.40, HPLC Rt 6.22 minutes, m.p. 177~179°C (decompos.). HR-ESI-MS: calculated for $C_{32}H_{39}NO_{16}$ MW 693.227, found 694. 227 $(M+H)^+$.

Cell Lines and Viability Assays

The MCF-7 human breast carcinoma cell line (American Type Culture Collection (ATCC), Manassas, VA) and its subline MCF-7Dox selected for resistance to doxorubicin were propagated in Dulbecco modified Eagle's medium (PanEco, Russia) supplemented with 5% fetal calf serum (BioWhittaker, Belgium), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C, 5% CO_2 in humidified atmosphere. The MCF-7Dox subline was cultured in the presence of 0.5 μM doxorubicin. The K562 human leukemia cell line (ATCC) and its variant K562i/S9 that expresses Pgp without drug selection after *MDR*1/Pgp gene transfer and immunoflow cytometry-based cell sorting of Pgp-positive cells[11] were cultured in RPMI-1640 (PanEco) with the same supplements as for breast carcinoma cells. The expression of cell surface Pgp in K562i/S9[11] and MCF-7Dox sublines was confirmed by immunoflow cytometry (data not shown). Cells in logarithmic phase of growth were used in all experiments. Anthracyclines, verapamil and vincristine (Sigma Chemical Co., Madison, WI) were dissolved in sterile water immediately before experiments.

The cytotoxicity of drugs was determined in a formazan [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazane (Sigma Chemical Co., St. Louis, MO)] conversion (MTT) test[12,13]. Briefly, cells (5×10^3 in 190 μl of culture medium) were plated into a 96-well plate (Becton Dickinson, Franklin Lakes, NJ). After 16 hours, cells were either left untreated (control) or treated with increasing concentrations of an anthracycline or vincristine (in duplicate) for 72 hours. The volume of added drugs was 10 μl. In experiments with MDR reversal, verapamil (10 μM) was used together with **1** or vincristine. After the completion of

drug exposure, 20 μl of 5 mg/ml aqueous solution of formazan were added into each well for an additional 3 hours. Formazan was dissolved in acidified DMSO, and the absorbance at λ=540 nm was measured on a Flow Multiscan plate reader (LKB, Sweden). Growth inhibition at given drug concentration was calculated as percentage of absorbance in wells with drug treated cells to that of untreated cells. Data are expressed as mean of four experiments with <15% error.

Results

Chemistry

Earlier it was shown that reductive alkylation of carminomycin (**3**) with aldehydes in the presence of $NaBH_3CN$ yields corresponding 13-(*RS*)-dihydrocarminomycin derivatives[14]. In order to protect the 13-CO group of the antibiotic from reduction 13-dimethyl ketal of 14-bromocarminomycin (**6**) was used as the starting compound. It was obtained by the method described[14,15]. To introduce the polyhydroxylated substituents, disaccharide melibiose (6-*O*-α-D-galactopyranosyl-D-glucose) (**7**) and monosaccharide D-galactose (**8**) were used. 3′-*N*-[α-D-galactoctopyranosyl-(1→6)-*O*-1-deoxy-D-glucit-1-yl]-14-hydroxycarminomycin (**10**) and 3′*N*-(1-deoxy-D-galact-1-yl)-14-hydroxycarminomycin (**12**) were obtained in 8% and 5% yields starting from **6** and **7** or **8**, respectively, with the use of $NaBH_3CN$ after hydrolysis of the intermediate bromoketals (**9**) and (**11**) (Scheme 1). In compound **10** D-galactose has the α-anomeric configuration.

Thin-layer chromatography (TLC) and HPLC analysis showed that compounds **10** and **12** were homogeneous and contained no admixtures of **3** or **4**. Under the conditions of drastic acid hydrolysis (1 N HCl, 105°C, 1 hour) the compounds **10** and **12** produced 14-hydroxycarminomycinone (aglycon), galactose was presented in the products of hydrolyses of **10** as demonstrated by TLC and paper chromatography where authentic compounds were used as standards. The NMR investigations identified all signals in aglycon and carbohydrate moieties (Table 1); mass-spectral data showed the correct molecular weights.

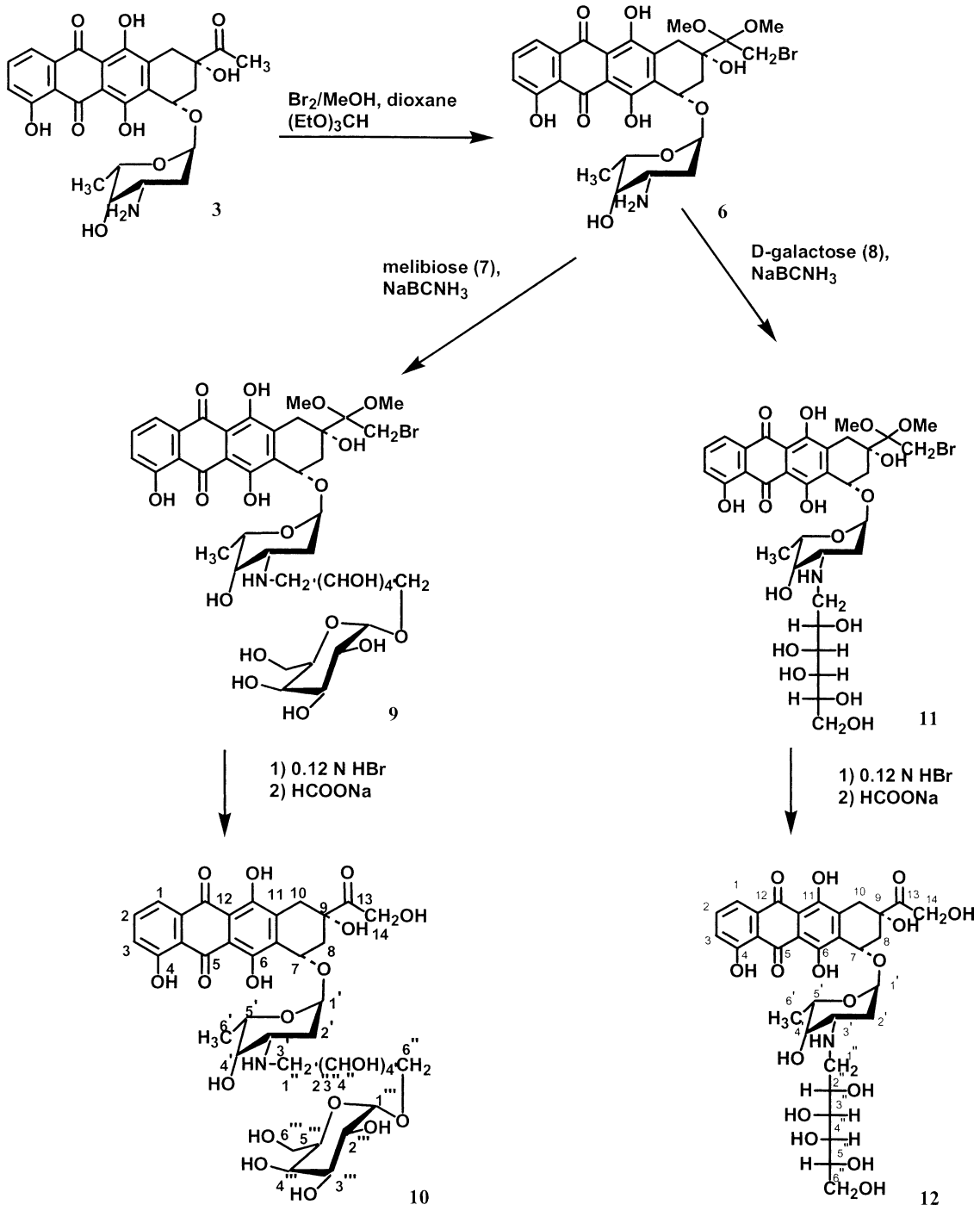
Biological Testing

The cytotoxicity of carminomycin (**3**), 14-hydroxycarminomycin (**4**) and the derivatives **10** and **12** for cultured human tumor cell lines was tested. It was compared with that of doxorubicin and the derivative of doxorubicin **5**. Cells were incubated with drugs for 72 hours to ensure the completion of late events in cell death. Table 2 shows the comparative potencies of these compounds against wild type breast carcinoma and leukemia cell lines and their MDR variants. Carminomycin caused cell death at lower concentrations than doxorubicin remarkably, **3** and **4** were toxic in the nanomolar (**3**) or low micromolar (**4**) ranges. Addition of polyhydroxylated residues to 14-hydroxycarminomycin (yielding **10** and **12**) resulted in a significant decrease of cytotoxicity (Table 2). However, both **10** and **12** were more potent than the doxorubicin derivative **5** containing polyhydroxylated substituent at 3′*N*-position of daunosamine (Table 2). These results demonstrate that carminomycin is more potent than doxorubicin for cultured tumor cells.

We next sought to investigate whether **3** and its derivatives were capable of circumventing Pgp-mediated MDR. Two chemotherapeutic drugs known to be transported by Pgp, doxorubicin and vincristine, were used to demonstrate MDR phenotypes in MCF-7Dox and K562i/S9 cells. Fig. 3 shows that each of our Pgp-expressing sublines was resistant to doxorubicin or vincristine compared to the wild type counterpart. This resistance could be reversed by verapamil, an inhibitor of Pgp-mediated transport[16], providing evidence for a Pgp-mediated MDR in K562i/S9 and MCF-7Dox sublines. In striking contrast, carminomycin showed similar activity against wild type and MDR cells. These data indicate that **3** can circumvent Pgp-mediated MDR.

Finally, we tested whether the carminomycin derivatives **4**, **10** and **12** retain this important feature. As shown in Table 2 and Fig 4A, **4** killed MCF-7 and MCF-7Dox cells with virtually equal potency. Furthermore, Pgp did not protect cells from death induced by **10** and **12**: IC_{50}s for these agents were similar for K562 and K562i/S9 cells (Table 2). Compound **12** was effective for the MCF-7 and MCF-7Dox cells as well (Table 2, Fig. 4B). Together, our data clearly demonstrate that 1) unlike doxorubicin, carminomycin and 14-hydroxycarminomycin are potent for tumor cells resistant to Pgp-transported chemotherapeutic drugs; 2) 3′-*N*-(1-desoxy-D-galactit-1-yl)-14-hydroxycarminomycin (**12**) and 3′*N*-[-α-D-(galactopyranosyl-(1→6)-*O*-D-1-desoxyglucit-1-yl]-14-hydroxycarminomycin (**10**), although less active than **3** or **4**, retain its efficacy for MDR cells.

Scheme 1



Discussion

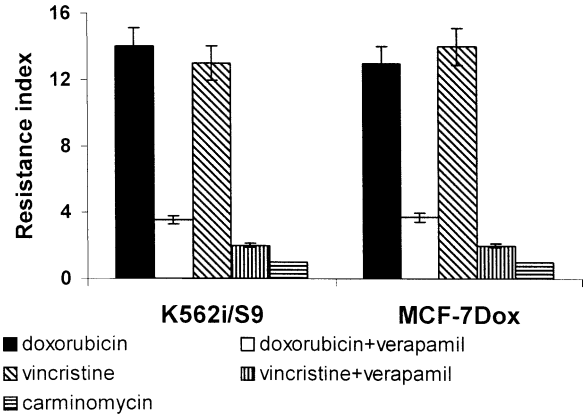
Growing amount of data support the role of Pgp as a general mechanism of cell resistance[17,18]. The fact that viability of K562 cells and their variant with Pgp alone (K562i/S9) was similar in the presence of **3** or **12** indicates that Pgp does not protect cells from death induced by these agents. More importantly, Pgp together with other mechanisms tentatively expressed in the doxorubicin-selected subline MCF-7Dox failed to confer resistance to **3** or **12**, suggesting that **3** and its analogs **4**, **10** and **12** can

Table 1. ^{1}H NMR spectra of compounds **10** and **12** in pyridine-d_5+CF_3COOD, rt; δ ppm.

Chemical shifts	Atom no.	10	12
Anthracyclinon part	1	7.81	7.85
	2	7.61	7.60
	3	7.31	7.29
	7	5.11	5.13
	8	2.48, 2.74	2.48, 2.73
	10	3.37, 3.49	3.35, 3.47
	14	5.35	5.33
Daunosamine part	1'	5.61	5.55
	2'	2.64	2.60
	3'	4.19	4.15
	4'	4.39	4.37
	5'	4.67	4.63
	6'	1.41	1.40
Polyole part	1"	3.85	3.92
	2"	4.82	4.92
	3"	4.54	4.57
	4"	4.39	4.15
	5"	4.12-4.59	4.45
	6"		4.63
D-galactose part	1"'	5.37	
	2"'	4.54	
	3"'	4.12-4.59	
	4"'		
	5"'		
	6"'a		
	6"'b		

circumvent an MDR phenotype(s) established in response to other anthracyclines. However, because verapamil did not restore completely the sensitivity of MCF-7Dox cells to **2** and vincristine (Fig. 3), resistance mechanisms other than Pgp in these cells may exist, although their role is likely to be minor. This, in turn, presumes that **3** kills MDR cells mainly due to the inability of Pgp to counter its intracellular accumulation.

Fig. 3. Influence of verapamil on resistance indexes to studied antitumor compounds.



Resistance index is the ratio of concentrations that caused a 50% growth inhibition of the Pgp-expressing subline versus respective wild type cell line (see also Table 2).

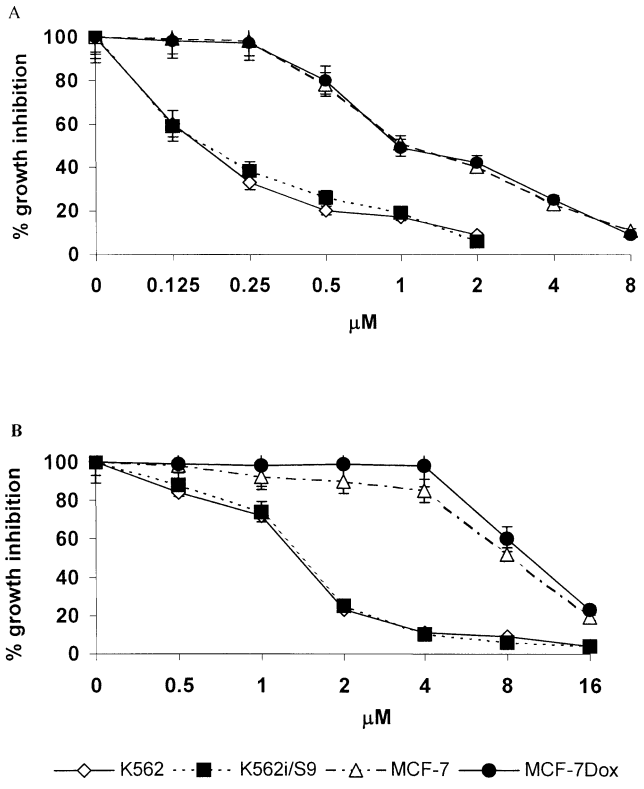
Table 2. Toxicity of anthracyclines for wild type (MCF-7, K562) and Pgp-expressing (MCF-7Dox, K562i/S9) cell lines.

	Compound	MCF-7	MCF-7Dox	R/S	K562	K562i/S9	R/S
Doxorubicin and its derivative	Doxorubicin	300*	3650	12.5	140	1990	14.0
	5	>32000	n.d.	n.d.	15650	n.d.	n.d.
Carminomycin and its derivatives	Carminomycin	90	90	1.0	60	60	1.0
	4	1180	1220	1.03	180	190	1.05
	10	n.d.	n.d.	n.d.	5850	14860	2.53
	12	9170	11380	1.24	1180	1220	1.03

*IC_{50} (nM), the concentration that caused 50% growth inhibition after 72 h of exposure.

Data are mean of 4 measurements with <15% error. R/S, resistance index (ratio of concentrations that caused a 50% growth inhibition of the Pgp-expressing subline versus respective wild type cell line). n.d., not determined.

Fig. 4. Activity of 14-hydroxycarminomycin (**4**) (A) and 3′*N*-(1-desoxy-D-galact-1-yl)-14-hydroxycarminomycin (**12**) (B) for wild type (K562, MCF-7) and Pgp-positive (K562i/S9 and MCF-7Dox) cells.



Chemical modifications of anthracyclines yielded the compounds capable of circumventing MDR[19~21]. Early observations with bacterial and tumor systems have shown the ability of carminomycin to accumulate in cells resistant to **1** and **2** and to kill these cells[22,23]. Importantly, addition of the polyhydroxylated substituent to the 3′-*N*-position of daunosamine moiety of carminomycin did not render carminomycin derivative **12** Pgp substrate. Therefore, addition of mono- or disaccharide aimed at increasing the amphiphilicity of anthracycline molecule should not alter the affinity of the derivatives to Pgp. However, this modification can produce the congeners less toxic for normal tissues.

Whatever the significance of Pgp, it is important to identify the intracellular factor(s) that may decrease the antitumor activity of carminomycins. Cellular drug resistance is not confined only to Pgp-mediated MDR but is rather a pleiotropic phenomenon[24]. In the course of their natural history tumor cells can accumulate mechanisms of irresponsiveness to physiological signals that control cell proliferation and differentiation. These mechanisms can also contribute to resistance to toxins including anticancer chemotherapeutics (intrinsic drug resistance). The Bcr/Abl gene rearrangement in K562 cells (that results in a constitutive activation of anti-apoptotic signaling) and lack of effector caspase-3 in MCF-7 cells are well-documented factors of resistance expressed prior to drug exposure[25~27]. Remarkably, **3** was toxic for these cell lines at nanomolar concentrations, implying that this compound could overcome the intrinsically altered response to xenobiotics. We are currently testing whether other mechanisms known to limit the efficacy of anthracyclines (such as p53 and anti-apoptotic Bcl-2 family proteins) can be circumvented by **3** and its derivatives. If proven, this would broaden the therapeutic potential of carminomycin or its derivatives for tumor cells with various phenotypes of multifactorial drug resistance.

In summary, we demonstrate that carminomycin, 14-hydroxycarminomycin and its polyole derivatives **10** and **12** are active against cultured human tumor cells and their sublines with Pgp-mediated MDR. Further studies are needed to evaluate the applicability of novel compounds for preclinical trials.

Acknowledgement

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