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Abstract

We have established an Adriamycin (ADM) -resistant small cell lung cancer (SCLC) cell line, SBC-3/ADM100, which shows multifactorial mechanisms of resistance to ADM, such as overexpression of P-glycoprotein, an enhanced detoxifying system and a decrease in topoisomerase II activity. In the present study, we confirmed that SBC-3/ADM 100 showed collateral sensitivity to methotrexate and TNP-351, a new antifolate, though this cell line showed a typical multidrug resistance (MDR) pattern. We also demonstrated a faster uptake and higher accumulation (1.3-fold) of TNP-351 in the SBC-3/ADM100 cells than those in the parent SBC-3 cells. These results explain one of the mechanisms for collateral sensitivity in the resistant cells. Furthermore, this cell line was found to have no cross-resistance to edatrexate and minimal cross-resistance to trimetrexate, 254-S (cisplatin analog), 5-fluorouracil and 4-hydroperoxyifosfamide. These drugs will have clinical importance in patients with SCLC who were previously treated with an ADM-containing regimen. Thus, antifolates, especially TNP-351 and edatrexate, can be expected to eradicate residual multidrug resistant SCLC cells selected by ADM.

KEYWORDS: Adriamycin-resistant cell line, antifolates, small cell lung cancer

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We have established an Adriamycin (ADM) - resistant small cell lung cancer (SCLC) cell line, SBC-3/ADM100, which shows multifactorial mechanisms of resistance to ADM, such as overexpression of P-glycoprotein, an enhanced detoxifying system and a decrease in topoisomerase II activity. In the present study, we confirmed that SBC-3/ADM100 showed collateral sensitivity to methotrexate and TNP-351, a new antifolate, though this cell line showed a typical multidrug resistance (MDR) pattern. We also demonstrated a faster uptake and higher accumulation (1.3-fold) of TNP-351 in the SBC-3/ADM100 cells than those in the parent SBC-3 cells. These results explain one of the mechanisms for collateral sensitivity in the resistant cells. Furthermore, this cell line was found to have no cross-resistance to edatrexate and minimal cross-resistance to trimetrexate, 254-S (cisplatin analog), 5-fluorouracil and 4-hydroperoxyifosfamide. These drugs will have clinical importance in patients with SCLC who were previously treated with an ADM-containing regimen. Thus, antifolates, especially TNP-351 and edatrexate, can be expected to eradicate residual multidrug resistant SCLC cells selected by ADM.

Key words: Adriamycin-resistant cell line, antifolates, small cell lung cancer

Introduction of intensive chemotherapy for the treatment of small cell lung cancer (SCLC) has resulted in significant improvements, with complete response rates ranging from 30 to 50 %. However, majority of the complete responders eventually relapse and only a small fraction of patients can achieve long-term disease-free survival (1). The development of drug resistance has been assumed to be responsible for such a dismal outcome (2). Accordingly, new drugs, which can eradicate residual resistant cells, are required to improve the curative treatment for SCLC. Adriamycin (ADM) is considered one of the most active drugs in the treatment of SCLC (3). We have established an ADM-resistant SCLC cell line (SBC-3/ADM100) *in vitro* (4) to test drugs which do not show cross-resistance but retain collateral sensitivity to residual resistant cells selected by ADM. The SBC-3/ADM100 cells showed multifactorial mechanisms of resistance to ADM (4). They include a) overexpression of MDR 1 mRNA and P-glycoprotein (P-gp), b) an increase in intracellular glutathione (GSH) and glutathione S transferase (GST)-π content, c) a decrease of topoisomerase II activity and d) longer doubling time.

A number of ADM-resistant cell lines have been reported previously, the majority of which showed a typical multidrug resistance (MDR) phenomenon and were resistant to different types of drugs such as vinca alkaloids, anthracyclines and taxol (5). However, some of them are reported to retain collateral sensitivity to a variety of drugs, and each MDR pattern differs from the others. Although the basis for this diversity has not yet been established, a variety of mechanisms responsible for the resistance and collateral sensitivity seem to be involved in each cell line.

In the present study, we investigated cross-resistance patterns of an ADM-resistant SCLC subline, SBC-3/ADM100, to clarify the mechanism of collateral sensitivity.

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Materials and Methods

Chemicals and reagents. Drugs used in this study were obtained from the following: ADM, vinorelbine, mitomycin C and 5-fluorouracil from Kyowa Hakko Co., Tokyo, Japan; daunomycin and pirarubicin from Meiji Seika Co., Tokyo; aclarubicin from Yamanouchi Pharmaceutical Co., Tokyo; mitoxantrone and methotrexate (MTX) from Lederle Japan, Tokyo; vinblastine, 254-S (cisplatin analog) and 4-hydroperoxyifosfamide (an active metabolite of ifosfamide) from Shionogi & Co., Osaka, Japan; teniposide from Bristol-Myers Squibb, NY, USA; carboplatin from Bristol-Myers Squibb K. K., Tokyo; bleomycin and peplomycin from Nippon Kayaku Co., Tokyo; TNP-351, (+)-N-[4-[3-(2, 4-diamino-7H-pyrrolo[2, 3-d]pyrimidin-5-yl)-propyl] benzoyl]-L-glutamic acid (6) and [^{14}C] TNP-351 from Takeda Chemical Ind., Osaka; edatrexate from Ciba-Geigy, Tokyo; trimetrexate from Dainippon Pharmaceutical Co., Osaka; cytarabin from Nippon Shinyaku Co., Kyoto, Japan. 3-[4,5-Dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Co., St. Louis, MO, USA.

Cell cultures. The parent cell line, SBC-3 (7), derived from bone marrow aspirates of an untreated patient with SCLC, an ADM-resistant subline of SBC-3 (SBC-3/ADM100) (4), an etoposide-resistant subline of SBC-3 (SBC-3/ETP) (8), and a cisplatin-resistant subline of SBC-3 (SBC-3/CDDP) (9) were established previously in our laboratory. They have been maintained in RPMI 1640 medium, supplemented with 10 percent fetal bovine serum (GIBCO BRL, Grand Island, NY, USA), penicillin-G (100units/ml) and streptomycin (100 μg/ml) (RPMI-FBS).

Tests for drug sensitivity. Drug sensitivity was evaluated by MTT assay (10) with a slight modification (11). The SBC-3/ADM100 cells were assayed within six months after cloning. The SBC-3/ETP and SBC-3/CDDP cells were assayed within two months after cloning. Briefly, cells from each cell line (SBC-3: 2,000 cells/well; SBC-3/ADM100, SBC-3/ETP and SBC-3/CDDP: 5,000 cells/well) were plated in quadruplicate in 96 Well Cell Culture Clusters (Coster, Cambridge, MA, USA) and incubated for 96h in the media-containing test agents. MTT was added to each well and 4h later the absorbance was measured. The concentration of each drug required to inhibit cell growth by 50 % (IC_{50}) was determined from the dose-response curve. Each experiment was repeated at least twice to confirm the results. In the preliminary tests, we confirmed that SBC-3/ADM100 cells showed more than 80-fold resistance to ADM, which was calculated from a ratio of IC_{50} values of SBC-3/ADM100 cells: IC_{50} values of SBC-3 cells to ADM.

Cell cycle distribution. Cell cycle distribution was determined by flow cytometric analysis as described by Krishan (12). Briefly, exponentially growing cells were harvested, washed twice with phosphate buffered saline, and centrifuged at room temperature. Cells were rapidly fixed with cold 70 % ethanol by vigorous vortexing. Cells were stained for DNA with propidium iodide (Sigma). Subsequently, cells were analyzed using Epics Profile II (Coulter Co., Hialeah, FL, USA) with Multicycle software (Coulter Co.). Propidium iodide was excited at 488nm and fluorescence was measured using a 630nm longpass filter.

Cellular influx and efflux of [^{14}C] TNP-351. The cell volume was estimated for determination of intracellular concentration of [^{14}C] TNP-351. Cells in a late exponential growth phase were counted by hemocytometer, and centrifuged at 7,500rpm for 5min (Kubota Hematcrit KH-120, Kubota, Tokyo, Japan) in a capillary tube. The cell volume was determined by cell number (cells/ml) and its cytocrit (%) (Cytocrit Method) in five separate experiments. The SBC-3 and SBC-3/ADM100 cells were incubated at a cell density of 1×10^6/ml in RPMI-FBS containing 10μM [^{14}C] TNP-351 at 37°C. At various time intervals, 0.2ml samples in triplicate were removed from the culture and filtered with a semiautomatic cell harvester (Labo Mash, Science Co. Ltd., Tokyo, Japan). The cells on the filter were solubilized by Clear-sol I (Nakarai Chemicals Ltd., Tokyo, Japan) and the residual radioactivity in the cells was determined by a liquid scintillation counter (Aloka LSC-700, Aloka Co. Ltd., Tokyo, Japan), and then intracellular drug concentration was calculated.

The efflux of the drug was measured subsequently. After incubation of the SBC-3 and SBC-3/ADM100 cells in the presence of 10μM [^{14}C]TNP-351 for 60min at 37 °C, the cells were centrifuged at 4°C, and the supernatant was completely removed. The cells were resuspended in drug-free medium at 37°C. At appropriate time intervals, 0.2ml samples in triplicate were withdrawn, and the residual radioactivity was determined as described above.

The initial uptake of [^{14}C]TNP-351 was analyzed as

described previously (13). [^{14}C]TNP-351 at a graded concentration (2.5, 5 and 10 μM) was added to the cell suspensions (2×10^6 cells/ml). They were incubated for 2.5 min, removed from the culture and filtered. The residual radioactivity in the cells was determined as described above. Influx Vmax and influx Km were determined from the line fitted by the least square method using a Lineweaver-Burk plot (14).

Results

Drug sensitivity. The SBC-3/ADM100 cells were completely cross-resistant to vinca alkaloids (vinblastine and vinorelbine) and teniposide, moderately cross-resistant to anthracyclines, mitomycin C, bleomycin, peplomycin and cytarabin, and minimally cross-resistant to 254-S (cisplatin analog), 4-hydroperoxyifosphamide and 5-fluorouracil (Table 1).

Table 2 shows collateral sensitivity of SBC-3/ADM100 to antifolates except for trimetrexate. Relative resistance was 0.52 for TNP-351, 0.70 for MTX, and 0.87 for edatrexate.

Table 3 shows cross-resistance patterns of the SBC-3/ETP and SBC-3/CDDP cells to antifolates. Among four different antifolates tested, trimetrexate showed the lowest cross-resistance when tested with the SBC-3/CDDP cells.

Table 1 Patterns of cross-resistance of an Adriamycin-resistant subline (SBC-3/ADM100)

Drugs	IC_{50} value (μM)[a] for		Relative resistance[b]
	SBC-3	SBC-3/ADM100	
Adriamycin	0.0188	1.93	103
Daunomycin	0.0360	1.14	31.7
Pirarubicin	0.0068	0.172	25.3
Aclarubicin	0.0064	0.053	8.28
Mitoxantrone	0.0187	0.186	9.95
Vinblastine	0.00230	0.580	252
Vinorelbine	0.00220	2.000	909
Teniposide	0.0266	4.55	202
254-S	0.776	1.264	2.43
4-HI	2.053	5.723	2.79
Mitomycin C	0.0393	0.380	9.53
Bleomycin	0.0153	0.128	8.36
Peplomycin	0.00797	0.0799	10.0
5-Fluorouracil	1.550	4.49	2.90
Cytarabin	0.0644	0.304	4.71

a: Values given represent the means of results obtained in two or more independent experiments.
b: IC_{50} (SBC-3/ADM100): IC_{50} (SBC-3). IC_{50}: Concentration to inhibit cell growth by 50%; ADM: Adriamycin.

Table 2 Patterns of cross-resistance of an ADM-resistant subline (SBC-3/ADM100) to four antifolates

Drug[a]	IC_{50} value (μM)[b] for		Relative resistance[c]
	SBC-3	SBC-3/ADM100	
Methotrexate	32.3 ± 14.1	18.4 ± 4.90	* 0.70 ± 0.24
Edatrexate	6.12 ± 2.78	4.72 ± 0.70	n.s. 0.87 ± 0.32
TNP-351	6.92 ± 3.66	3.34 ± 1.57	* 0.52 ± 0.15
Trimetrexate	154.1 ± 114.9	292.8 ± 147.9	** 4.23 ± 2.25

a: The molecular structures of the four antifolates are shown in Fig. 3.
b: Values given represent the means ± SD of results obtained in five independent experiments.
c: IC_{50} (SBC-3/ADM100 cells): IC_{50} (SBC-3 cells). Statistical significance, compared to the parent cells, by paired Student's *t*-test. **: $P < 0.02$; *: $P < 0.05$; n.s.: not significant. IC_{50}, ADM: See Table 1.

Table 3 Patterns of cross-resistance of etoposide-resistant (SBC-3/ETP) and cisplatin-resistant (SBC-3/CDDP) sublines to four antifolates

Drugs	Degree of relative resistance[a] (± SD[b]) for	
	SBC-3/ETP	SBC-3/CDDP
Methotrexate	1.02 ± 0.30	1.45 ± 0.50
Edatrexate	1.43 ± 0.81	2.22 ± 0.89
TNP-351	2.15 ± 1.54	3.78 ± 2.18
Trimetrexate	2.48 ± 0.76	1.04 ± 0.40

a: IC_{50} (SBC-3/ETP or IC_{50} SBC-3/CDDP cells): IC_{50} (SBC-3 cells) in each experiment.
b: Values given represent the means ± SD of results obtained in five independent experiments. IC_{50}: See Table 1.

Cell cycle distribution. Cell cycle distribution in percent (mean ± standard deviation obtained in three separate experiments) determined by flow cytometric analysis was as follows: percent distributions of G0/1, S and G2/M phases were 51.4 ± 5.7, 40.1 ± 4.6, and 8.7 ± 2.1, respectively, in the SBC-3 cells, and 67.9 ± 6.5, 25.6 ± 4.06, and 6.5 ± 2.95, respectively, in the SBC-3/ADM100 cells. In the SBC-3/ADM100 cells, the proportion of G0/1 phase was increased and that of the S phase decreased significantly ($P < 0.02$).

Intracellular influx and efflux of [^{14}C] TNP-351. The cell volume of the SBC-3 cells (1,744 ± 358 μm^3) exceeded that of the SBC-3/ADM100

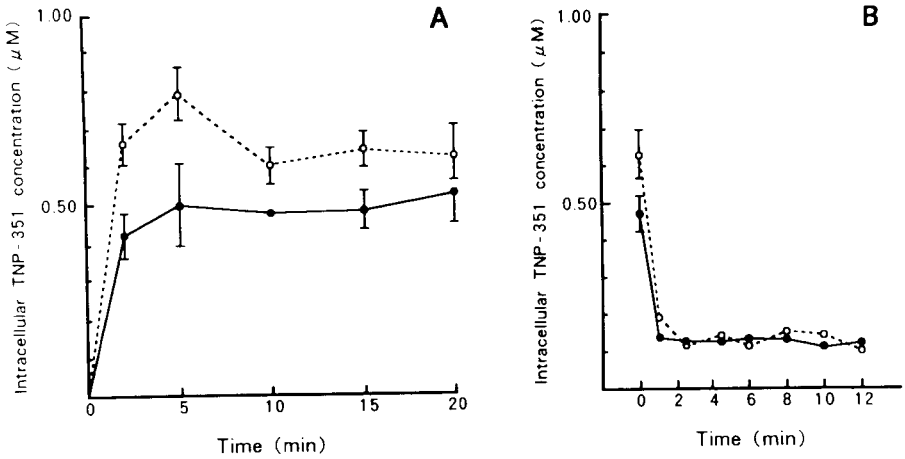

Fig. 1 The time course of cellular influx and efflux of [^{14}C] TNP-351 in SBC-3 and SBC-3/ADM100. Cellular influx (A) and efflux (B); (●): SBC-3; (○): SBC-3/ADM100. [^{14}C] TNP-351 at 10 μM was added to cell suspensions of both cell lines at 0 time. Serial samples were withdrawn at the time indicated and the cellular drug concentrations were determined as described in the Materials and Methods section. Each point represents the mean ± SD in three experiments. ADM: Adriamycin.

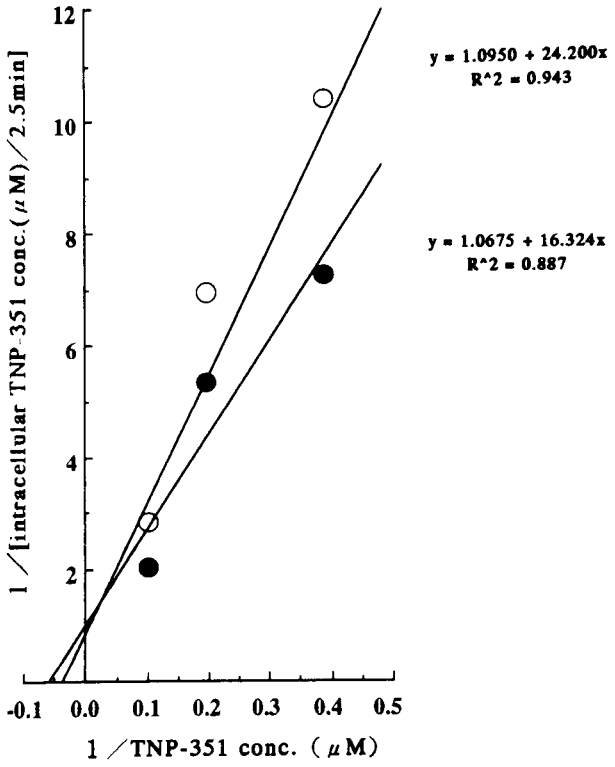

Fig. 2 Lineweaver-Burk plot for the initial rate of influx of [^{14}C] TNP-351 in the SBC-3 cells and the SBC-3/ADM100 cells. (●): SBC-3 cells; (○): SBC-3/ADM100 cells. For determining Vmax and Km, the line was fitted by the least squares method. Each point represents the mean of three experiments. ADM: See Fig. 1.

cells (1,030 ± 398 μm^3) at a significant level ($P < 0.02$). Since the SBC-3/ADM100 cells showed the highest collateral sensitivity to TNP-351, TNP-351 was selected for cellular influx and efflux study to clarify mechanisms of the collateral sensitivity. Fig. 1 indicates a representative experiment of cellular influx (A) and efflux (B) of [^{14}C] TNP-351. For both cell lines [^{14}C] TNP-351 was incorporated into the cells at an early phase within 5 min after incubation; thereafter, concentrations of the drug reached a plateau phase. The influx of TNP-351 was very rapid in both cell lines, when compared to that of MTX in Meth A and P388 cells (15). Intracellular accumulation of the drug in the SBC-3/ADM100 cells was significantly higher than that in the SBC-3 cells at any time tested ($P < 0.02$). Intracellular accumulation of the drug in the SBC-3 and SBC-3/ADM100 cells in the plateau phase, after the addition of 10 μM TNP-351, was 0.49 μM and 0.63 μM, respectively. The accumulation of the drug in the resistant cells was approximately 1.3-fold higher than that in the parent cells.

For both cell lines, the efflux of the drug from the cells was more rapid than we expected; the intracellular concentration decreased to 0.13 μM within 4 min after the replacement to drug-free RPMI-FBS. Although in initial efflux at 1 min the intracellular concentration of the drug in the SBC-3/ADM100 cells was higher than that in the SBC-3 cells, the initial efflux rate (μM/1 min) of TNP-

351 in the parent cells was equal to that in the SBC-3/ADM100 cells (71 %).

The initial influx rate (μM/2.5 min) at 2.5, 5 and 10 μM in the SBC-3/ADM100 cells was higher than that in the SBC-3 cells. The influx Vmax values were 1.10 and 1.07 μM/2.5 min, and the influx Km values were 22.1 and 15.3 μM for the parent and resistant cells, respectively (Fig. 2). The SBC-3/ADM100 cells showed almost the same Vmax values as the SBC-3 cells, and lower influx Km values than the SBC-3 cells.

Discussion

We have established an ADM-resistant SCLC cell line, SBC-3/ADM100, which shows a typical cross-resistance pattern as observed in MDR cells. Various ADM-resistant cell lines have been reported and they showed typical MDR patterns. However, some of them were reported to show no cross-resistance or collateral sensitivity to a number of drugs, such as mitomycin C, MTX (16), cytarabin (16, 17), melphalan, actinomycin D (18), bleomycin and cisplatin (19). In the present study, however, we demonstrated moderate cross-resistance to mitomycin C, cytarabin and bleomycin in the SBC-3/ADM100 cells. Although the basis for this discrepancy has not been established, factors such as tumor type, methods of cytotoxicity assessment (clonogenic assay or MTT assay, incubation time of the drug), the degree of ADM-resistance and the date of cytotoxicity assay after cloning may all contribute to this difference.

In the present study, the SBC-3/ADM100 cells showed collateral sensitivity to antifolates; TNP-351, MTX and edatrexate but not trimetrexate. To clarify the mechanism of this collateral sensitivity we demonstrated a decreased percentage distribution of the S phase in the SBC-3/ADM100 cells as compared with the parent SBC-3 cells. Nonetheless, alteration of cell kinetics will not account for the collateral sensitivity because antifolates affect DNA synthesis, especially in the S phase. Herman *et al.* (20), Bhalla *et al.* (16) and Klohs *et al.* (21) also demonstrated collateral sensitivity to MTX in ADM-resistant cell lines. Concerning the mechanism, Herman *et al.* (20) suggested that the collateral MTX sensitivity was related to a decrease of dehydrofolate reductase and an increase of MTX accumulation in the ADM-resistant cells.

TNP-351, a new antifolate (Fig. 3), showed the strongest activity of growth inhibition among the four

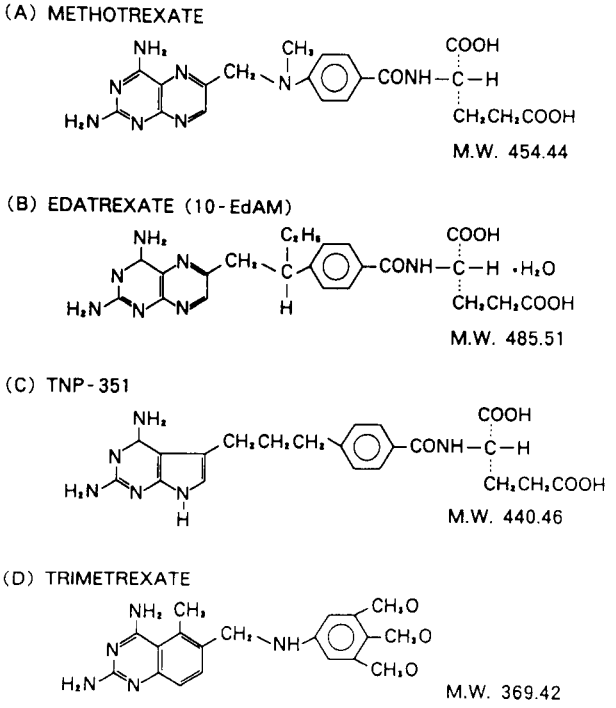

Fig. 3 Molecular structures of the four antifolates. (**A**) methotrexate, (**B**) edatrexate, (**C**) TNP-351, (**D**) trimetrexate.

antifolates tested, and a stronger activity *in vivo* than MTX against solid tumor cell lines including Meth A, Colon 26 and Lewis Lung cells (22). After a rapid incorporation into Meth A cells, mainly through the same pathway as MTX, TNP-351 is converted to polyglutamates by folylpolyglutamate synthetase and accumulated at a higher concentration than MTX (15).

We demonstrated a faster initial uptake rate and higher accumulation of TNP-351 in the SBC-3/ADM100 cells than the parent SBC-3 cells. The same Vmax values and lower Km values in SBC-3/ADM100 indicate that TNP-351 at a low concentration, which is clinically achievable, can enter the resistant cells easily in comparison with the parent cells. These results explain one of the mechanisms for collateral sensitivity in the resistant cells.

Among four antifolates, the SBC-3/ADM100 cells failed to show collateral sensitivity only to trimetrexate. As trimetrexate has greater lipophilicity than MTX, one possible explanation is that it can enter the cell by diffusion rather than by a MTX transport system. Klohs *et al.* (21) reported that a pleiotropically resistant P388 cell line selected by ADM showed cross-resistance to

trimetrexate and other lipophilic antifolates *in vivo*. They demonstrated a decrease in trimetrexate accumulation in the resistant cells. Arkin *et al.* (23) and Takemura *et al.* (24) also reported that 200-fold trimetrexate-resistant cells with classical MDR pattern overexpressed MDR 1 mRNA and P-gp. On the other hand, MOLT-3/MTX 10,000 cells with 10,000-fold resistance to MTX did not overexpress P-gp (25). These results suggest that P-gp may influence the resistance of only lipophilic trimetrexate, but not MTX. Therefore, the SBC-3/ADM100 cells with overexpression of P-gp (4) may be resistant to trimetrexate and the SBC-3/CDDP cells without overexpression of P-gp (9) may have the highest sensitivity to trimetrexate.

But the SBC-3/ETP cells with a similar mechanism of resistance to the SBC-3/ADM100 cells (8) showed no collateral sensitivity to antifolates. Development of this drug resistance may be the result of various mechanisms of resistance and the role of each mechanism to each drug may be different in individual cell lines.

The SBC-3/ADM100, SBC-3/ETP and SBC-3/CDDP cells expressed collateral sensitivity or minimal cross-resistance to antifolates. Especially TNP-351 and edatrexate showed stronger growth inhibitory activity on the SBC-3/ADM100 cells than MTX. When antitumor efficacies of these drugs to SCLC are confirmed in clinical studies, they can be expected to eliminate residual resistant cells.

These observations suggest that a) a combination of ADM and antifolates may have a synergistic effect, b) antifolates, especially TNP-351 and edatrexate, may eradicate the residual resistant cells after treatment with ADM, and c) antifolates may be useful for more effective therapeutic strategies in the treatment of SCLC.

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