Cancer Chemother Pharmacol (2006) 57: 811–818
DOI 10.1007/s00280-005-0118-0

ORIGINAL ARTICLE

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In vivo antitumor efficacy and cardiotoxicity of novel anthracycline ID6105 (11-hydroxy-aclacinomycin X, Hyrubicin)

Received: 4 June 2005 / Accepted: 18 August 2005 / Published online: 21 September 2005

Abstract Hybrid biosynthetic approach produced a new anthracycline ID6105 (11-hydroxyaclacinomycin X, Hyrubicin), which has potent antitumor activities against a broad range of cancer cell lines. Like other anthracyclines, ID6105 has the inhibitory effects on DNA synthesis as well as topoisomerase II. As preclinical studies of ID6105, we investigated ID6105's efficacy on human tumors, and cardiotoxicity. In human tumor xenografts, the ID6105's antitumor effects were greater than other anticancer drugs. ID6105 induced tumor regression in Hep G2 human hepatoma model, and slowed down the tumor growth rates in several tumor models. Doxorubicin-refractory tumors such as PC-3, DU-145, and CX-1 were sensitive to ID6105, and the growth of EKVX, lung cancer, which did not respond to paclitaxel, was also inhibited by ID6105, but tumor mass in CFPA, MCF7, and HCT-15 was not reduced by ID6105. The cardiotoxicity of ID6105 has also been assessed in rats. ID6105 did not induce any remarkable histopathological changes in hearts, and its lipid peroxidation in rat cardiac muscles did not occur as much as doxorubicin, indicating that the cardiotoxicity of ID6105 is remarkably lower than that of doxorubicin. Taking all into account, our results suggest that ID6105 would be a promising candidate for a novel anthracycline chemotherapeutic agent.

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Keywords ID6105 · Hyrubicin · Anthracycline · Xenograft · Cardiotoxicity

Abbreviations ID6105: 11-hydroxyaclacinomycin X · DOX: Doxorubicin · MDA: Malonaldehyde · IV: Intravenous · TGI: Concentration of drug inducing total growth inhibition

Introduction

The anthracycline antibiotics derived from a *Streptomyces*, such as daunorubicin [2], doxorubicin [4], has been one of the most prevalent anticancer agents in the treatment of cancer patients by its broad spectrum of activity [2, 5, 19]. However, their medical application has been refrained mainly due to its serious side effects, including a cumulative, dose-related cardiomyopathy associated with progressive and irreversible heart failure [20]. Many new anthracycline analogues have been developed with intensive efforts during the past 20 years through a biosynthetic or syntheses [1, 16] but no such problems resolved as yet.

The aclacinomycin X, 7-(O-rhodosaminyl-deoxyfucosyl-redosyl)-aklavinone, and 11-hydroxyaclacinomycin X are novel aclacinomycin analogues as previously described [12, 13]. They were obtained from *Streptomyces galilaeus* ATCC 3113. In particular, 11-hydroxyaclacinomycin X was transformant of this strain containing the aclavinone 11-hydroxylase gene, *dnrF* [12]. Two novel aclacinomycin analogues, especially 11-hydroxyaclacinomycin X, showed a strong cytotoxic activity against human tumor cells [13]. In this paper, we investigated therapeutic activities of 11-hydroxyaclacinomycin X, named ID6105 on 13 human tumor xenograft models and compared ID6105-induced cardiotoxicity with doxorubicin's in aspects of lipid peroxidation and histopathological changes, as preclinical studies

Materials and methods

Chemicals

ID6105 was synthesized by KRIBB (Korea Research Institute Bioscience and Biotechnology, Taejon, Korea). ID6105 was dissolved in 10 mM sodium acetate buffer (pH 4.5) containing 5% maltose and stored in −20°C. The commercial chemotherapeutic agents are dissolved as follows: Doxorubicin hydrochloride was dissolved in sterile saline; paclitaxel was dissolved in 12.5% EtOH/ 12.5% Cremophor/75% saline (soluble); mitomycin C was prepared in 0.9% saline; gemcitabine was prepared in saline. These drugs were kept in 4°C and reconstituted just before use. The Fig. 1 shows the structure of ID6105.

Cell lines

For human tumor xenograft models, human breast cancer (MCF7), colon cancer (CX-1, HCT-116, HCT-15), prostate cancer (PC-3, Du-145), ovary cancer (SK-OV-3, NIH: OVCAR-3), lung cancer (LX-1, A549, EKVX), pancreas cancer (CFPAC-1) were cultured. All cell lines were grown and maintained at 37°C, 5% CO_2 in Dulbeco's Modified Eagles Medium (Hyclone, USA) with 10% fetal bovine serum (Hyclone, USA).

Animals

All test animals in these studies were provided by Charles River (Orient, Seoul, Korea). Female BALB/c-nu mice weighing 18∼22 g were for xenograft models. Male SD rats weighing 220∼250 g were used in cardiotoxicity tests. All animals were maintained in specific pathogen-free condition with 12-h day/night schedule and fed with sterile food and R/O water ad libitum. Animal experiments were approved by Animal Ethics Committee at the Ildong pharmaceuticals Co. and performed under the guidelines of Korea Food and Drug Administration (KFDA).

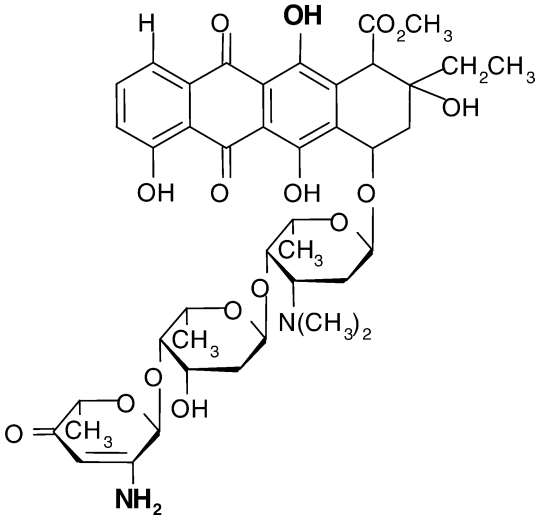

Fig. 1 Structure of ID6105 (11-hydroxyaclacinomycin X)

Influence of ID6105 on DNA synthesis

HeLa cells (10^5 cells/well) were cultured in 24-well plates for 24 h. ID6105 (0.1, 0.3, 1 μg/μl) was added and the plates was cultured further with [^{3}H] thymidine for 1, 2, and 3 h. After incubation, cells were detached with trypsin-EDTA and precipitated with cold TCA. The inhibition rate of DNA synthesis was calculated from the percentage of incorporation of the [^{3}H] thymidine in drug-treated versus untreated control cells.

Topo II inhibition assay

Topo II inhibition was assayed with a Topo II drug screening kit (TopoGEN, Columbus, OH). The assays were carried out according to the manufacturer's methods. In brief, the assays were performed in 25 μl total reaction buffer containing 10× buffer, pRYG supercoiled DNA (0.25 μg), human topoisomerase II and drugs. Control drugs were etoposide (cleavable complex form assay) and aclacinomycin A (relaxation assay). The mixtures were incubated at 37°C for 30 min and terminated by the addition of SDS and proteinase K. Samples were loaded onto 1% agarose gels and electrophoresed. Gels were stained with 0.5 μg/μl of EtBr for 20 min and photographed. In cleavable-complex formation assays, samples were loaded onto agarose gels containing EtBr for better resolution of nicked open circles and linear forms of DNA.

In vivo tumor models

The solid tumors were induced by the following procedure. The cultured tumor cells were collected and reconstituted in sterile cold PBS. 6×10^7 cells per mouse implanted subcutaneously into the right axillary region of mouse.

Treatment and assessing antitumor activity

For human tumor xenograft models, animals were treated repeatedly. The treatment began when tumor was palpable (100∼200 mm^3 or 30∼40 mg). The chemotherapy of ID6105 was scheduled to administer consecutively for 5 days (3 mg/kg, i.v.). Tumor volume(mm^3) was measured twice a week. Tumor volume (mm^3) was estimated from measurements of the length (L) and the width (W) of each tumor with a vernier caliper (mm) according to the following formula: $L\times W^2/2$

The efficacies of drugs were also expressed as T–C values which mean the difference of tumor doubling time in the treated and control.

Malonaldehyde (MDA) assay

ID6105 (1, 2, 3 mg/kg) or Doxorubicin (2 mg/kg) was administered to rats intravenously for 5 days. After 14 days, their hearts were isolated and washed with saline. The isolated hearts were weighed and homogenized with 1.15% KCl. One hundred and thirty microliter of homogenates was added to a mixture containing 80 μl of 1% H_3PO4, 260 μl of 0.67% thiobarbituric acid. The mixture was boiled for 45 min and immediately cooled in ice. Extracts with 1.03 μl *n*-butanol were centrifuged at 3,000 rpm for 15 min. The absorbance of its supernatant was measured at 535 and 520 nm with a microplate reader (Quant, Bio-Tek, USA). MDA concentrations in the samples were calculated by a standard calibration curve of 1, 1, 3, 3-tetraethoxypropane prepared in the same manner. Each measurement was performed in triplicate.

Electron microscopic study

We injected ID6105 (2 mg/kg) and Doxorubicin (2 mg/kg) intravenously for consecutive 5 days. After 14 days, the hearts of rats were isolated and preparation of tissue block was performed according to Forrest et al. [7] and Zhou et al. [21]. Cardiac tissues cut into 1 mm^3 were fixed with 2.5% glutaraldehyde and then washed several times with PBS and secondarily fixed with 1% osmium tetroxide for 40 min. After double fixation, the specimens were dehydrated with a graded EtOH series. The samples were finally embedded in Epon mixture. Representative areas of each lesion were sectioned at approximately 1-mm thickness and stained with 1% toluidine blue solution. Selected areas were trimmed further for thin sectioning and stained with uranyl acetate and lead citrate. The sections were examined under an electron microscope (Hitachi H-600 electron microscope, Japan).

Results

Influence of ID6105 on DNA synthesis

As shown in Fig. 2, ID6105 blocked DNA synthesis in dose-dependent manner. The inhibition rate of 1 μg/μl was about 40% for 1 h, and after 3 h, it increased to 90%.

Topo II inhibition assay

We investigated whether ID6105's inhibitory effect on DNA synthesis was mediated by the suppression of topoisomerase II. As a result, ID6105 seems to induce a catalytic topo II inhibition. The results are shown in Fig. 3. Although high dose of ID6105 (100 μg/μl) slightly accumulated linear DNA derived from the cleavable complex in cleavable complex form assay (Fig. 3A), in relaxation assay (Fig. 3B) additions of ID6105 resulted in dose-dependent inhibition of topoisomerase II activity of which the intensity was almost the same as that by aclacinomycin A.

Studies in human tumor xenograft models

We examined ID6105's antitumor efficacy in 13 human tumor xenograft models. Firstly, we tried to compare with doxorubicin against eight tumor models, followed by experiments with five commercial drugs against additional five tumor models. We injected 3 mg/kg of ID6105 for 5 days in all cases. As shown in Fig. 4, ID6105 presented effective activities on eight tumor models tested. Its activity was greater than doxorubicin, except in SK-HEP-1 and SK-OV-3 models where they showed an equivalent response to doxorubicin. Average tumor inhibition rate of ID6105 on last day was about 40% (data not shown). Table 1 summarized the results from the secondary experiments in which we compared with other commercial agents. The results indicated that ID6105 had effects on EKVX, HepG2 tumors, in which the T–C values of ID6105, of 16.5 and 16.2 days, were apparently longer than paclitaxel and doxorubicin of the respective 2.2 and 0.2 days. Additionally, in HepG2 hepatoma model, 7 out of 10 animals dosed with ID6105 showed a complete regression effect while one was partial, proving its predominant effects over doxorubicin. And it seemed to be moderately effective in CFPAC-1 models, 6.3 days in T–C, while MCF7 was slightly inhibited (5 days) and HCT-15 was refractory to ID6105 (1.4 days).

Cardiotoxicity studies

Lipid peroxidation of hearts

ID6105 (1, 2, 4 mg/kg) or Doxorubicin (2 mg/kg) was administered to rats intravenously for 5 days. After 14 days, their hearts were isolated and MDA assays were performed. Although ID6105 increased the levels of lipid peroxidation in a dose-dependent manner, its degrees showed significantly lower than the doxorubicin-treated group (1, 2 mg/kg) (Fig. 5).

Histological examination of cardiac tissue

In the histological studies, doxorubicin (2 mg/kg) induced loss of striated muscle, intercalated disc, and swelling and ablation of outer membrane in mitochondria. But no histopathological changes were found in ID6105 (2 mg/kg) group (Fig. 6).

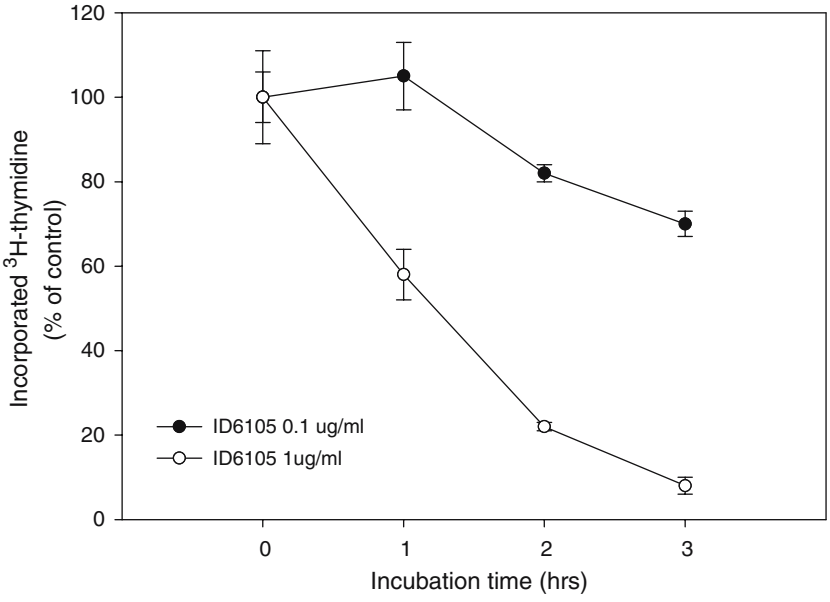

Fig. 2 The effects of ID6105 on DNA biosynthesis in HeLa cells. The cells were incubated with variable concentrations of ID6105 for 1, 2, 3 h with [^{3}H] thymidine. [^{3}H] thymidine incorporation into the acid-insoluble fraction of HeLa was determined. Each *plot* represents the mean ± SE 1 μg/μl of ID6105 inhibited about 90% of DNA synthesis in HeLa for 3 h

Discussion

ID6105 (11-hydroxyaclacinomycin X) is a new anthracycline produced by *Streptomyces galilaeus* ATCC 3113 transformed with akalvinone 11-hydroxylase gene (*dnrF*). ID6105 is 11-hydroxyaklavinone with trisaccharides moiety containing two amino sugars—rhodosamine and rednose [12, 13]. The functions of amino sugars were reported to increase cytotoxic activity and DNA-binding affinity in doxorubicin [22, 23]. Hwang et al. [10] demonstrated that in aclacinomycin A, a second generation of anthracycline which has an aklavinone with one amino sugar [17], 11-hydroxlation of aklavinone increased in vitro cytotoxicity against tumor cells. Therefore, having structural merits of both doxorubicin and aclacinomycin A, ID6105 is expected to surpass both drugs in antitumor activities.

The ID6105's mechanism of action has been postulated in various aspects. Anthracyclines can intercalate

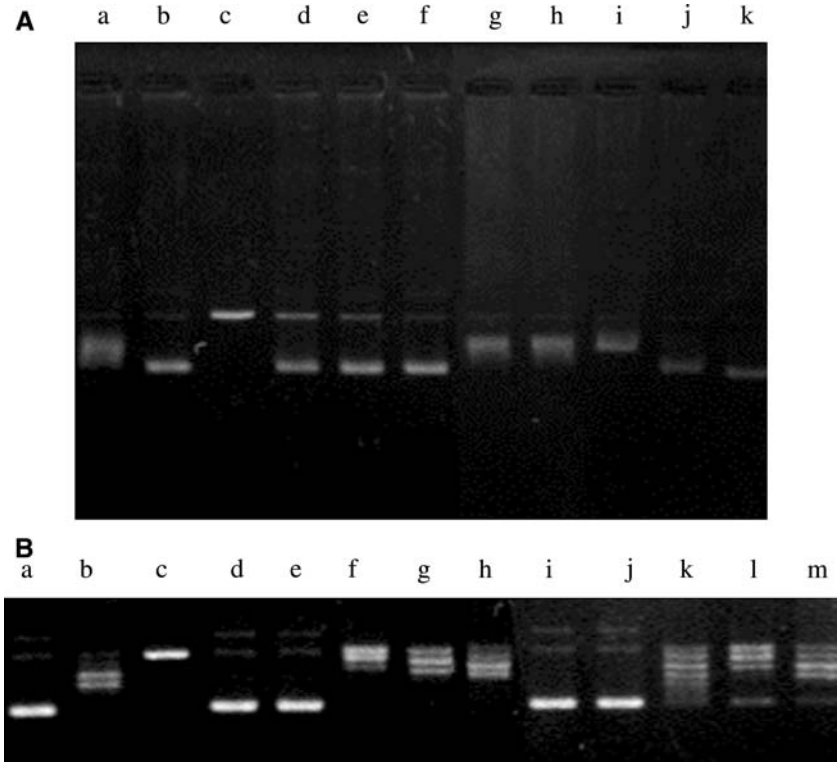

Fig. 3 Agarose gel of Topoisomerase II assay. **A** Cleavable complex assay (Lane a: no enzyme control, Lane b: enzyme control, Lane c: linear DNA, Lane d∼f: etoposide 100, 20, 40 μM, Lane g∼k: ID6105 100, 20, 4, 0.8, 0.16 μM), ID6105 has a slight effect on accumulation of cleavable complex of plasmid DNA. **B** Relaxation assay (Lane a: no enzyme control, Lane b: enzyme control, Lane c: linear DNA, Lane d∼h: aclacinomycin A 100, 20, 4, 0.8, 0.16 μM, Lane i∼m: ID6105 100, 20, 4, 0.8, 0.16 μM) ID6105 inhibited topoII activity as much as aclacinomycin A

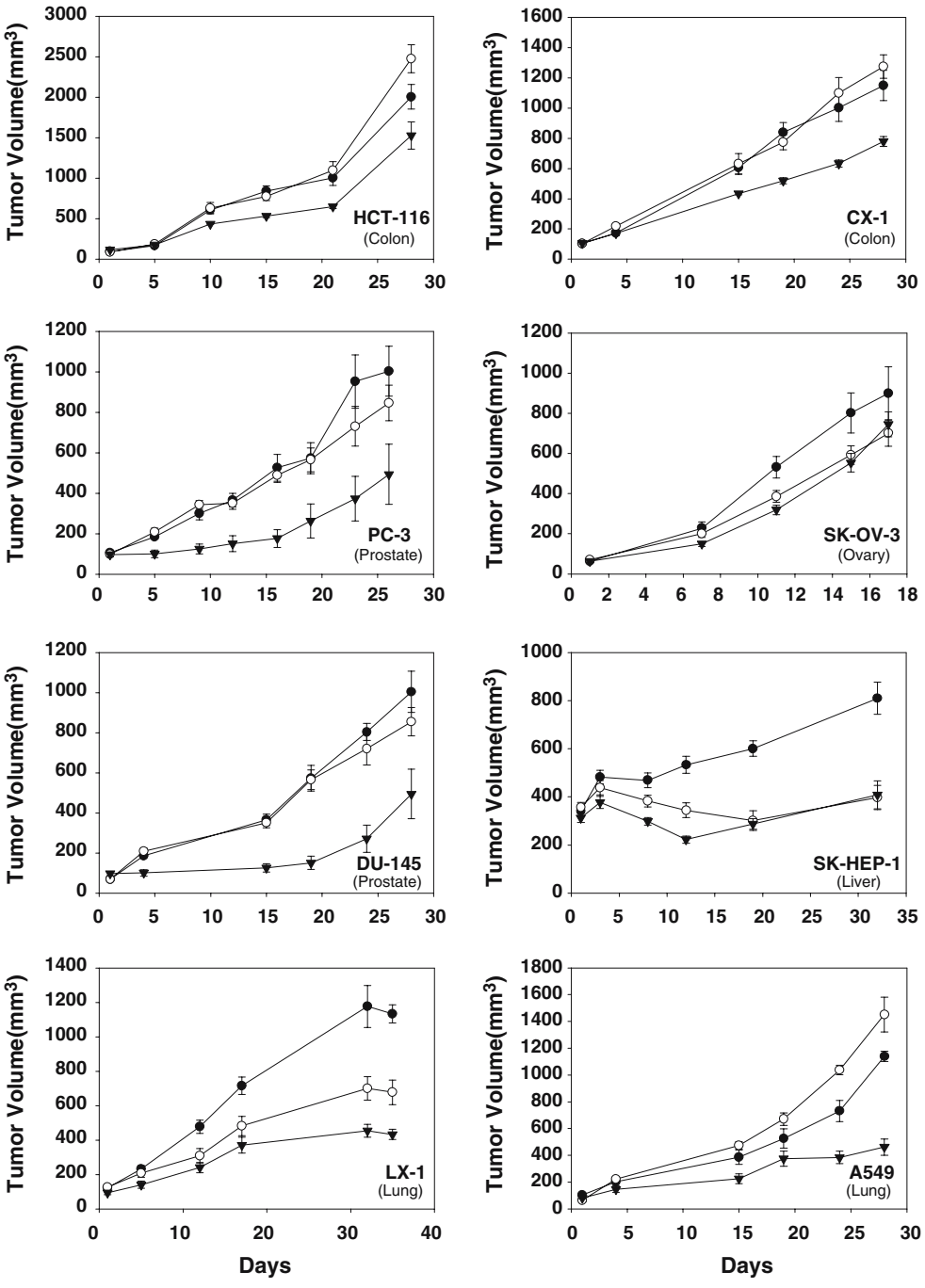

Fig. 4 Growth curves for groups of mice carrying eight different human xenografts. Tumors were induced in nude mice by s.c. inoculation of tumor cells. Intravenous treatment started when tumor mass reached 100∼200 mm^3. Groups were administered for 5 days with: 10 mM sodium acetate buffer control, pH4.5 (*filled circle*); 3 mg/kg ID6105 (*inverted triangle*); and 3 mg/kg doxorubicin (*open circle*). The number of animals was at least six in each group

between DNA base pairs, possibly bind covalently to DNA, and inhibit topoisomerase I or II [3, 9]. Like these drugs, ID6105 could block the DNA synthesis as well as topoisomerase II activity. As shown in Fig. 3, ID6105 seemed to have a catalytic topoisomerase II inhibitory activity. Its mechanistic pattern of blockade was more similar to aclacinomycin A than etoposide. However, it is difficult to find out its exact mechanism, considering unclear mechanism of the anthracyclines that still remained in considerable controversy for a long time. Recently we tried to find ID6105-induced apoptosis in several tumor cells.

In the previous report [13], in vitro cytotoxicities of ID6105 were tested by the DTP (Developmental Therapeutic Program) of National Cancer Institute. They reported a remarkable cytotoxicity and high sensitivity of ID6105 to human cancer cells. In comparison with doxorubicin data in DTP, ID6105 was about 20 times more potent than doxorubicin in average TGI (total growth inhibition concentration) value.

In vivo studies, ID6105 also had positive results on treatment of tumors. In human xenograft models, we firstly compared the efficacy of ID6105 with doxorubicin in eight tumor models, and then with several commercial

Table 1 Antitumor activities of compounds in human tumor xenograft models

Tumor	Group	Dose (mg/kg/day)	Route	Schedule	Tumor regression			DT[b]	T–C[c]
					Partial	Complete	Duration[a]		
HCT-15	CTRL	–	i.v.	QDX5	0	0	–	7.4	–
	Mitomycin C	3	i.p	Q4DX3	0	7	14.8	>35	>27.6
	ID6105	3	i.v.	QDX5	0	0	–	8.8	1.4
EKVX	CTRL	–	i.v.	QDX5	0	0	–	25.3	–
	Paclitaxel	12	i.v.	QDX5	0	0	–	27.5*	2.2
	ID6105	3	i.v.	QDX5	0	0	–	41.8*	16.5
CFPAC-1	CTRL	–	i.v.	QDX5	0	0	–	4.3*	–
	Gemcitabine	180	i.v.	Q3DX4	0	2	8.4	22.4	18.1
	ID6105	3	i.v.	QDX5	0	0	–	20.6	6.3
Hep G2	CTRL	–	i.v.	QDX5	0	0	–	12.7	–
	Doxorubicin	1.5	i.v.	QDX5	–	–	–	12.8	0.2
		0.75	i.v.	QDX5	0	2	-	11.1	−0.6
	ID6105	3	i.v.	QDX5	1	7	7.7	17.8	16.2
MCF7	CTRL	–	i.v.	QDX5	–	–	–	8*	–
	Paclitaxel	12	i.v.	QDX5	0	0	–	>27.0*	>19.0
	ID6105	3	i.v.	QDX5	0	0	–	13*	5

The study was designed to evaluate the antitumor efficacy of compound ID6105 against human tumor xenografts s.c. implanted in female nude mice as 30∼40 mg tumor fragments. There were 10 animals in the control and treatment groups. The tumor implants grew to median size of 120 mg before treatment CTRL, 10 mM Sodium acetate buffer (pH 4.5)

[a]D, duration of tumor regression (days)

drugs- paclitaxel, mitomycin C, and gemcitabine, doxorubicin in five models. The schedule and dose of ID6105 was determined from preliminary experiments, in which 5-day consecutive administration was chosen for better efficacy and lower systemic toxicity, and 3 mg/kg was MTD for this schedule. In all models tested, EKVX and HepG2 showed the most sensitive to ID6105. We observed a complete regression effect on HepG2 xenografts for 7.7 days which was refractory to doxorubicin. Also in other tumor models, we could find better activities of ID6105 than the reference groups. However, ID6105 did not induce any changes in comparison with the vehicle group in MCF7 models. These results confirmed that our new compound had a different characteristic of antiproliferative activity from doxorubicin and its activity was more potent than other chemotherapeutics in some tumor models.

Cardiotoxicity is a well-recognized clinical problem and most of its serious adverse effects caused to limit the use of all anthracyclines [11, 18]. Anthracyclines produce

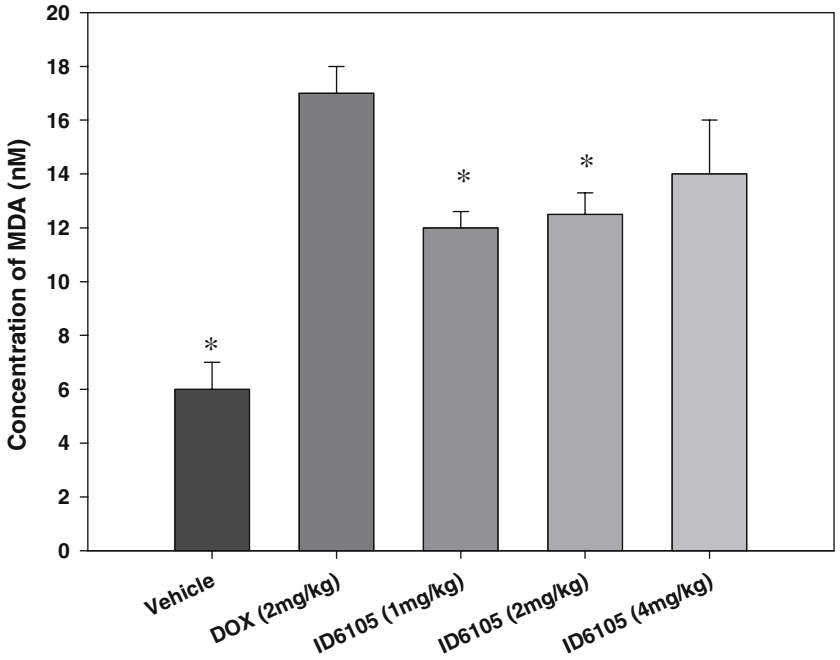

Fig. 5 Lipid peroxidation of male rats treated withQDX5. The level of malondialdehyde) in cardiac tissues was evaluated after acute ID6105 and doxorubicin intoxication. Rats were injected i.v. with QDX5 schedule. *MDA*, malondialdehyde; *DOX*, Doxorubicin. *:vs. doxorubicin group at $P < 0.05$

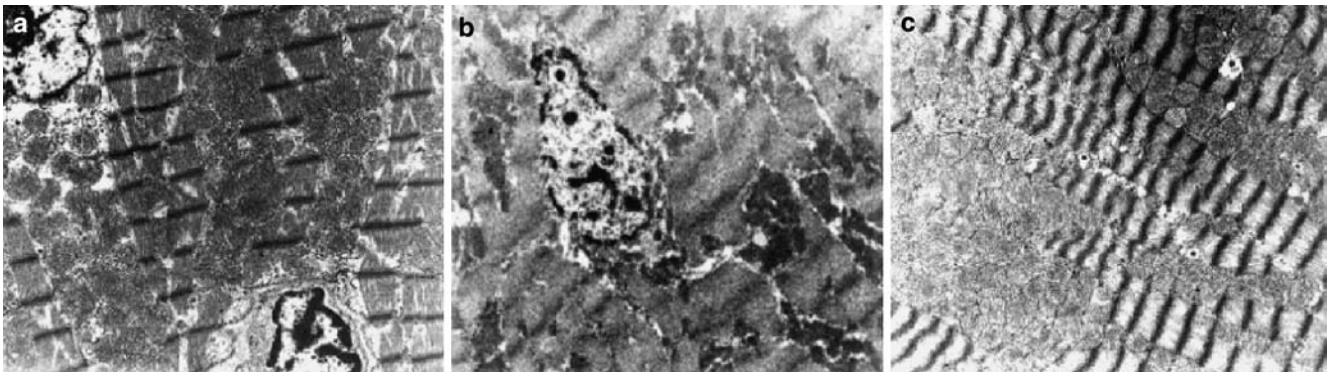

Fig. 6 Electron microscopic findings of myocardium of rat treated with QDX5. **a** Blank, 10 mM sodium acetate buffer: normal arrangement of myofibrils and shape of mitochondria; **b** Doxorubicin-treated, 2 mg/kg: myofibrils are sparse, and intercalated disks are irregular. Also marked proliferation of mitochondria of various sizes is observed. A part of the myocardium has undergone necrosis; **c** ID6105-treated, 2 mg/kg: relatively well preserved myofibrils and mitochondria. Original magnification: ×6,000

a superoxide free radical [6] which gives irreversible damages to normal cells by lipid peroxidation. The products of lipid peroxidation such as malondialdehyde (MDA) can increase the cell membrane permeability to hurt membrane proteins, thus inactivating receptors and membrane-bound enzymes in cardiac muscle cells. The endomyocardial biopsy experiments of doxorubicin presented characteristic changes, including extensive depletion of myofibrillar bundles, myofibrillar lysis, distortion and disruption of the Z-lines, mitochondrial swelling and particularly swelling and disruption of the sarcoplasmatic reticulum, leading to intramyocyte vacuolisation [8, 14, 15]. The levels of MDA in ID6105-treated groups (qdX5, 1, 2 mg/kg) were significantly lower than doxorubicin group (qdX5, 2 mg/kg). Our finding on the cardiac tissues of rats by electron microscopy revealed that 2 mg/kg of ID6105 (qdX5) did not adversely affect to any normal conditions but the same dose of doxorubicin induced a loss of striated muscle, intercalated disc, and swelling and ablation of outer membrane in mitochondria. These results suggested that the cardiotoxicity of ID6105 was lower than doxorubicin at an effective dose.

In conclusion, our findings suggest that ID6105 is a promising anti-cancer agent with many structural advantages over doxorubicin on its cytotoxic effects through in vitro and in vivo tests and favorable profiles of its cardiotoxicity, However, we need to evaluate further in a preclinical study.

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