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ORIGINAL ARTICLE

Apoptosis induced by ID6105, a new anthracycline (11-hydroxyaclacinomycin X, Hyrubicin), and its anti-tumor effects on experimental tumor models

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Abstract A new anthracycline ID6105 (11-hydroxyaclacinomycin X, Hyrubicin), which has potent antitumor activities against a broad range of cancer cell lines, was produced by hybrid biosynthetic approach. We investigated ID6105-induced apoptosis and in vivo efficacy on experimental tumors, and also defined its optimal dosing schedule. From PARP cleavage assay and caspase-3 activation assay, we found that ID6105 can induce apoptosis in tumor cells and its ability was superior to doxorubicin. In human tumor xenograft models, ID6105 showed greater antitumor effects on SW620 and NCI-H23 than doxorubicin. The 1 mg/kg of ID6105 treatment reduced size of tumor by 93% in NCI-H23 model whereas doxorubicin (2 mg/kg) showed only 39% inhibition rate. In SW620 model, 0.3 mg/kg of ID6105 proved to be comparable to 2 mg/kg of doxorubicin. Testing with several dosing schedule such as qd10, qd5, and q4d3, we decided intravenous qd5 treatment was an optimal schedule as a dose regimen of ID6105. In conclusion, ID6105 is a potent apoptosis-inducing anthracycline and effective in treatment of tumors with qd5 dosing schedule.

Keywords ID6105 · Hyrubicin · Anthracycline · Apoptosis · Xenograft · Biopsied cancer cell

Abbreviations

ID6105	11-hydroxyaclacinomycin X
Dox	Doxorubicin
IR	Inhibition rate

Introduction

The anthracycline antibiotics derived from *Streptomyces*, such as daunorubicin [1], doxorubicin [2], were introduced into clinical practice more than 20 years ago. Since then, with characteristic profiles including broad spectrum of activity, they have been one of the most prevalent anticancer agents in the treatment of cancer patients [1, 3, 4]. However, due to its cumulative and dose-related cardiomyopathy [5], their medical application has been refrained. New strategies have been designed to circumvent these side effects including alterations in scheduling, administration of cardio-protective agents and advanced methods for monitoring cardiac abnormalities, as well as development of less toxic and powerful analogues [6–8], no such problems have been resolved as yet.

The aclacinomycin X, 7-(*O*-rhodosaminyl-deoxyfucosyl-redosyl)-aklavinone, and 11-hydroxyaclacinomycin X are novel aclacinomycin analogues as

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previously described [9, 10]. They were obtained from *Streptomyces galilaeus ATCC 31133*. In particular, 11-hydroxyaclacinomycin X was transformant of this strain containing the aclavinone 11-hydroxylase gene, *dnrF* [10]. Two novel aclacinomycin analogues, especially 11-hydroxyaclacinomycin X, showed a strong cytotoxic activity against human tumor cells [9]. We reported the pharmacokinetic characteristics of ID6105 in rats and dogs, which indicated that ID6105 was rapidly cleared from the blood and transferred to tissues, and the majority of ID6105 appears to be excreted in the bile [11, 12]. In addition, the MDA assay and histopathological studies showed that the cardiotoxicity induced by ID6105 was less serious than that by doxorubicin [13].

In the present study, we investigated the ID6105's ability to induce apoptosis and measured antitumor activities of ID6105 in vivo xenograft models, and also tried to find out its appropriate dose regimen.

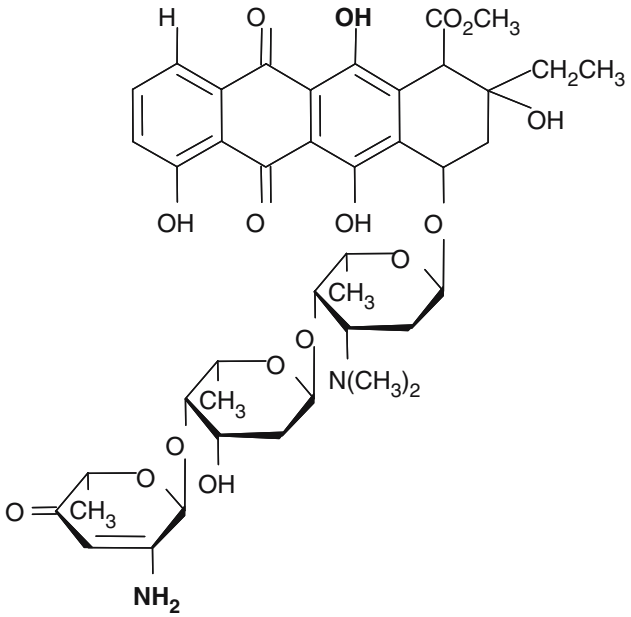

Fig. 1 Structure of ID6105 (11-hydroxyaclacinomycin X)

Materials and methods

Chemicals

ID6105 was manufactured by KRIBB (Korea Reasearch Institute Bioscience and Biotechnology, Taejon, Korea). Doxorubicin hydrochloride was supplied by IlDong Pharmaceutical Co.,Ltd (Seoul, Korea). ID6105 was dissolved in 10 mM sodium acetate buffer (pH 4.5) containing 5% maltose and stored in –20°C. Doxorubicin hydrochloride was kept in 4°C and diluted in sterile saline. All drugs were dissolved and reconstituted just before use. Figure 1 shows the structure of ID6105.

Cell lines

For human tumor xenograft models, human colon cancer (SW620), prostate cancer (PC-3), and lung cancer (NIH-H23) were cultured. Human lung cancer (A549) and human colon cancer (HCT-116) were used in apoptosis studies. All cell lines were grown and maintained at 37°C, 5% CO_2 in Dulbeco's Modified Eagles Medium (Hyclone, UT, USA) with 10% fetal bovine serum (Hyclone).

Animals

All animals in these studies were provided by Charles River (Orient, Seoul, Korea). Female BALB/c-*nu* mice weighing 20–25 g, were used for xenograft models. 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 Pharmaceutical Co. and performed under the guidelines of Korea Food & Drug Administration (KFDA).

Western blot

Cells (HCT-116 and A549) were pre-incubated at 37°C in 5% CO_2 for 24 h before treatment. After the incubation with drugs for 16 h, the cells were harvested, washed with PBS and lysed for 30 min at 4°C in a lysis buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors (Sigma, St Louis, MO, USA). The amount of protein in each sample was measured by BCA method. The lysates (70 μg) were separated by 8% SDS-PAGE and transferred to a PVDF membrane. The blot was incubated overnight with a polyclonal antibody to PARP from Cell Signaling Technology Inc., followed by goat anti-rabbit antibody coupled to peroxidase antibody. Bound antibody was visualized by ECL (Amersham Biosciences, Buckinghamshire, England).

Estimation of caspase-3 activity

A549 cells were pre-incubated at 37°C in 5% CO_2 for 24 h before treatment. After the incubation with drugs for 16 h, the cells were harvested and washed with

PBS. By centrifugation, the cell pellet was collected and reconstituted in PBS (9×10^6 cells/sample). Caspase-3 activity was estimated with an ApoProbe Caspase-3 Fluorescent Assay Kit (Peptron, Seoul, Korea) according to the manufacturer's protocol. Caspase-3 activity was expressed as a ratio of the fluorescence of the treated relative to negative controls.

In vivo tumor models

The cultured tumor cells were collected and reconstituted in sterile cold PBS. 5×10^6 cells per mouse were implanted s.c. into the right axillary region of mouse. Only those animals with tumor of 100–200 mm^3 were used.

Treatment and assessing antitumor activity

The treatment began when tumor was palpable (100–200 mm^3). The chemotherapy was administered according to the respective dose regimens. When the tumors of the controls reached 1,000 mm^3, the study was terminated, and the inhibition rates (IR) were calculated as follows:

$$\text{Inhibition rate, IR (\%)} = 100 \times (C - T)/C$$

where T is the average tumor volume of the treated and C the average tumor volume of the vehicle.

The tumor volume 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: $L \times W^2/2$

Results

PARP cleavage assay

120 nM of ID6105 cleaved PARP, but PARP cleavage was barely detected with 360 nM of doxorubicin in A549. In HCT-116 cell line, PARP cleavage appeared with 40 nM of ID6105 and 440 nM of doxorubicin, respectively. The positive control was 10 nM camptothecin and negative control was DMSO (Fig. 2).

Evaluation of the caspase-3 activity

The caspase-3 activity was increased after treatment with ID6105 for 16 h in A549 cells (Fig. 3). The caspase-3 activity increased 1.2-fold at 120 nM of ID6105, but doxorubicin did not make any changes. At this concentration, ID6105 induced PARP cleavage in A549.

ID6105 induced 1.5-fold induction of caspase-3 activity at 240 nM, while doxorubicin only 1.1-fold induction, which was not significant.

From both results, we found that ID6105 induced apoptosis, whose activity was greater than doxorubicin.

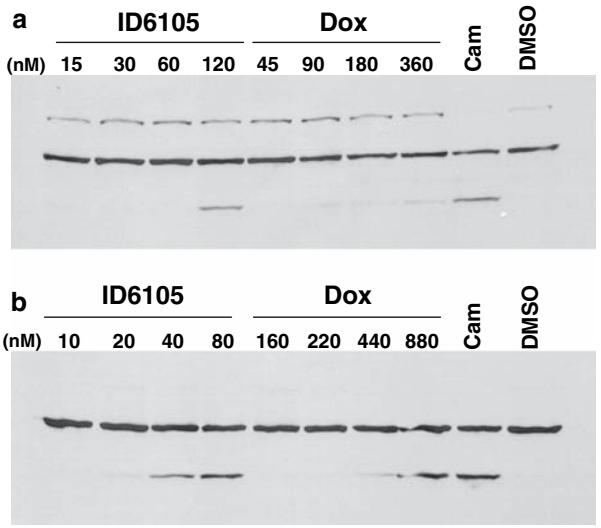

Fig. 2 The effects of ID6105 on PARP cleavages in A549 and HCT-116. The cells were treated with ID6105 or doxorubicin at the indicated concentrations for 16 h (**a** A549, **b** HCT-116). The camptothecin and DMSO were used as a positive and negative controls, respectively. *Dox* doxorubicin, *Camp* 10 nM camptothecin

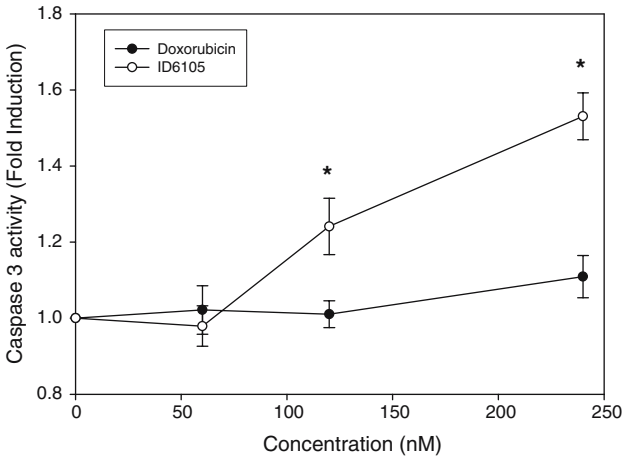

Fig. 3 Caspase-3 activity in A549 cells treated with ID6105. The cells were treated for 16 h with ID6105 or doxorubicin at the indicated concentrations. The results were expressed as a ratio of the fluorescence of the treated relative to negative control (DMSO). *Significantly different from doxorubicin group, $P < 0.05$ by t test

In vivo studies

Efficacy study

Table 1 summarized the results from two xenograft models—SW620, NCI-H23. We injected both drugs for consecutive 10 days intraperitoneally. The high dose of ID6105 decreased the size of tumor by 93% in NCI-H23 model whereas doxorubicin (2 mg/kg) showed only 39% inhibition rate. In the other model, SW620, 0.3 mg/kg of ID6105 proved to be comparable to 2 mg/kg of doxorubicin.

Schedule-defining study

The dose regimens tested were qd10, qd5, and q4d3. Figure 4 presented the results. Qd10 and qd5 schedule were the most effective, however, the others—Dox and q4d3—did not reduce the tumor size. As the IRs of qd10 and qd5 were similar to each other (data not shown), we adopted qd5 schedule as an ID6105's dosing schedule.

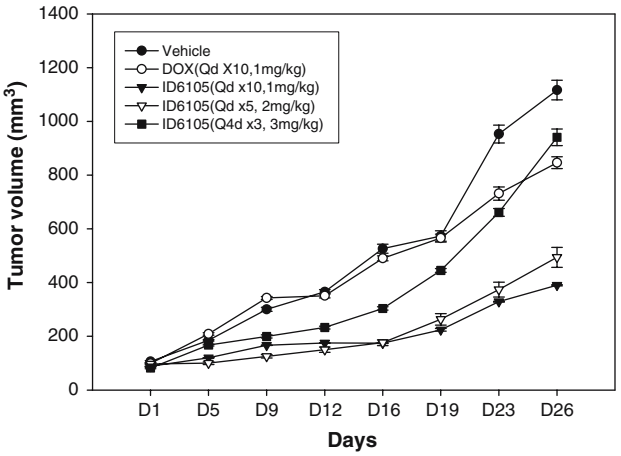

Fig. 4 Growth curves for groups carrying PC-3 tumors treated with different schedules. Tumors were induced in nude mice by s.c. inoculation of tumor cells. Intravenous treatment started when tumor mass reached 100–200 mm^3. Groups were administered with designated schedules respectively. The number of animals was at least six in each group. Vehicle was 10 mM sodium acetate buffer (pH 4.5)

Discussion

ID6105 (11-hydroxyaclacinomycin X), as a new anthracycline produced by *S. galilaeus ATCC 31133* has a remarkable cytotoxicity and very high sensitivity to various human tumor cell lines [9]. Its TGI (total growth inhibition concentration) was reported to be about 20 times more potent than doxorubicin. In terms of cardiotoxicity, a well-recognized clinical problem of anthracyclines, the biochemical and histopathological studies revealed that ID6105 induced milder cardiac damage than doxorubicin [13–15]: the levels of MDA in ID6105-treated groups were significantly lower than doxorubicin, and 2 mg/kg of ID6105 did not adversely affect to normal cardiac tissues. In addition, having structural merits of both doxorubicin and aclacinomycin A such as two amino sugars and 11-hydroxylation of aklavinone, ID6105 showed excellent antitumor efficacy in several human tumor xenograft models [13].

Although the exact mechanism of ID6105 was still unknown, we previously suggested that it blocks DNA synthesis as well as topo II activity like other anthracyclines, and has similar pattern to aclacinomycin A, a catalytic topoisomerase II inhibitor [13, 16, 17]. In this study, to investigate ID6105-induced apoptosis, we measured PARP cleavage and caspase-3 activation in comparison with doxorubicin. PARP is a nuclear enzyme present in higher eukaryotes and some lower eukaryotes, whose cleavage during apoptosis was first described by Kaufmann et al. [18] in tumor cells, and produces an 89 kDa C-terminal fragment and the 24 kDa N-terminal fragment [19]. As the cleavage of PARP is a sensitive parameter of apoptosis, it is a very useful assay for measuring apoptosis [20]. Because its main effectors were known to be caspases and granzyme B, the detection of the 89 kDa fragment of PARP signifies activation of caspase-3-like activity. In PARP cleavage assay, 120 nM of ID6105 induced PARP cleavage in A549 while 40 nM of ID6105

Table 1 Antitumor acitivities of drugs in human tumor xenograft models

	IR (%)			
	ID6105 (mg/kg)			Dox (mg/kg)
	0.1	0.3	1	2
SW620	35	61*	–	50
NCI-H23	19	31	93*	39

The study was designed to evaluate the antitumor efficacy of compound ID6105 against human tumor xenorafts s.c. implanted in female nude mice. There were ten animals in the control and treatment groups. The drugs were administered intraperitoneally for 10 days. The tumor implants grew to 100–200 mm^3 before treatment. When the tumors of the controls reached 1,000 mm^3, the study was terminated, and the IRs were calculated

Dox doxorubicin

Inhibition rate, IR (%) = $100\times(C-T)/C$, where T is the average tumor volume of the treated and C the average tumor volume of the vehicle

*Significantly different from Dox group, $P < 0.05$ by t test

cleaved PARP in HCT-116. But doxorubicin did not induce PARP cleavage in both cell lines as much as ID6105 (Fig. 2.) and the caspase-3 activities induced by ID6105 were significantly higher than that in the same dose of doxorubicin group. These results indicated that by inducing PARP fragmentation in tumor cells and effectively activating caspase-3, in a greater level than doxorubicin, ID6105 can kill tumor cells by apoptosis.

In vivo studies, ID6105 also had inhibited growth of experimental tumors. Two human tumor xenografts—SW620 and NCI-H23—were shrunk by intraperitoneal ID6105 treatment. The tumor size of the ID6105-treated group was reduced up to 7% of the non-treated in NCI-H23 model, and 39% in SW620 model, respectively.

To define the optimal dosing schedule of ID6105, we made use of several dose regimens—qd10, qd5, and q4d3 in PC-3 xenograft model. The qd10 and qd5 schedules were superior to q4d3 schedule in efficacy. But, due to excessive loss of the body weights with qd10 schedule (data not shown), we adopted qd5 treatment as a dosing schedule of ID6105 for drug compliance. From pharmacokinetic studies of ID6105 for rats, we found that it did not accumulate in the body and excreted through bile following single or qd5 i.v. treatment, which implied that ID6105 could be treated in few cycles—qd5 $\times$ 2 or may be qd5 $\times$ 3.

Taking all into account, we suggest that ID6105 is a promising anti-cancer agent with potent apoptosis-inducing effects on tumors, and its in vivo activity was the greatest with qd5 schedule.

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