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Kyoung-Hwa Lim · Hun-Sik Kim · Yong-Man Yang
Sang-Deuk Lee · Won-Bae Kim · Junnick Yang
Jae-Gahb Park

Cellular uptake and antitumor activity of the new anthracycline analog DA-125 in human cancer cell lines

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Abstract To predict the clinical usefulness of DA-125, a newly developed doxorubicin analog, we compared its antitumor activity against 20 different human cancer cell lines with that of doxorubicin using the MTT in vitro chemosensitivity test. We also measured and compared the cellular uptake of this drug and doxorubicin in two cancer cell lines and their doxorubicin-resistant sublines. In the MTT test, DA-125 showed lower IC_{50} values than doxorubicin for 14 of 20 cell lines. DA-125 was more potent than doxorubicin for hepatocellular cancer cells with high *mdr 1* expression. Among cancer cells from the stomach and colon, DA-125 was more potent than doxorubicin in 12 of 14 cell lines. We also investigated the cross-resistance of this drug with doxorubicin using four doxorubicin-resistant cancer cell sublines. Except in one cell line, there was very low cross-resistance. Cellular drug-uptake experiments were performed for two gastric cancer cell lines and their doxorubicin-resistant sublines. In this experiment, DA-125 was found to be very rapidly and completely converted to its active metabolite, M1, in the culture media. After this conversion, M1 was incorporated into these cancer cells more rapidly and reached higher intracellular concentrations than doxorubicin, suggesting that DA-125 (as M1) could achieve earlier and higher levels of intracellular accumulation than doxorubicin in its target tissues from the bloodstream. As a possible alternative antineoplastic agent to doxorubicin, DA-125 awaits further evaluation for its antitumor activity and toxicity.

Key words Doxorubicin analog · DA-125 · Cellular uptake · Multidrug resistance · MTT test · Cross-resistance

Abbreviations *DOX* Doxorubicin · *DA-125* (8*s*, 10*s*)-8-(3-aminopropanoyloyacetyl)-10-{(2,6-dideoxy-2-fluoro-α-L-talopyranosyl)oxy}-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5, 12-naphthacenedione · *ME2303* (8*s*,10*s*)-8-(6-carboxyhexanoyloxyacetyl)-10-{(2,6-dideoxy-2-fluoro-α-L-talopyranosyl)oxy}-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione · *M1* 7-*O*-(2,6-dideoxy-2-fluoro-α-L-talopyranosyl) adriamycinone · *MTT* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoline bromide · *MDR* multidrug resistance · IC_{50} drug concentration that inhibits cell growth by 50%

Introduction

Doxorubicin, an anthracycline antibiotic, is a widely used anticancer chemotherapeutic agent and is highly active against many tumors [5, 12]. However, its use is restricted due to its undesirable side effects, such as myelosuppression and cardiotoxicity [7]. The development of multidrug resistance (MDR) [13, 26] is another major obstacle to the satisfactory therapeutic application of this drug. Efforts have therefore been made to synthesize or isolate new anthracycline derivatives that are less toxic and more active against tumors expressing the MDR phenotype.

A new fluorine-containing anthracycline derivative, ME2303, was developed by Meiji Seika Kaisha Ltd. (Tokyo, Japan) [30, 31]. It is a 2-fluoroglycoside of doxorubicin [7-*O*-(2,6-dideoxy-2-fluoro-α-L-talopyranosyl)adriamycinone-14-hemipimerate] and has shown more potent antitumor activity in several in vivo and in vitro studies [9, 10, 19]. ME2303 is hydrolyzed by

K.-H. Lim · Y.-M. Yang · J.-G. Park (✉)
Laboratory of Cell Biology, Cancer Research Institute and Cancer Research Center, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-744, Korea Tel: (82-2) 760-3380; Fax: (82-2) 742-4727

H.-S. Kim
Department of Pharmacology, College of Medicine, Chungbuk National University, Gaesin-dong 48, Cheongju, Chungcheongbuk-do 360-763, Korea

S.-D. Lee · W.-B. Kim · J. Yang
Research Laboratory, Dong-A Pharmaceutical Company, Ltd., 47-1 Sanggal-ri, Kiheungup, Yongin-gun, Kyunggi-do 449-900, Korea

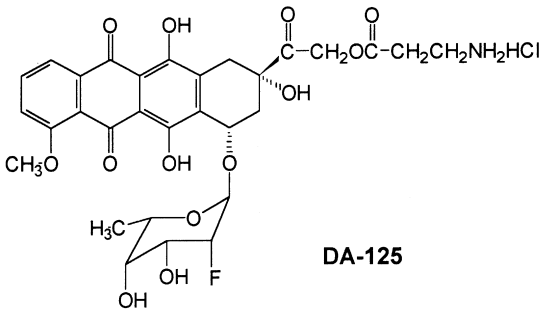

Fig. 1 Chemical structure of DA-125

serum esterase to its active metabolite, 7-*O*-(2,6-dideoxy-2-fluoro-α-L-talopyranosyl)adriamycinone (M1) [10]. M1 is reported to be more active than ME2303 and is taken up rapidly into tumor cells, reaching a high intracellular concentration [16]. The higher antitumor activity of ME2303 was thus attributed to this esterolytic conversion process to M1 [16].

The Dong-A Pharmaceutical Company Research Laboratory (Yongin, South Korea) has recently developed another fluorine-containing doxorubicin analog, DA-125 [(8s, 10s)-8-(3-aminopropanoyloyacetyl)-10-{(2,6-dideoxy-2-fluoro-α-L-talopyranosyl) oxy}-7,8,9,10-tetrahydro -6, 8, 11-trihydroxy-l-methoxy-5, 12-naphthacenedione], which is a water-soluble prodrug (β-alanine derivative) of M1 (Fig. 1). After intravenous administration, DA-125 is rapidly converted in the plasma to M1 (the only active metabolite), with a degradation half-life of 0.54 min in humans [27, 28, 34]. M1 is further metabolized to M2 and M3, both of which are then converted to M4 [17, 27]. Both in vitro and in vivo preclinical tests of DA-125 showed that its antitumor activity was superior to that of doxorubicin (unpublished results). DA-125 was also reported to have lower cardiotoxicity and hematotoxicity than doxorubicin [2, 3].

In the present study, to predict its clinical usefulness as a new antineoplastic agent, we compared the antitumor activity of DA-125 with that of doxorubicin in 20 different human cancer cell lines derived from carcinomas of the stomach, liver, and colon using the MTT in vitro cytotoxicity test. For these cell lines the levels of *mdr 1* gene expression were also measured by mRNA slot-blot analysis. These values were compared with the IC_{50} values obtained in the MTT cytotoxicity test for DA-125 and doxorubicin so as to determine whether there might be any correlation between the multidrug-resistance (MDR) phenotype and chemosensitivity to these two drugs. In addition, we evaluated its cross-resistance to doxorubicin by comparing the antitumor activity of doxorubicin against four doxorubicin-resistant cancer cell sublines with that of DA-125. Finally, the intracellular accumulation of DA-125 and its metabolites by two cancer cell lines and their doxorubicin-resistant sublines was measured and compared with that of doxorubicin.

Materials and methods

Drugs and chemicals

Doxorubicin hydrochloride was obtained from commercial sources (Adriamycin; Adria Laboratories, Inc., Columbus, Ohio). DA-125 was synthesized and provided by the Research Laboratory of Dong-A Pharmaceutical Co. Ltd. These drugs were dissolved or diluted in phosphate-buffered saline (PBS) just prior to their use. MTT, trichloroacetic acid (TCA), NaN_3, and dimethylsulfoxide (DMSO) were supplied by Sigma Chemical Co. (St. Louis, Mo.).

Cell lines and culture conditions

A total of 20 human cancer cell lines originating from gastric (SNU-1, SNU-5, SNU-16, SNU-484, SNU-620, SNU-638, and SNU-719), hepatocellular (SNU-182, SNU-354, SNU-387, SNU-368, SNU-398, and SNU-423), and colonic (SNU-C2A, SNU-C4, SNU-C5, SNU-61, SNU-81, SNU-175, and SNU-1047) carcinomas were used in this study. They were established at the Cancer Research Center and the Cancer Research Institute of Seoul National University College of Medicine, Seoul, Korea. The general characteristics of some cell lines (SNU-1, -5, -16, -C2A, -C4, -C5, -182, -354, -387, -368, -398, and -423) have been reported elsewhere [21, 22, 25]. Descriptions of the establishment and characterization of other cell lines are in preparation (Park et al., manuscript in preparation). Cells were grown in RPMI-1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Logan, Utah) in a humidified incubator at 37 °C under an atmosphere comprising 5% CO_2 and 95% air.

Four doxorubicin-resistant cancer cell sublines (SNU-1/DOX250, SNU-16/DOX250, SNU-C4/DOX1000, and SNU-C2A/DOX1000) were derived from their parental cell lines by continuous exposure to increasing concentrations of doxorubicin (up to 250 ng/ml for SNU-1 and -16 and 1,000 ng/ml for SNU-C2A and -C4).

mdr 1 RNA measurement

Expression of the *mdr 1* gene was measured by slot-blot analysis using total RNA. The human *mdr 1* cDNA, MDR pHDR5A [33], was used as a probe. Total RNA was extracted by the guanidinium thiocyanate method with some modifications [6]. RNA samples were denatured at 68 °C for 15 min in a mixture of formamide and formaldehyde and were applied to nitrocellulose filters using a slot-blot kit (Shleicher & Schuell Co., Minifold II). Four different concentrations (10, 3, 1, and 0.3 μg) of total RNA samples were loaded on duplicate filters. After baking in a vacuum oven for 2 h at 80 °C, the filters were prehybridized at 42 °C for 4 h in a solution containing 50% formamide, 5 × SSC, 50 m*M* sodium phosphate (pH 6.5), 5 × Denhardt's solution, and salmon-sperm DNA (200 μg/ml). The filters were then probed with ^{32}P-radiolabeled *mdr 1* and β-actin probes. The specific activity of labeling was 3×10^8 cpm/μg DNA, and the denatured probes were added to make 5×10^6 cpm/ml of hybridization solution. After hybridization for 18 h at 42 °C, the filters were washed, dried, and then autoradiographed with Kodak XAR-5A films at −70 °C for 5 days. Hybridization with a β-actin RNA probe was used to correct for RNA loading. The autoradiograms were analyzed and quantitated by video densitometer (model 620; Bio-Rad Laboratories, Richmond, Calif.). The autoradiographic signal densities were compared with those of KB-3-1 and KB-8-5 cell lines. A value of 1 U was assigned to the signal given by 10 μg of KB-3-1 RNA, and 30 U was assigned to the signal given by 10 μg of KB-8-5 RNA [8]. According to these arbitrary units, the expression level of *mdr 1* was classified as either "low" for values below 5 U, "intermediate" for values of 5–30 U, or "high" for values above 30 U.

MTT cytotoxicity assay

A colorimetric assay using the tetrazolium salt MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide) was used to assess the cytotoxicity of DA-125 and doxorubicin. The MTT assay was performed as previously described [4, 18, 20]. The optimal number of cells to be plated into 96 wells was determined by preliminary studies such that cells were in the exponential growth phase at the end of the 4-day period of incubation. A single-cell suspension was prepared and the cell density was measured. An equal number of cells were inoculated into each well, to which drugs or PBS (for 100% survival control) were added. For each drug, five to ten concentrations were used, spanning a range of 3–5 log concentrations. After 4 days of culture, 0.1 mg (50 μl of a 2-mg/ml solution) of MTT (Sigma Chemical Co., St. Louis, Mo.) was added to each well and then incubated at 37 °C for a further 4 h. Plates were centrifuged at 450 *g* for 5 min at room temperature and the media was then aspirated, with care being taken not to disturb the formazan crystals at the bottom of the wells. Next, 150 μl of DMSO (Sigma) was added to each well to solubilize the formazan crystals. The plates were read immediately at 540 nm on a scanning multiwell spectrophotometer (Bio-Tek Instruments Inc., Burlington, Vt.). All experiments were performed three times and the mean absorbance values for each drug concentration were calculated. The IC_{50} value was defined as the drug concentration that produced a 50% reduction of absorbance at 540 nm in drug treated cells as compared with untreated controls.

Determination of intracellular drug uptake

Intracellular drug uptake into the cell was determined by fluorometry as described by Kunimoto et al. [14, 15]. Tumor cells were harvested, washed with RPMI-1640 medium, and suspended in RPMI-1640 medium containing 10% FBS and antibiotics. For uptake experiments, cell suspensions ($2–5 \times 10^6$/ml, 1 ml) were incubated with drug solution (30 μ*M*) at 37 °C. Uptake was terminated by centrifugation at 12,000 *g* for 30 s in a microcentrifuge. After being washed with cold PBS, the cells were suspended in PBS and then disrupted by ultrasonication. Drugs taken into the cells were extracted from 0.8 ml of the sonicated cell suspension by the addition of 2 ml of 40% TCA (Sigma, St. Louis, Mo.) followed by overnight incubation. The incubated mixtures were centrifuged and the fluorescence intensity of the supernatant at 550 nm was determined using a spectrofluorophotometer (RF-510, Shimadzu) with an excitation wavelength of 470 nm. The molar concentrations of drugs were calculated by comparison with those of standard solutions treated in the same manner. For more precise determination of the relative contribution of DA-125 and its metabolites (M1, M2, M3, and M4) to the fluorescence we measured, we also analyzed in separate experiments under the same conditions the culture fluid and cell extracts by a high-performance liquid chromatograph (HPLC) connected with a fluorescence detector (Linear fluor LC304, Linear, Reno Nev. USA), which was set at an excitation wavelength of 488 nm and an emission wavelength of 556 nm. The detailed procedures of HPLC analysis were as described in previous reports [17, 28].

Results

mdr 1 mRNA expression of human cancer cell lines used in this study

Among the 20 cell lines used in this study, *mdr 1* expression by 12 lines (SNU-1, -5, -16, -C2A, -C4, -C5, -182, -354, -368, -387, -398, and -423) had previously been reported [23, 24]. For eight other cell lines and four doxorubicin-resistant sublines (SNU-1/DOX250, SNU-16/DOX250, SNU-C2A/DOX1000, and SNU-C4/DOX1000) we measured *mdr 1* mRNA levels using the slot-blot procedure to reveal any relationship between *mdr 1* expression and chemosensitivity to the two drugs compared in this study, DA-125 and doxorubicin. The results, expressed in three categories (low, intermediate, high) as described in Materials and methods, are presented in Tables 1 and 2 together with data from previous studies.

No gastric cancer cell line showed high or intermediate *mdr 1* expression (Table 1). Among seven colonic cancer cell lines, one (SNU-C4) and two (SNU-61 and -1047) were high- and intermediate-level *mdr 1* expressors, respectively (Table 1). As previously reported [24], two of six hepatocellular cancer cell lines (SNU-354 and -356) were high in *mdr 1* expression.

Comparison of antitumor activities of doxorubicin and DA-125

Table 1 shows the cytotoxicity of doxorubicin and DA-125 against 20 human cancer cell lines as determined by MTT assay. The results represent mean IC_{50} values ± SD for three experiments. In 10 of 20 cancer cell lines the cytotoxicity of DA-125 was more than twice that of doxorubicin (Potency ratios > 2.0). In six cell lines, DA-125 showed lower cytotoxicity than doxorubicin (potency ratios < 1.0). The other four cell lines showed a slightly higher level of resistance to doxorubicin (potency ratios 1.0–1.5). The differences in IC_{50} values were most striking in the case of SNU-C5, followed by SNU-1047 (potency ratios 22 and 10.05, respectively), both of which derive from colorectal cancers. Several colorectal (SNU-C4, -C5, -1047) and hepatocellular cell lines (SNU-354, -368, -387) displayed considerable resistance to doxorubicin, showing IC_{50} values higher than 1.0 μg/ml. For these cells, especially those from colorectal cancers, DA-125 was more active than doxorubicin.

Among the six hepatocellular carcinoma cell lines, differences found between the IC_{50} values recorded for the two drugs were much greater in cells with high levels of *mdr 1* gene expression (SNU-354 and -368) than in those expressing low levels of *mdr 1* (Table 1). There seemed to be no such close correlation among cancer cell lines from the stomach and colon. For example, SNU-C5, SNU-1, and SNU-620, in which *mdr 1* expression was low, showed large differences between the IC_{50} values noted for the two drugs (Table 1).

Cross-resistance of DA-125 to doxorubicin in doxorubicin-resistant sublines

Table 2 shows the results obtained from the in vitro cytotoxicity assays of doxorubicin and DA-125 against two gastric and two colorectal cancer cell lines and their doxorubicin-resistant sublines. In doxorubicin-resistant sublines of gastric origin (SNU-1 and -16), *mdr 1*

Table 1 Comparison of the in vitro antitumor activity of doxorubicin and DA-125 in several human cancer cell lines

Cell lines	*mdr 1* Expression[a]	IC_{50} (mean ± SD, ng/ml)		Potency ratio[b]
		Doxorubicin (A)	DA-125 (B)	A/B
Stomach:				
SNU-1	L [23]	14.7 ± 0.58	2.33 ± 2.31	6.29
SNU-5	L [23]	62.7 ± 23.2	43.7 ± 13.4	1.44
SNU-16	L [23]	12.4 ± 5.25	43.7 ± 23.5	0.28
SNU-484	L[c]	67.5 ± 30.2	50.4 ± 36.2	1.34
SNU-620	L[c]	356 ± 293	71.0 ± 34.2	5.02
SNU-638	L[c]	90.7 ± 63.2	22.0 ± 13.9	4.12
SNU-719	L[c]	224 ± 18.6	265 ± 9.07	0.85
Colon:				
SNU-C2A	L [23]	426 ± 300	327 ± 247	1.30
SNU-C4	H [23]	1,580 ± 683	278 ± 61.8	5.69
SNU-C5	L [23]	4,620 ± 4,520	210 ± 160	22.0
SNU-61	I[c]	407 ± 260	313 ± 151	1.30
SNU-81	L[c]	793 ± 321	259 ± 195	3.06
SNU-175	L[c]	33.3 ± 25.8	16.3 ± 7.23	2.04
SNU-1047	I[c]	1,300 ± 177	129 ± 101	10.1
Liver:				
SNU-182	L [24]	123 ± 110	314 ± 272	0.39
SNU-354	H [24]	18,650 ± 5,520	2,230 ± 478	8.38
SNU-368	H [24]	33,300 ± 34,520	4,750 ± 4,060	7.02
SNU-387	L [24]	1,990 ± 1,380	2,200 ± 478	0.90
SNU-398	L [24]	120 ± 17.6	171 ± 74.5	0.71
SNU-423	L [24]	90.0 ± 106	243 ± 177	0.37

[a]Determined by slot-blot analysis of mRNA: *L* low (below 5 U), *I* intermediate (5–30 U), *H* high (above 30 U) levels of *mdr 1* mRNA expression (see Materials and methods for details)

[b]Ratio of IC_{50} values for the two drugs (doxorubicin/DA-125)

[c]Data obtained in the present study

Table 2 Comparison of IC_{50} and relative resistance values obtained for doxorubicin and DA-125 in doxorubicin-resistant sublines

Cell lines	*mdr 1* Expression[a]	IC_{50} (mean ± SD, ng/ml)	
		Doxorubicin	DA-125
SNU-1	L [23]	14.7 ± 0.58	2.33 ± 2.31
SNU-1/DOX250	L[c]	712 ± 130	156 ± 53.2
Relative resistance[b]		48.57	66.71
SNU-16	L [23]	12.4 ± 5.25	43.7 ± 23.5
SNU-16/DOX250	L[c]	462 ± 201	74.9 ± 9.04
Relative resistance		37.39	1.71
SNU-C2A	L [23]	426 ± 300	327 ± 247
SNU-C2A/DOX1000	H[c]	> 100,000	1,660 ± 305
Relative resistance		> 100	5.08
SNU-C4	H [23]	1,579 ± 683.2	278 ± 61.8
SNU-C4/DOX1000	H[c]	> 100,000	1,356 ± 265.9
Relative resistance		> 100	4.88

[a]Determined by slot-blot analysis of mRNA: *L* low (below 5 U), *I* intermediate (5–30 U), *H* high (above 30 U) levels of *mdr 1* mRNA expression (see Materials and methods for details)

[b]IC_{50} values for the resistant cell line/parent cell line of each drug

[c]Data obtained in the present study

expression levels were low, suggesting that other resistance mechanisms are involved. All the doxorubicin-resistant sublines proved to have > 35-fold higher IC_{50} values for doxorubicin than did their parental cell lines. When the IC_{50} values recorded for the two drugs were compared, the SNU-1 subline resistant to doxorubicin (SNU-1/DOX250) was 48.6 times more resistant to doxorubicin and 66.7 times more resistant to DA-125 than its parental cell line (SNU-1). In contrast, the SNU-16 subline resistant to doxorubicin (SNU-16/DOX250) was only 1.7 times more resistant to DA-125, whereas the cells were 37.4 times more resistant to doxorubicin. As a result, in the doxorubicin-resistant subline of SNU-16, DA-125 was more active than doxorubicin, whereas in the parental cell line, doxorubicin was more active (Table 1; potency ratio reversed from 0.28 to 6.17). Both

of the colorectal cell sublines resistant to doxorubicin (SNU-C2A/DOX1000 and SNU-C4/DOX1000) were high-level *mdr 1* expressors (Table 2). They were about 5 times more resistant to DA-125 and over 100 times more resistant to doxorubicin. Hence, except for SNU-1/DOX250, which showed a high degree of cross-resistance to DA-125, the others showed very low degrees of cross-resistance to doxorubicin and DA-125. In the case of SNU-1/DOX250, however, the IC_{50} value recorded for DA-125 (155.7 ng/ml) was also much lower than that noted for doxorubicin (712.3 ng/ml).

Cellular uptake of DA-125 and doxorubicin

The uptake of DA-125 by SNU-1, SNU-16, SNU-1/DOX250, and SNU-16/DOX250 cells in RPMI-1640 medium was compared with that of doxorubicin. In preliminary experiments, 99.9% of DA-125 was demonstrated to be hydrolyzed to M1, the only active metabolite, within 3 min in the culture media used in this study. The half-life for this conversion was 15.3 s. Subsequent conversion processes of M1 to other metabolites were very slow, the elimination half-life of M1 being 40.5 h. These data clearly show that after the addition of DA-125, the major chemical form taken up by cells is M1, not DA-125 or other metabolites such as M2, M3, or M4. As shown in Fig. 2A, B, DA-125 (after conversion to M1) was incorporated into SNU-1 and SNU-1/DOX250 cells more rapidly than doxorubicin, and it maintained much higher intracellular concentrations in both cell lines. Intracellular concentrations of DA-125 reached the saturation point at 120 min after the beginning of its uptake in both cell lines, but doxorubicin continued to accumulate slowly in both lines. Accumulation levels observed for DA-125 at 120 min were 4.6 and 5.0 times higher than those seen for doxorubicin in SNU-1 and SNU-1/DOX250, respectively. To verify that the fluorescence obtained in DA-125 uptake experiments was emitted by M1, we also analyzed the cell extract after drug uptake with HPLC and fluorescence detector. Almost all the fluorescence was due to M1; other inactive metabolites were not detectable.

In experiments with SNU-16 and its doxorubicin-resistant subline, treatment with DA-125 resulted in a more rapid and higher intracellular accumulation of fluorescence than did incubation with doxorubicin (Fig. 2C,D). In comparison with SNU-1 and SNU-1/DOX250,DA-125 reached its saturation point much faster (30 min) in SNU-16 and SNU-16/DOX250. Both drugs reached lower levels in doxorubicin-resistant sublines (SNU-1/DOX250 and SNU-16/DOX250) than

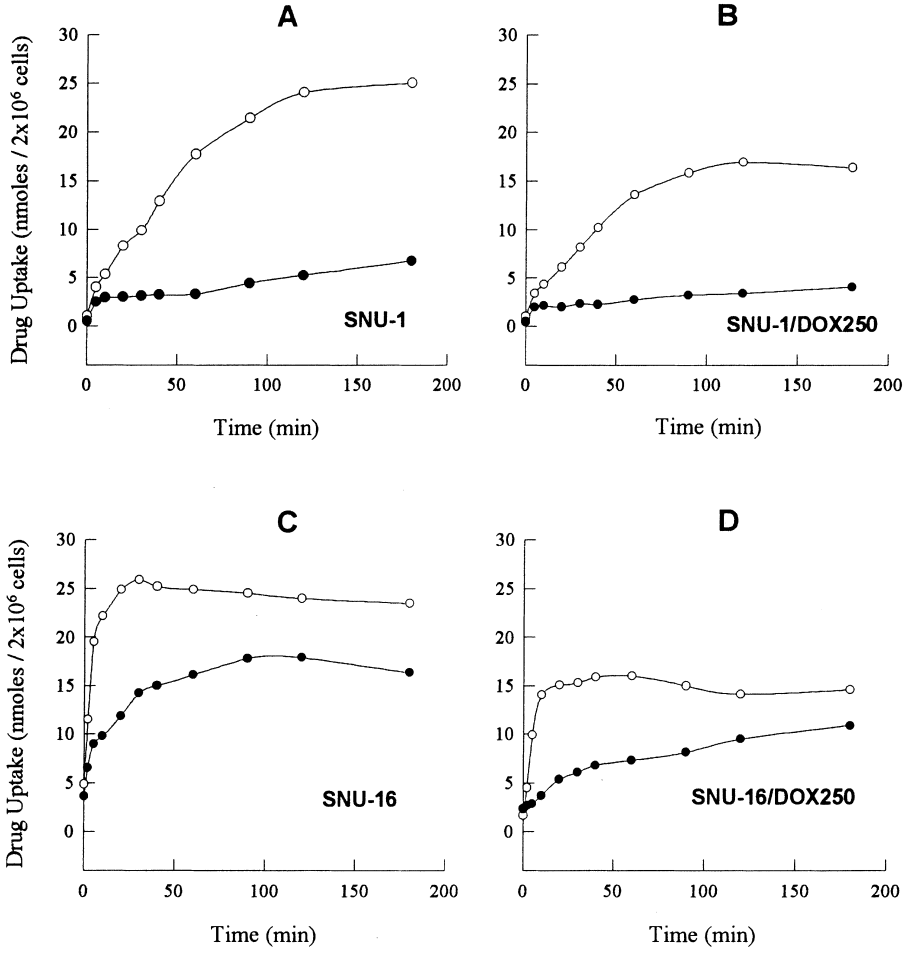

Fig. 2A–D Drug uptake of DA-125 (○) and doxorubicin (●) by **A** SNU-1, **B** SNU-1/DOX250, **C** SNU-16, and **D** SNU-16/DOX250 cells. Drug uptake was determined by incubation of a cell suspension (2×10^6 cells/ml, 1 ml) with a drug solution (30 μM) in RPMI1640 medium. The amount of drug taken up by cells was measured by fluorescence

in their nonresistant parental cell lines. In cell extracts of SNU-16 and its doxorubicin-resistant sublines, however, other metabolites (M2 and M4) were also found by HPLC analysis in separate experiments (data not shown). In these cells the composition of M1 was lowest at 180 min of uptake (79% for SNU-16 and 83% for SNU-16/DOX250). Considering the relative molar fluorescence efficacy values of these metabolites (M1, 1.00; M2, 1.06; M4, 1.21), errors in fluorometric measurement in DA-125 uptake experiments were 22.9% for SNU-16 and 17.8% for SNU-16/DOX250, respectively. This means that M1, the only active metabolite of DA-125, accounted for only 77.1% and 82.2% of the fluorescence, respectively. When these errors were taken into account, M1 reached 1.16 and 2.3-fold higher levels than doxorubicin at 20 min after the DA-125 uptake experiment in SNU-16 and SNU-16/DOX250, respectively. Hence, the intracellular accumulation of M1 was also more rapid and higher than that of doxorubicin in these cell lines.

Discussion

Fluorine-containing anthracyclines, containing 2-fluoroglycosides, were developed to confer resistance to chemical hydrolysis, to increase their antitumor activity, and to decrease the cardiotoxicity of doxorubicin [30]. 7-*O*-(2,6-Dideoxy-2-fluoro-α-L-talopyranosyl)adriamycinone (M1) was one of such compounds showing weak toxicity, but it was sparingly soluble in water, rendering it unsuitable for parenteral use. This poor water solubility of M1 prompted the need to develop water-soluble prodrugs. ME2303 is another such drug; its hydrolysis in the liver yields M1 [10]. In in vitro chemosensitivity tests, M1 is more cytotoxic than ME2303 [16]. However, the conversion process from ME2303 to M1 seems to be important for the in vivo antitumor activity of ME2303 [10].

To increase the water solubility of M1, C-14 positions were modified with various amino acids at the research laboratory of the Dong-A Pharmaceutical Company (Yongin, Korea). Among them, a β-alanine derivative of M1 (DA-125) was selected for further development because of its chemical stability, strong antitumor activity, and weak toxicity. In in vivo screening tests using L1210 and P388 leukemia-bearing mice, DA-125 showed antitumor activity superior to that of doxorubicin (unpublished results). After intravenous administration, DA-125 is very rapidly hydrolyzed in plasma to M1 [17, 27, 34]. In rats, DA-125 has rapidly disappeared from plasma after intravenous administration, and the concentration of M1, its active metabolite, has reached its peak level within 1 min [27]. M1 is then converted to other metabolites – M2, M3, and M4 [28]; it is the only active metabolite showing antitumor activity equivalent to that of DA-125 and low cardiac toxicity [3] and has been found to be accumulated in tissues, including those of the liver, lung, kidney, and intestine [17, 34].

For prediction of the clinical usefulness of this new anthracycline, DA-125 and doxorubicin were compared in this study for their in vitro antitumor activity against 20 human cancer cell lines derived from gastric, colorectal, and hepatocellular carcinomas. *mdr 1* Gene expression was also measured by mRNA slot-blot analysis for these cells, and these values were compared with the IC_{50} values obtained from MTT cytotoxicity tests of DA-125 and doxorubicin in an attempt to see if there might be any correlation between multidrug resistance and chemosensitivity to these two drugs. We also tested four doxorubicin-resistant cancer cell sublines for their cross-resistance to both drugs. In addition, we measured and compared the cellular uptake and accumulation of DA-125 and doxorubicin in two cancer cell lines and their doxorubicin-resistant sublines to evaluate the kinetics of both drugs in control and doxorubicin-resistant cancer cell sublines.

The results of the in vitro chemosensitivity test performed in this study indicate that for some cancer cells the antitumor activity of DA-125 is higher than that of doxorubicin (Table 1). In tests against various human cancer cell lines, IC_{50} values recorded for DA-125 were lower than those noted for doxorubicin in 14 of 20 cell lines. IC_{50} values obtained for DA-125 are generally lower in cells from gastric carcinomas, especially those with poor differentiation (SNU-1, -5, -16) [22, 23]. Among six hepatocellular carcinoma cell lines, differences between the IC_{50} values recorded for the two drugs were much greater in cells with high levels of *mdr 1* gene expression (SNU-354 and -368). On the other hand, other hepatocellular cancer cell lines showing little difference in resistance to the two drugs were reported to be low-level *mdr 1* expressors (Table 1). Thus, it could be concluded that DA-125 is more potent than doxorubicin against hepatocellular cancer cells with high levels of *mdr 1* expression. It should also be noted, however, that the IC_{50} values recorded for DA-125 against these two cell lines (SNU-354 and -368) were too high for effective use of this analog in clinical settings. There seems to be no such close correlation among cancer cells lines from the stomach and colon (Table 1). For these cells, DA-125 seemed to be generally more cytotoxic than doxorubicin, regardless of their level of *mdr 1* expression.

A comparison of the cytotoxicity of the two drugs in doxorubicin-resistant sublines of two stomach and two colorectal cancer cells showed that DA-125 was not cross-resistant to doxorubicin in three cell lines (Table 2). There was one exception, however, SNU-1/DOX250 was 66.7 times more resistant to DA-125 than its parental cell line, but in this case the resistant subline was nonetheless 4.6 times more resistant to doxorubicin than to DA-125. These results indicate that DA-125 is generally much more cytotoxic than doxorubicin to doxorubicin-resistant cancer cells. From the results obtained with doxorubicin-resistant colorectal cancer cell sublines, DA-125 would be expected to be relatively insensitive to *mdr 1*-gene-mediated MDR. Another fluorine-containing anthracycline, ME2303, has also been

reported to have low-degree cross-resistance to doxorubicin, being effective against several MDR cell lines in vitro [32].

Intracellular drug-uptake studies showed that DA-125, after nearly complete and immediate conversion to M1 in the culture fluid employed in this study, was more rapidly taken up by the cancer cells studied and achieved higher intracellular concentrations than doxorubicin. In SNU-1 the large difference in intracellular accumulation observed between DA-125 (M1) and doxorubicin (Fig. 2A) is reflected in their IC_{50} values (Table 1; potency ratio 6.29). In SNU-16, however, the difference was quite small (1.16 times higher), and this small difference could partially explain the relative resistance of this cell line to DA-125 (potency ratio 0.28; Table 1). The finding that the uptake of M1, the active metabolite of DA-125, into cells occurs at a faster rate and to a higher level than does that of doxorubicin, suggests that DA-125 (as M1) can be incorporated into its target tissues from the bloodstream more rapidly than doxorubicin.

In a previous study it was demonstrated that DA-125 was taken up by P388 mouse leukemia cells faster than doxorubicin and that it accumulated at a level 2–7 times higher [29]. In that investigation, DA-125 was also found to have mechanisms of action similar to those of doxorubicin but had stronger DNA strand-breaking activity than doxorubicin, especially for double-strand scission, in tumor cells [29]. DA-125 also showed stronger activity for inhibition of nucleic acid synthesis in tumor cells [29]. This finding of higher biochemical activity in these cells could be explained by the observation that DA-125 reached higher intracellular concentrations than did doxorubicin [11, 29].

Preclinical toxicity tests on DA-125 have revealed that this drug is less cardiotoxic than doxorubicin [3]. Another report indicates that DA-125 produces less severe hematotoxicity than doxorubicin [2]. DA-125 has also been reported to cause less local irritation to the injection site [1].

The results of this study, together with other reports on the antitumor activity, pharmacokinetic properties, and toxicity of this new anthracycline derivative, indicate that DA-125 is a possible alternative anthracycline antineoplastic agent to doxorubicin. DA-125 is now under phase II clinical trial in Korea and awaits further evaluation. It is expected that DA-125 could be used in place of doxorubicin for tumors that are doxorubicin-resistant or show high levels of *mdr 1* expression.

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