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Intracellular Carboxyl Esterase Activity Is a Determinant of Cellular Sensitivity to the Antineoplastic Agent KW-2189 in Cell Lines Resistant to Cisplatin and CPT-11

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KW-2189, a novel antitumor antibiotic belonging to the duocarmycins, possesses marked DNA-binding activity upon activation by carboxyl esterase to its active form, DU-86. Three duocarmycins, KW-2189, DU-86 and duocarmycin SA, were active against the cisplatin (CDDP)-resistant human non-small cell lung cancer cell lines PC-9/CDDP and PC-14/CDDP, and the multidrug-resistant human small cell lung cancer cell line H69/VP. However, HAC2/0.1, a CDDP-resistant human ovarian cancer cell line which is also resistant to CPT-11 because of decreased intracellular activation of CPT-11, was about 12.8-fold more resistant to KW-2189. HAC2/0.1 was not resistant to other duocarmycins as compared to its parental cell line, HAC2. There was no difference between HAC2 and HAC2/0.1 with regard to the intracellular accumulation of KW-2189. Addition of 130 mU/ml of carboxyl esterase to the culture medium did not influence the sensitivity of HAC2 cells to KW-2189. However, the sensitivity of HAC2/0.1 cells to KW-2189 was enhanced to the level of HAC2. These results suggest that HAC2/0.1 is less potent than HAC2 in activating KW-2189. The carboxyl esterase activity of whole-cell and microsomal extracts from HAC2/0.1 was approximately 60% of that from HAC2. The cell-free experiment revealed that KW-2189 bound to DNA more efficiently in the presence of HAC2 than HAC2/0.1 cell extract. It was concluded that decreased intracellular carboxyl esterase activity in HAC2/0.1 cells caused decreased intracellular conversion of KW-2189 to its active form, thus producing resistance to KW-2189. The decreased conversion of CPT-11 to SN-38 in HAC2/0.1 cells might be explained by decreased carboxyl esterase activity.

Key words: KW-2189 — Carboxyl esterase — Duocarmycin — Drug resistance — CPT-11

The duocarmycins (DUMs), antitumor antibiotics produced by *Streptomyces sp.*, are structurally similar to CC-1065, which is one of the most cytotoxic cyclopropylpyrroloindoles.[1] KW-2189, a water-soluble derivative of duocarmycin, is more effective than the so-called "key agents," adriamycin and cisplatin (CDDP), against many kinds of murine and human cancer cell lines *in vivo*.[2] Furthermore, the delayed lethal toxicity to mice, which prevented CC-1065 from being used clinically, was not observed with KW-2189. KW-2189 was selected for further investigation in clinical trials because of its superior water solubility, stability and outstanding antineoplastic activity. We have reported previously that carboxyl esterase activated KW-2189, probably by removing the N-methylpiperazine side chain to augment DNA-binding potency and cytotoxicity, and that DNA strand breakage, as evaluated by the alkaline elution method, was also enhanced by esterase.[3] Arkin and Ohnuma[4] reported that carboxyl esterase enhanced the cytotoxic activity of KW-2189 and that exposure of cells to KW-2189 for 4 h or longer induced DNA damage. Kobayashi *et al.*[2] demonstrated that the major molecule adducted to DNA in cells treated with KW-2189 for 12 h was DU-86, the structure of which is similar to the expected structure of activated KW-2189, which lacks an N-methylpiperazine side chain (Fig. 1). Thus, although the antineoplastic activity, metabolic drug activation and DNA-binding potency of KW-2189 have been intensively investigated, the cellular factors governing sensitivity to KW-2189 are less well understood. While we were examining whether KW-2189 was active against drug-resistant cells using CDDP-resistant and multidrug-resistant cell lines established in our laboratory, we found that only one CDDP-resistant human ovarian cancer cell line, HAC2/0.1, showed cross-resistance to KW-2189. We reported previously that this cell line was resistant to CPT-11, a camptothecin-derived prodrug, because of decreased conversion of CPT-11 to its active metabolite, SN-38.[5] HAC2/0.1 cells remain sensitive to SN-38. Similarly HAC2/0.1 was resistant to KW-2189 but sensitive to DU-86. These results led to the hypothesis that there might be specific phenotypic differences between HAC2 and HAC2/0.1 cells responsible for their common sensitivity to the two prodrugs, KW-2189 and CPT-11. Based

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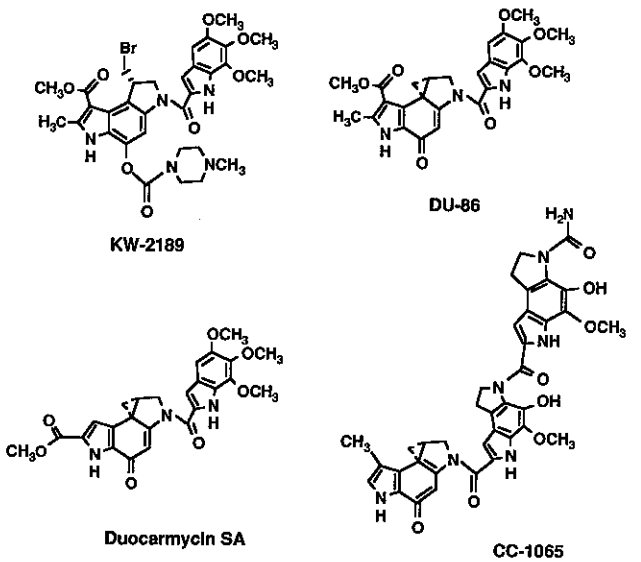

Fig. 1. Structures of duocarmycins and CC-1065.

on this idea, we have investigated the mechanisms of resistance of HAC2/0.1 cells to KW-2189. The importance of intracellular drug-activation as a determinant of cellular sensitivity to KW-2189 and CPT-11 will be discussed.

MATERIALS AND METHODS

Chemicals KW-2189, DU-86, duocarmycin SA and [^{3}H]KW-2189 (3.9 Ci/mmol) were supplied by Kyowa Hakko Kogyo, Tokyo. KW-2189 was dissolved in 5% glucose solution and stored at −80°C. DU-86 and duocarmycin SA were dissolved in absolute ethanol. There was no radioactivity in the N-methylpiperazine side chain of [^{3}H]KW-2189. Hog liver esterase was obtained from Boehringer Mannheim Yamanouchi, Tokyo. RPMI 1640 and phosphate-buffered saline without metal salts (PBS(−)) were purchased from Nissui, Tokyo. pBR322 DNA was from Nippon Gene, Tokyo. Other chemicals were purchased from Sigma Chemical Company, St. Louis, MO., unless otherwise stated.

Cell lines and culture HAC2 is a human ovarian cancer cell line. HAC2/0.1 is a drug-resistant subline of HAC2 selected by CDDP. HAC2/0.1 is resistant not only to CDDP but also to CPT-11 because of the decreased intracellular conversion to SN-38.[5] PC-9 and PC-14 are human non-small cell lung cancer cell lines. PC-9/CDDP and PC-14/CDDP are CDDP-resistant cell lines derived from PC-9 and PC-14, respectively.[6,7] H69 is a human small cell lung cancer cell line. H69/VP is a multidrug-resistant cell line that overexpresses mdr-1 mRNA, and is derived from H69 cells, selected by VP-16.[8] All the cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Cytosystem, Australia) plus penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37°C.

MTT assay The MTT assay was performed as described previously.[9] Cells were seeded into 96-well microplates and incubated with drug for 96 h in the presence or absence of carboxyl esterase (130 mU/ml).

Drug accumulation study Drug accumulation was studied according to the method of Versantvoort *et al.*[10] Log phase cells were washed and resuspended at a density of 5×10^5 cells/ml in warm fresh culture medium. DNase I (10 U) was added to the medium to prevent the formation of extracellular drug-DNA adducts. The cells were incubated with [^{3}H]KW-2189 and 4 h after treatment, drug accumulation was stopped by dispensing 0.8-ml aliquots into 50 ml of ice-cold PBS(−). After two cold washes, the cells were lysed with 1% SDS at 37°C for 1 h. All of the samples were transferred to liquid scintillation fluid (Clear-sol I, Nacalai Tesque), and radioactivity was measured using an LS6000TA scintillation counter (Beckmann, Tokyo).

Preparation of microsomal and whole-cell extract The microsomal extract was prepared as described previously with some modifications.[11] Log phase cells (5×10^8 cells) were washed with ice-cold PBS(−) and centrifuged for 10 min at 5000*g*. The precipitate was suspended in 3 ml of 0.25 *M* sucrose, homogenized, and centrifuged at 16,000*g* for 30 min. The sediment was discarded and the supernatant was centrifuged at 105,000*g* for 60 min at 2°C. The sediment (microsomal fraction) was suspended in 0.1 *M* Tris-HCl at pH 8.5. This microsomal fraction was disrupted in an ice bath using an ultrasonic homogenizer to reduce the size of the microsomal particles. Digitonin (final concentration: 15 mg/ml) was added to this and the mixture was stirred for 60 min to solubilize the carboxyl esterase. After centrifugation for 2 h at 105,000*g*, the supernatant contained the bulk of the carboxyl esterase activity. The samples were aliquoted and stored at −80°C. To prepare the whole-cell extract, the packed cells were homogenized in 0.1 *M* Tris-HCl at pH 8.5 followed by treatment with digitonin.

Measurement of carboxyl esterase activity The carboxyl esterase activity was determined as described previously.[11] In brief, the reaction mixture (1 ml) containing 1 m*M* *para*-nitrophenyl acetate, 50 μg of whole-cell extract or 12.5 μg of microsomal extract and 100 m*M* Tris-HCl (pH 8.2) was incubated at room temperature. The optical density at 405 nm was measured continuously for 2 min to detect the metabolite, *para*-nitrophenol, using a U-3210 spectrophotometer (Hitachi, Tokyo).

Table I. Antineoplastic Activity of Duocarmycins (IC_{50})[a]

Cell line	CDDP (μg/ml)	(RR)[b]	KW-2189 (nM)	(RR)	DU-86 (nM)	(RR)	Duocarmycins SA (nM)	(RR)
PC-9	3.0±0.2[c]		76.5±11.8		0.31±0.03		0.044±0.005	
PC-9/CDDP	33.0±2.60	(11.0)	94.7±7.60	(1.24)	0.61±0.13	(1.97)	0.074±0.015	(1.68)
PC-14	2.7±0.52		75.2±5.50		0.35±0.05		0.046±0.009	
PC-14/CDDP	30.4±1.6	(11.3)	113.0±8.80	(1.50)	0.58±0.04	(1.66)	0.081±0.016	(1.76)
H69	2.8±0.42		54.1±4.40		ND[d]		ND	
H69/VP	ND		42.8±4.8	(0.79)	ND		ND	
HAC2	0.12±0.03		1.48±0.53		1.10±0.31		0.13±0.04	
HAC2/0.1	0.65±0.22	(5.4)	19.0±6.90	(12.8)	0.45±0.12	(0.41)	0.05±0.01	(0.38)

a) Drug concentration to reduce cell growth by 50% of control.
b) Relative resistance calculated as IC_{50} for resistant cells/IC_{50} for parental cells.
c) Mean±SD from three independent experiments.
d) Not determined.

Drug-DNA adduct formation The reaction mixture (100 μl) containing plasmid pBR322 (500 ng), 200 μM [^{3}H]KW-2189, and hog carboxyl esterase (1300 mU/ml) or 10 μg of HAC2 or HAC2/0.1 cell extract was incubated at 30°C. After the indicated incubation periods, the reaction was stopped by ethanol precipitation. The precipitates were dissolved in 100 μl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and then extracted with phenol. The resulting solution was added to scintillation fluid and the retained radioactivity was measured.

RESULTS

Cytotoxic activity of DUMs against drug-resistant cells The activity of KW-2189 against drug-resistant human cancer cells was examined in pairs of drug-sensitive and drug-resistant cell lines. KW-2189 was active against the CDDP-resistant lung cancer cells, PC-9/CDDP and PC-14/CDDP, and the multidrug-resistant lung cancer cell line, H69/VP. Among the drug-resistant cell lines, only HAC2/0.1 showed 12.8-fold cross-resistance to KW-2189 (Table I). To examine whether HAC2/0.1 was cross-resistant to other DUMs, the antineoplastic activity of DU-86 and duocarmycin SA was examined in all of the cell lines. DU-86 and duocarmycin SA were active against all of the drug-resistant cell lines including HAC2/0.1. While the order of relative cytotoxic activity of the three duocarmycins was KW-2189<DU-86< duocarmycin SA in most cell lines, the IC_{50} value of KW-2189 against HAC2 was almost the same as that of its active form, DU-86. It might be reasonable to consider that HAC2 was hypersensitive to KW-2189 rather than that HAC2/0.1 was resistant to KW-2189.

Accumulation of KW-2189 into HAC2 and HAC2/0.1 To elucidate the mechanisms of the resistance of HAC2/0.1 cells to KW-2189, the intracellular accumulation of different concentrations of KW-2189 was examined. This examination was carried out in culture medium containing 10% fetal bovine serum because conversion of KW-2189 to DU-86 was not induced by the fetal bovine serum.[2] In each cell line, KW-2189 accumulated in a concentration-dependent manner (Fig. 2). At each concentration of KW-2189, there was no significant difference in drug accumulation between HAC2 or HAC2/0.1 cells.

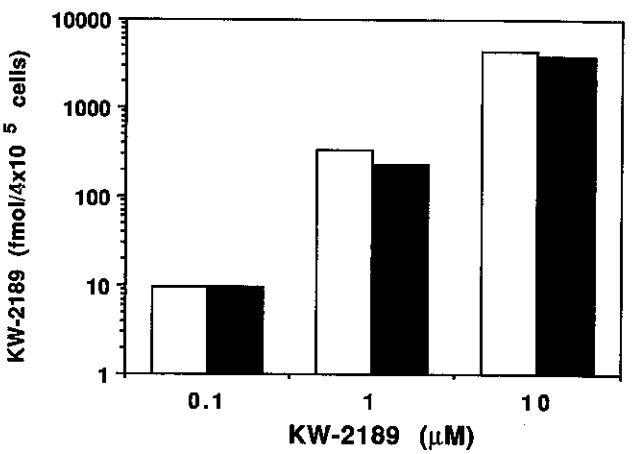

Fig. 2. KW-2189 accumulation in HAC2 and HAC2/0.1 cells. HAC2 (white bars) and HAC2/0.1 (dark bars) were incubated with 0.1, 1.0 or 10.0 μM [^{3}H]KW-2189 for 4 h. After two cold washes, the cells were lysed and radioactivity was measured. The values given are the averages of two independent experiments.

Carboxyl esterase augments the sensitivity of HAC2/0.1 to KW-2189 The cytotoxic activity of KW-2189 was enhanced by the non-cytotoxic activity of extracellular carboxyl esterase in both a time- and a dose-dependent manner.[3] The effect of extracellular carboxyl esterase on the cytotoxic activity of KW-2189 against HAC2 and HAC2/0.1 was examined. Unexpectedly, the sensitivity of HAC2 to KW-2189 was not significantly affected by esterase. However, the sensitivity of HAC2/0.1 to KW-

2189 was significantly enhanced by extracellular carboxyl esterase. In the presence of 130 mU/ml carboxyl esterase, HAC2/0.1 showed almost the same sensitivity as HAC2 (Fig. 3). This result suggested that HAC2/0.1 is resistant to KW-2189 but not resistant to the activated KW-2189. This idea was supported by data which demonstrated that HAC2 and HAC2/0.1 were equally sensitive to DU-86, the active metabolite of KW-2189. The addition of carboxyl esterase did not enhance the antineoplastic activity of CDDP, DU-86 or duocarmycin SA in these two cell lines (data not shown).

Intracellular carboxyl esterase activity To examine whether HAC2 and HAC2/0.1 have different abilities to activate KW-2189, their carboxyl esterase activity in whole-cell and microsomal extracts was determined. The carboxyl esterase activities in whole-cell and microsomal extracts in HAC2/0.1 were 57% and 62.5%, respectively of that in HAC2 cells (Table II).

Efficient adduct formation by HAC2 cell extracts To examine the effect of the cellular extract on the DNA-binding activity of KW-2189, the plasmid DNA was incubated with KW-2189 in the presence of hog carboxyl esterase (1300 mU/ml) or whole-cell extracts from HAC2 or HAC2/0.1 cells. Although KW-2189 itself formed drug-DNA adducts, more efficient adduct formation was

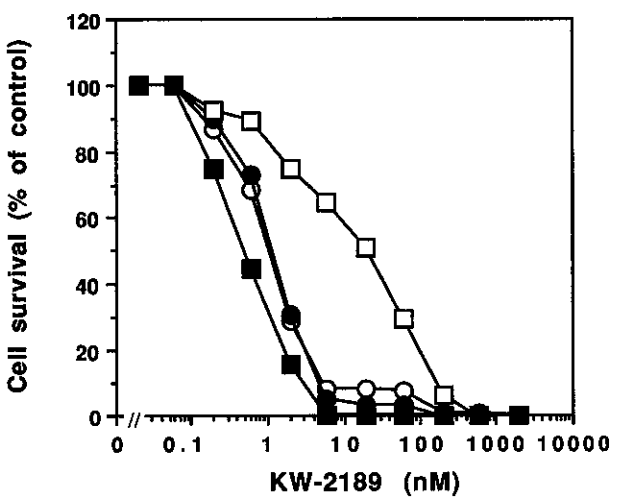

Fig. 3. The effect of carboxyl esterase on the cytotoxic activity of KW-2189 in HAC2 (circles) and HAC2/0.1 (squares) cells. Cells were treated with KW-2189 for 96 h in the absence (open symbols) or presence (solid symbols) of 130 mU/ml carboxyl esterase. The values given are the means of three independent experiments.

Table II. Carboxyl Esterase Activity in HAC2 and HAC2/0.1

Cell line	Whole cells (mU/mg protein)	Microsomes
HAC2	1349±190[a)]	2209±189
HAC2/0.1	766±126	1381±96

a) Mean±SD from three independent cell preparations.

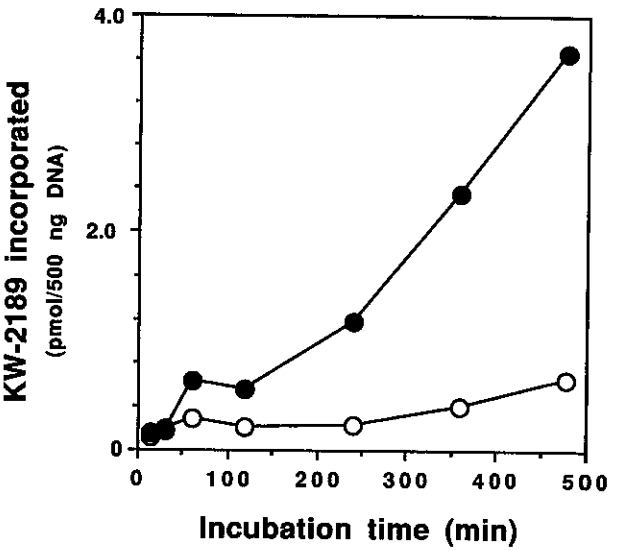

Fig. 4. Formation of DNA adducts by KW-2189. pBR322 (500 ng) was treated with 200 μM [^{3}H]KW-2189 in the presence (solid circles) or absence (open circles) of hog liver esterase (1300 mU/ml). After the indicated incubation period, DNA was ethanol-precipitated and extracted with phenol. The values are fractions of radioactivity retained in the aqueous phase.

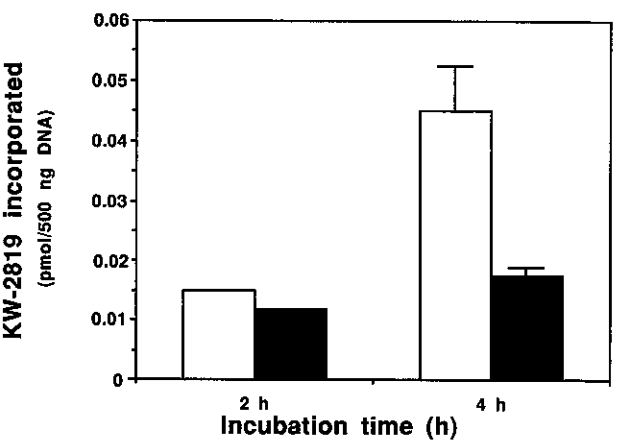

Fig. 5. Formation of drug-DNA adducts in cell extracts. pBR322 (500 ng) was treated with 200 μM [^{3}H]KW-2189 and 10 μg of cell extract from HAC2 (white bars) or HAC2/0.1 cells (dark bars). Values are the means of 2 experiments (for 2-h treatment) or 3 experiments (for 4-h treatment)±SD.

observed in the presence of hog carboxyl esterase (Fig. 4). The difference in the amount of drug-DNA adduct formation was little in 2-h incubation, but became greater in a time-dependent manner up to 8 h incubation. When the cell extracts were used, there was no significant difference in drug-DNA adduct formation following a 2-h incubation. However, after a 4-h incubation there were more drug-DNA adducts in the presence of the HAC2 extract than the HAC2/0.1 extract (Fig. 5). As KW-2189 bound to DNA more efficiently after it was metabolized to DU-86, these results indicate that the different capability of drug-DNA adduct formation by the two cell extracts was caused by the different intracellular esterase activities of these cell lines.

DISCUSSION

KW-2189, together with other DUMs, was active against drug-resistant cell lines such as H69/VP, PC-9/CDDP and PC-14/CDDP. H69/VP is a typical multidrug-resistant cell line. PC-9/CDDP and PC-14/CDDP are resistant to CDDP because of their decreased drug accumulation. PC-14/CDDP expresses less Na^+,K^+-ATPase, which might serve to transport CDDP into the cells.[7] The data presented here suggest that P-glycoprotein and Na^+,K^+-ATPase have no role in the transport of DUMs. Among the drug-resistant cells, a CDDP-resistant human ovarian cancer cell line, HAC2/0.1, showed cross-resistance to KW-2189 and was not resistant to other DUMs. From these results we hypothesized two mechanisms of resistance to KW-2189 in HAC2/0.1: 1) decreased intracellular accumulation of KW-2189, and 2) decreased intracellular conversion of KW-2189 to DU-86. The former hypothesis was based on the possibility that KW-2189 and other DUMs might be transported by different mechanisms. Drug-accumulation studies revealed that KW-2189 accumulation was not the determinant of sensitivity in these cell lines. It was noted that HAC2 was equally sensitive to KW-2189 and DU-86, and that this sensitivity was not influenced by extracellular esterase. It is possible that the HAC2 cells could metabolize KW-2189 very efficiently, thus diminishing the effects of extracellular carboxyl esterase. In a previous paper we proposed that intracellular carboxyl esterase activity might be one of the factors that influences the sensitivity of cancer cells to KW-2189.[3] We have demonstrated that the intracellular carboxyl esterase activity in HAC2/0.1 cells was approximately 60% of that in HAC2 cells. We have also shown that fewer drug-DNA adducts were formed in the presence of HAC2/0.1 than HAC2 extract. Thus the decreased carboxyl esterase activity in HAC2/0.1 cells resulted in less efficient activation of KW-2189 to DU-86, leading to less drug-DNA adduct formation. The difference in the amount of drug-DNA adducts induced by HAC2 or HAC2/0.1 extracts increased time-dependently (Fig. 5). This suggests that the difference in the amount of drug-DNA adducts between HAC2 and HAC2/0.1 cells may be magnified during longer incubation, such as 4-day exposure in the MTT assay. Based on these results, it can be concluded that decreased carboxyl esterase activity in HAC2/0.1 cells is primarily responsible for their resistance to KW-2189.

The complete reversal of the resistance of HAC2/0.1 to KW-2189 by extracellular carboxyl esterase seemed to occur by complementing the decreased intracellular conversion of KW-2189 to DU-86. This suggests that intracellular metabolic bioactivation is critical to the antineoplastic activity of KW-2189. Hence, quantification of carboxyl esterase activity in the neoplastic tumor and also in normal tissues will be of value in clinical investigations to predict the antitumor effect and the organ-specific adverse effects of KW-2189.

We have reported previously that carboxyl esterase may be responsible for the activation of CPT-11 to its active form, SN-38.[12] The reason why HAC2/0.1 cells showed resistance to CPT-11 but not to SN-38 might be the decrease in intracellular carboxyl esterase activity in this cell line. It is possible that the same esterase metabolizes both the methylpiperazinylcarbonyloxy moiety of KW-2189 and the piperidinopiperidinocarbonyloxy moiety of CPT-11.[13] Because both KW-2189 and CPT-11 are promising drugs, it seems to be important for future investigations to identify the carboxyl esterase that is responsible for metabolizing them.

The resistance to CDDP was not reversed by carboxyl esterase, suggesting that the decreased intracellular carboxyl esterase activity in HAC2/0.1 cells is not responsible for their resistance to CDDP. The reasons why the CDDP-resistant cells showed decreased carboxyl esterase activity remains unclear. To clarify the relationship between CDDP-resistance and the esterase activity, we compared the esterase activity between other parental and CDDP-resistant pairs such as PC-9, PC-14, PC-9/CDDP, and PC-14/CDDP, but the esterase activity in PC-9 and PC-14 cells was too low to measure in our assay system. One possible explanation is that during establishment of the resistant cells in CDDP-containing medium, a population of the CDDP-resistant cells acquired the phenotype of decreased carboxyl esterase activity without losing their resistance to CDDP. The mutation-proneness of cancer cells[14] and the mutagenic activity of CDDP[15] could account for these phenotypic changes. Although we have no data to support this possibility, the fact that some drug-resistant cell lines have different phenotypes, unrelated to their drug-resistance, from their parental cell lines may have some relevance to our hypothesis.

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