Anti-Cancer Drugs 1994, **5**, pp. 343–354

Cytotoxic activity of 7-*N*-(2-((2-(-γ-L-glutamylamino)-ethyl)dithio)ethyl)-mitomycin C and metabolites in cell lines with different resistance patterns

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In this study the activity of KW-2149 and two of its metabolites, M-16 and M-18, was measured against cell lines with different types of resistance. The influence of these metabolites and of the exposure time on the cytotoxic efficacy of KW-2149 was investigated. Against the human ovarian carcinoma cell lines, AOvC and A2780, KW-2149 was more active than mitomycin C (MMC), with an IC_{50} of, respectively, 3.4 nM and 9.82 μM for KW-2149 and 18.2 nM and 67.71 μM for MMC. Activity of M-18 was significant against both cell lines and was comparable with that of KW-2149. Against an MMC-resistant cell line, $AOvC^{MMC}$, the resistance factor (RF) for KW-2149 was 3.1 versus 8.0 for MMC. Tested against a cisplatin-resistant cell line, $AOvC^{CDDP}$, KW-2149 had a RF of 7.7 versus 2.4 for MMC. Increasing the exposure time from 1 to 8 h decreased the RF for KW-2149 from 7.7 to 3.0. In an MDR mediated resistant cell line, $A2780^{mdr+}$, prolongation of exposure time increased RF for KW-2149 and MMC but decreased RF for M-18 from 7.0 at 1 h to 5.3 at 8 h. Tested against a rat colon carcinoma cell line CC531, KW-2149 and M-18 again demonstrated superior or equal activity compared with MMC, IC_{50} being, respectively, 0.6, 2.1 and 2.6. Here again M-18 showed an aberrant sensitivity pattern, as its activity decreased with *mdr-1* expression in contrast to the other mitomycins. Our data confirm the activity of KW-2149 as an agent with equal or superior activity as compared with MMC. It is concluded that the metabolite M-18 can contribute to the activity of KW-2149. Efficacy of both KW-2149 and its metabolites increases with increasing exposure times. The increments of exposure time appeared even as a means to overcome resistance in some instances.

Key words: Exposure time, KW-2149, M-16, M-18, mitomycin C, multidrug resistance.

Introduction

Mitomycin C (MMC) is a naturally occurring antitumor antibiotic with activity against a variety of experimental tumors.[1] It is being used in the treatment of carcinomas of the breast, lung and gastrointestinal tract.[2] The cytotoxicity of the antitumor antibiotic is considered to operate through mono- or bifunctional alkylation after reductive activation of the drug. However, the generation of oxygen radicals might well contribute to the overall cytotoxicity of MMC.[3] As an alkylating agent MMC has a different structure from the other classical alkylating agents. MMC is considered to have three potentially active groups; the quinone, the urethane and the aziridine groups. MMC is believed to bind to DNA via these groups leading to mono- or bifunctional alkylation. Tomasz *et al.* elegantly demonstrated the importance of the reduction of the quinone ring preceding cross-linking of activated MMC with the DNA double strand.[4,5]

For MMC used in conventional doses, the dose-limiting toxicity is bone marrow suppression, in particular cumulative delayed onset neutropenia and thrombocytopenia. Even with an intermittent schedule giving MMC once every 6–8 weeks, the hematological toxicity remains dose-limiting. Other toxicities seem to be in part dependent on total cumulative dose, as appeared to be the case for renal toxicity. For pulmonary toxicity the dose–effect relationship is less clear.

Development of MMC analogs has been directed towards compounds with an increased therapeutic index. Because the '7' position controls the reduction potential of the quinone ring and the intramolecular reductive activation of MMC could be achieved with substituents at C-7-N, various MMC derivatives with symmetrical and unsymmetrical disulfide-containing substituents at this position have been developed.[6–8] The observation that the quinone of MMC was easily reduced by a thiol with subsequent decomposition led to the development of analogs capable of intramolecular reductive activation of the quinone ring.

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One of these analogs is 7-*N*-(2-((2-(γ-L-glutamyl-amino)-ethyl)dithio)ethyl)-mitomycin C (KW-2149)[9]. KW-2149 has a γ-L-glutamylcystamino group at the C-7-N of the maternal compound MMC. The antitumor activity of KW-2149 has been evaluated in murine tumor models and in human tumor lines both *in vitro* and *in vivo*. In murine tumor cell lines activity of KW-2149 was similar to that of MMC except for the sarcoma line M5076 and the P388 leukemia, where superior activity was found with KW-2149. Against MMC-resistant leukemia cells P388/MMC, KW-2149 demonstrated significant activity. In human tumor xenografts, MMC and KW-2149 showed comparable activity.[10,11] Reduced bone marrow toxicity in mice has been reported.[12]

We were able to demonstrate that KW-2149 is converted in cancer patients into M-16 and M-18 (Figure 1). Both metabolites were present in patient plasma within a few minutes upon start of KW-2149 treatment. Production of M-16 and M-18 is considerable, and pharmacokinetic data demonstrate longer elimination half-lives of M-16 and M-18 than for KW-2149.[13] This has implications for *in vitro* activity testing which preferably should be performed in both the presence and absence of the naturally occurring metabolites in order to determine any possible synergistic/antagonistic interaction between the agents mentioned. Reports on activity of the metabolites as a function of concentration and exposure time are lacking.

In this report the cytotoxic activity of single agent KW-2149, M-16 and M-18, as well as of mixtures with the agents, against cell lines with different types of resistance are described.

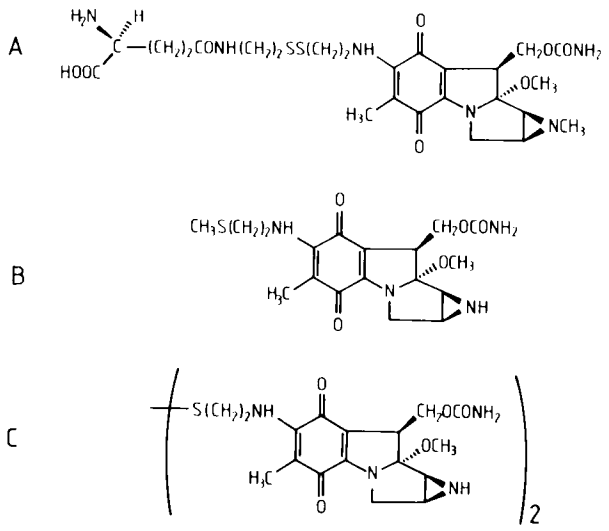

Figure 1. Structure of KW-2149(A), M-16(B) and M-18(C).

The influence of the exposure time is highlighted not only on the cytotoxic activity of the agents themselves, but also on resistance factor (RF) values determined in the different systems, in accordance with data on MMC.[14,15] The RF is the ratio of the IC_{50} of the drug resistant sublines and the IC_{50} of the reference line, i.e. the parental line.

Materials and methods

Cell lines and culture conditions

Human cell lines were cultivated in tissue culture flasks (Falcon, Becton Dickinson, Belgium) and grown in a monolayer while maintained in Dulbecco's modified Eagle's medium (Gibco Ltd, Paisley, UK) supplemented with 10% fetal calf serum. (Gibco Ltd, Paisley, UK), aspartic acid (0.1 mM) and glutamic acid (0.3 mM). They were cultured without antibiotics and were regulary tested for Mycoplasma infection.

All cell lines were treated similarly and cultures were maintained at 37°C in a humidified atmosphere of CO_2/air (5%/95%). RF data are based on a 1 h exposure time unless mentioned otherwise and calculated at the IC_{50} level.

The human ovarian cell line COV413.B was established from biopsy specimens from a patient with untreated advanced ovarian cancer.[16] Cisplatin resistance was obtained in these cells by continuous exposure to different concentrations of cisplatin.[16] In our hands these cells are designated AOvC and $AOvC^{CDDP}$. Cisplatin resistance was maintained by continuous exposure to cisplatin.[16,17] The RF of $AOvC^{CDDP}$/AOvC to cisplatin was 5.1.

The multidrug resistant (MDR) human ovarian cell line, $A2780^{AD}$, and its drug sensitive parental line, A2780, were kindly provided by R Ozols through P Borst at the University of Amsterdam.[18,19] From here on the cell lines are referred to as $A2780^{mdr-}$ and $A2780^{mdr+}$. The RF values for vincristine and adriamycin in our hands were 600 and 100, respectively. The MMC-resistant cell line was selected from the parental line AOvC by repeated exposure to MMC. AOvC cells in log-phase grown without antibiotic pressure were exposed for 2 h to 1 μM MMC. Treated cells were cultured in fresh medium until survivors resumed log-phase growth, at which time cells were passaged. The MMC treatments ranged from 2 to 4 weeks in the initial five exposure cycles to a stable 10 days

interval following cycle 10. From here on the cell line is denoted as $AOvC^{MMC}$. RF for MMC in the $AOvC^{MMC}$/AOvC system was 8.6.

Rat colon cancer cell lines CC531 were grown in 5% fetal calf serum, with other culture conditions comparable to those of human cells as mentioned above. CC531 is a rat colon cancer cell line used as reference and was kindly provided by RL Marquet through P Kuppen (University of Leiden). The multidrug resistant $CC531^{mdr+}$ is continuously exposed to 0.2 μM colchicin (Sigma, St Louis, MO), which is added fresh to the medium after every subculture. The cisplatin-resistant cell line $CC531^{CCDP}$ is continuously exposed to 0.75 μM cisplatin (Sigma). $CC531^{mdr+}$ displays the classical MDR phenotype with resistance to colchicin (RF: 87), vinblastin (RF: 90), doxorubicin (RF: 29) and actinomycin D (RF: 2.2).[20] Resistance to colchicin and daunorubicin can be reversed by addition of 3.3 μg/ml verapamil (RF: 6.8 and RF: 2.1, respectively). $CC531^{CDDP}$ is resistant to cisplatin and MMC as well, RF values of $CC531^{CDDP}$/CC531 were 7.1 and 4.9 for CDDP and MMC, respectively.

Cytotoxic assay

The survival parameters after exposure with MMC, KW-2149, M-16 and M-18 were determined by a slightly modified clonogenic assay.[16] Cells were exposed to different concentrations of KW-2149, M-16 and M-18, and to different exposure times.[14,15] The following combinations were tested: single KW-2149, M-16 and M-18; KW-2149 + M-16, KW-2149 + M-18, and KW-2149 + M-16 + M-18. For mixtures tested, the IC_{50} of the parent compound and $10^{-1} \times IC_{50}$ of the metabolites were combined. Incubation times chosen were 1, 2, 4, 6 and 8 h.

For the monolayer colony-forming assay, 250 cells were seeded in 6-well tissue culture plates and after 48 h the attached cells were exposed to the drug.[16] Plates were washed twice immediately after exposure and then fresh medium was added. The plates were kept in a humidified incubator for 11 days at 37°C in 5% CO_2 in air. The plates were fixed by methanol and the colonies were stained with Giemsa for counting. Sensitivity of the treated cells for a drug was expressed as the fraction of surviving colonies relative to untreated control culture × 100%. The concentration of a drug at which colony formation is 50% inhibited as compared with colony formation of untreated cells (IC_{50}) is used as the parameter of sensitivity for the drug. All these experiments were performed in triplicate. Standard deviation is not reported but was always 10% or less.

Statistical analysis

Data analysis included comparisons between the IC_{50} per exposure time used between cell lines tested and was performed by one-way ANOVA with Scheffe's procedure for multiple comparisons. For comparisons between the linear parts of the log dose–survival curves of the cell lines of interest, the Friedman two-way ANOVA test was used. For all tests $P < 0.05$ was taken as the level of significance.

Results

All cell lines used were grown as uniform monolayer cultures on plastic. The human cell lines had doubling times between 19 and 30 h for plastic adherent growth under the conditions described. The CC531 cell lines had doubling times between 34 and 38 h. All cells grew stably with respect to morphology, doubling time and DNA content. To investigate the sensitivity of cell lines for the compound of interest, the monolayer colony-forming assay was preferred. The log concentration–survival curves obtained with the agents of interest always had an S-shape with a step linear part generally between 10 and 90% survival.

Cytotoxicity of KW-2149, M-16, M-18 and MMC in the ovarian cancer cell lines

Activity in the $AOvC^{MMC}$/AOvC system. The MMC resistant human ovarian cancer cells showed also some resistance against KW-2149 (Table 1), with RF values for KW-2149 and MMC of 3.1 and 8.6, respectively. Cytotoxicity of KW-2149 was significantly higher in both cell lines over the molarities tested

Table 1. IC_{50}[a] and RF of MMC, KW-2149, M-16 and M-18 in the $AOvC^{MMC}$/AOvC cell system (t_{exp} = 1 h)

	IC_{50} $AOvC^{MMC}$ (nM)	IC_{50} AOvC (nM)	RF (–)
MMC	156.5	18.2	8.6
KW-2149	10.6	3.4	3.1
M-16	2.2×10^3	6.7×10^2	3.3
M-18	11.1	3.8	2.9

[a]The given concentration of the IC_{50} is the mean of three experiments.

as compared with MMC. Cytotoxicities of KW-2149, M-16, M-18 and MMC were exposure time dependent in both cell lines; in the AOvC^{MMC} cells prolongation of t_{exp} from 1 to 8 h resulted in a 30-fold decrease of IC_{50} for KW-2149 and a 10-fold decrease for MMC (Figure 2). Prolongation of t_{exp} increased the RF of MMC in the $AOvC_{MMC}$/AOvC system whereas the RF of KW-2149 and M-16 remained unchanged (Figure 3). The RF for M-18 increased with t_{exp} from t_{exp} = 1 to 3 h; further prolongation of t_{exp} up to t_{exp} = 8 h did not further influence the RF.

Combinations of KW-2149 with its metabolites demonstrated increased activity for those combinations in which M-18 was included: IC_{50} KW-2149 + 0.1 × IC_{50} M-18 and IC_{50} KW-2149 + 0.1 × IC_{50} M-16 + 0.1 × IC_{50} M-18 resulted in a significant decrease of survival of AOvC^{MMC} cells from 50% to 38% and 36%, respectively (t_{exp} = 1 h). Addition of 0.1 IC_{50} M-16 to IC_{50} KW-2149 did not decrease the 50% survival significantly as compared with the IC_{50} level of KW-2149 in the AOvC^{MMC} cells (i.e. 10.6 nM). Furthermore, the difference of survival between KW-2149 + M-18 and KW-2149 + M-16 + M-18 was negligible. Therefore it is concluded that the contribution of M-16 to the cytotoxicity of KW-2149 in AOvC^{MMC} cells is minimal. In the AOvC control cells a similar pattern was observed.

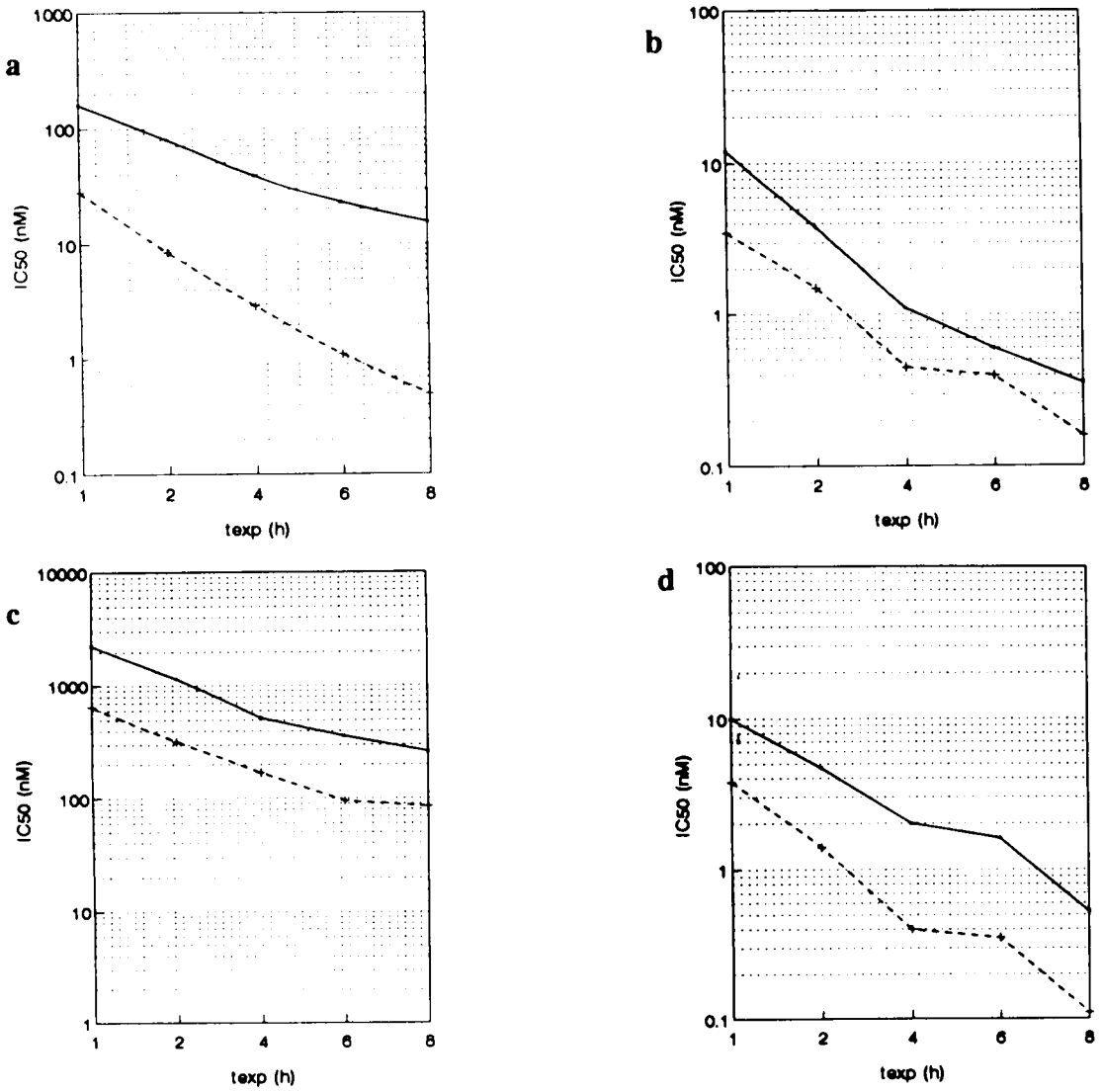

Figure 2. Activity of KW-2149, M-16, M-18 and MMC in the AOvC^{MMC}/AOvC system as a function of exposure time. Activity is expressed as the IC_{50} for each drug (nM) as a function of exposure time (h). (a) MMC, (b) KW-2149, (c) M-16 and (d) of M-18 in the AOvC cell line (+) and the AOvC^{MMC} (●) cell line.

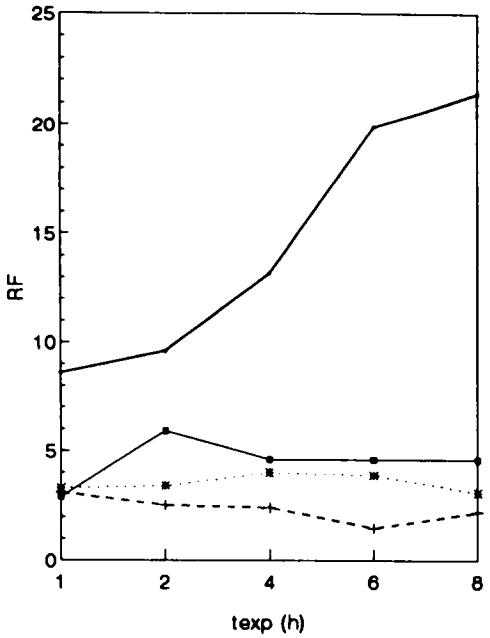

Figure 3. Influence of exposure time on RF in the AOvC^{MMC}/AOvC system for KW-2149 (+), M-16 (*), M-18 (■) and MMC (●).

Activity in the AOvC^{CDDP}/AOvC system. A marked decrease of activity of KW-2149 was observed in cisplatin-resistant human ovarian cancer cells: IC_{50} was 26.1 versus 3.4 nM in control cells (Table 2, RF = 7.7). MMC was less active than KW-2149: IC_{50} was 43.2 nM. The RF of MMC, however, appeared to be lower (Table 2, RF = 2.4). M-18 was the most active compound in cisplatin-resistant cells, but again cross-resistance was observed (RF = 3.6). M-16 was hardly hindered by cisplatin resistance with RF = 1.3; however, it was the compound with the lowest activity.

All mitomycins demonstrated t_{exp}-dependent activity in AOvC^{CDDP} cells; for MMC and M-18 this was noted over the whole range of t_{exp} tested (Figure 4). The RF of KW-2149 decreased markedly with t_{exp}; from RF = 7.6 at t_{exp} = 1 h to RF = 3.0 at t_{exp} = 8 h. The RF of M-18 decreased from 3.6 at t_{exp} = 1 h to 1.7 at t_{exp} = 8 h. The data of RF versus t_{exp} of the mitomycins tested demonstrate that cisplatin mediated cross-resistance can be partly overcome by an increase of exposure time at the cellular level (Figure 5).

Combinations including M-18 demonstrated a marked decrease of the 50% survival level as compared with single agent KW-2149 in cisplatin-resistant cells: 33% for the triple combination and 38% for IC_{50} KW-2149 + 0.1 × IC_{50} M-18. M-16 was concluded to be ineffective in increasing KW-2149 cytotoxicity, as in the MMC-resistant ovarian cancer cells.

Table 2. IC_{50}[a] and RF of MMC, KW-2149, M-16 and M-18 in the AOvC^{CDDP}/AOvC[b] cell system (t_{exp} = 1 h)

	IC_{50} AOvC^{CDDP} (nM)	IC_{50} AOvC (nM)	RF (–)
MMC	43.2	18.2	2.4
KW-2149	26.1	3.4	7.7
M-16	8.7×10^2	6.7×10^2	1.3
M-18	13.5	3.8	3.6

[a]The given concentration of the IC_{50} is the mean of three experiments.
[b]The RF for cisplatin in this system is 5.1.

Activity in the A2780^{mdr+}/A2780 system. The IC_{50} levels for MMC, KW-2149 and M-18 in the A2780 cell system were considerably higher than those obtained in the AOvC cell line. For M-16, however, the IC_{50} level was lower (Table 3). Cytotoxicity of KW-2149, M-16 and MMC was hardly affected by an increased PgP 170 expression as the RF ranged from 1.15 to 1.80. Contrary to these findings, cytoxicity of M-18 was a 7-fold lower in the A2780^{mdr+} cells, indicating a sensitivity of M-18 for *mdr* gene expression, i.e. the PgP 170 protein. The IC_{50} levels of all compounds of interest decreased with t_{exp} (Figure 6); a 3.3-fold decrease was noted for KW-2149 in A2780 control cells and for MMC in the A2780^{mdr+} cells a 3.2-fold decrease was found. The RF of M-16 and MMC was stable over t_{exp} tested (Figure 7). The RF of KW-2149 increased from 1.8 to 3.3 with t_{exp} from 1 to 6 h, whereas the RF of M-18 decreased from 7.6 at t_{exp} = 1 h to RF = 5.3 at t_{exp} = 8 h.

Addition of 0.1 × IC_{50} M-16 and/or 0.1 × IC_{50} M-18 to IC_{50} of KW-2149 had no marked effect on the survival of A2780 cells: survival was 47.1 to 50.7%. In the A2780^{mdr+} cells, however, the percentage of survival upon exposure to the triple combination

Table 3. IC_{50}[a] and RF of MMC, KW-2149, M-16 and M-18 in the A2780^{mdr+}/A2780[b] cell system (t_{exp} = 1 h)

	IC_{50} A2780^{mdr+} (μM)	IC_{50} A2780 (μM)	RF (–)
MMC	91.83	67.71	1.34
KW-2149	17.64	9.82	1.80
M-16	0.35	0.30	1.15
M-18	106.89	15.27	7.6

[a]The given concentration of the IC_{50} is the mean of three experiments.
[b]The RF for adriamycin is 100 and for vinblastin is 600.

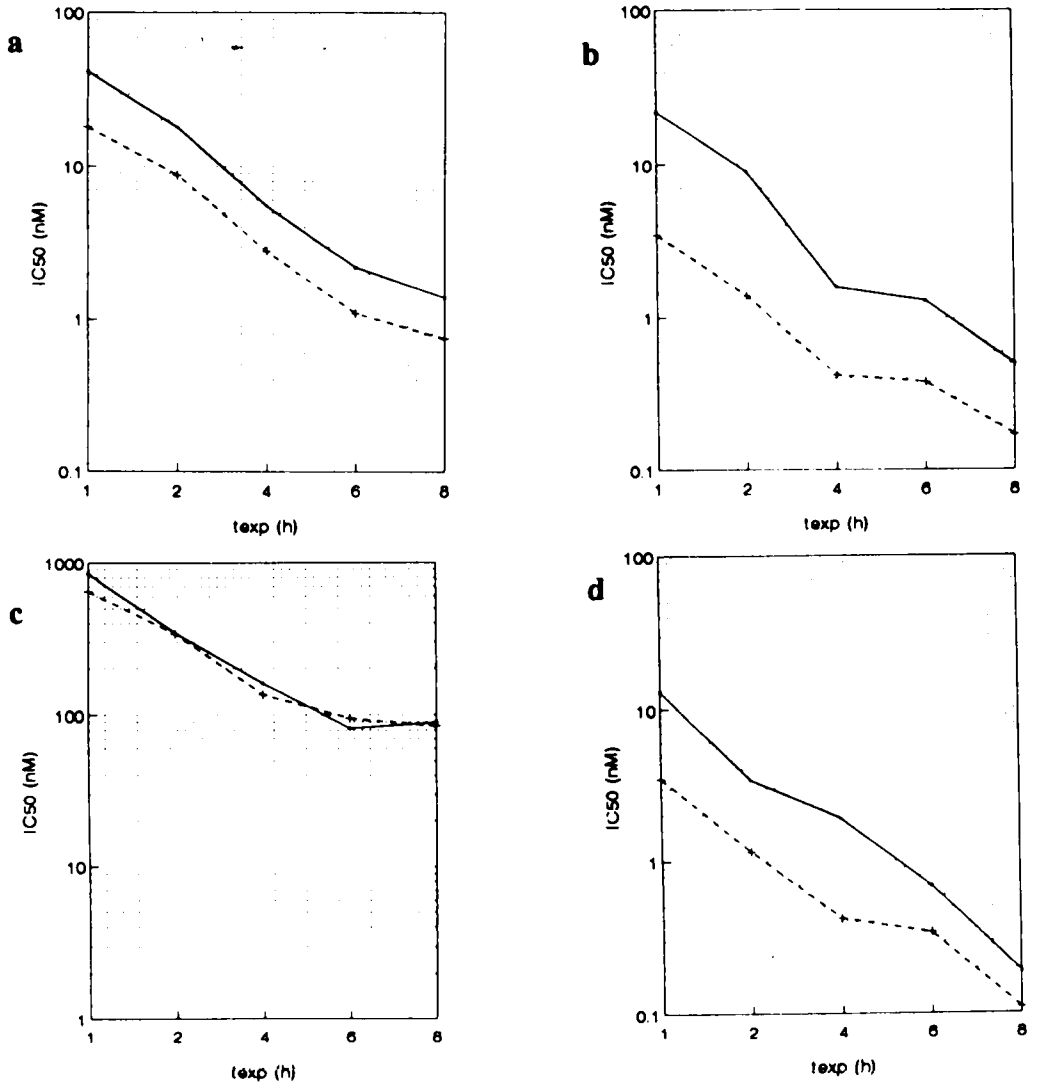

Figure 4. Activity of KW-2149, M-16, M-18 and MMC in the AOvC^{CDDP}/AOvC system. Activity is expressed as the IC_{50} for each drug (nM) as a function of exposure time (h). (a) MMC, (b) KW-2149, (c) M-16 and (d) M-18 in the AOvC^{CDDP} (●) and the AOvC (+) cell line.

was decreased to 18.2%. This is far below the calculated survival percentage of 1.2 × IC_{50} KW-2149 in A2780 cells, i.e. 42%. It is concluded therefore that M-16 + M-18 synergistically operate with KW-2149 in A2780^{mdr+} cells.

Cytotoxicity of KW-2149, M-16, M-18 and MMC in the CC531 colon cancer line

Activity in the CC531^{CDDP}/CC531 system. Resistance to cisplatin CC531 cells was induced by continuous exposure to cisplatin with an increase in concentrations over 2 years. The RF for cisplatin with t_{exp} = 1 h was 7.4. There was no marked change of the RF in the CC531^{CDDP}/CC531 system upon alteration of t_{exp} of cisplatin in the range of 1–8 h (6.8 < RF < 8.2). The IC_{50} at t_{exp} = 1 h in CC531^{CCDP} cells was 53.6 μM and in CC531 cells IC_{50} was 7.2 μM. Remarkably, the RF of KW-2149 in the CC531^{CDDP}/CC531 system was almost twice the RF for CDDP: 13.7 (Table 4). A similar RF was calculated for M-16, i.e. 11.7, whereas for M-18 and MMC the RF values were lower, i.e. 3.4 and 5.0, respectively. M-18 demonstrated a higher cytotoxicity in CC531^{CDDP} cells than KW-2149 (IC_{50} = 7.1 versus 8.1 μM for M-18 and KW-2149, respectively). All mitomycins tested demonstrated a decrease of IC_{50} with t_{exp} (Figure 8), which was linear in an IC_{50}–t_{exp} relationship for KW-2149 and

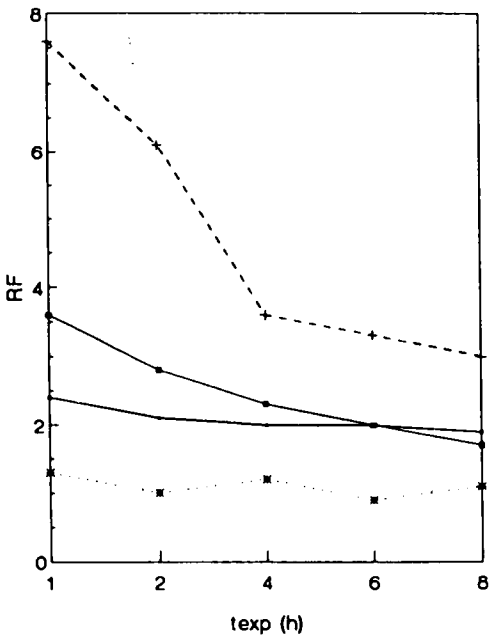

Figure 5. Influence of exposure time on RF in the AOvC^{CDDP}/AOvC system for KW-2149 (+), M-16 (*), M-18 (■) and MMC (●).

M-16. For MMC and M-18, an optimum of efficacy for prolongation of t_{exp} (dE/dt_{exp} = max) was found at t_{exp} = 4 h. The IC_{50}–t_{exp} relationship of MMC had a comparable decrease for both cell lines, therefore the RF was rather stable over t_{exp} tested (Figure 9). The RF of KW-2149 decreased from t_{exp} 1 to 2 h and gradually from t_{exp} 2 to 8 h. A marked increase of RF was found for M-16 from RF = 10.5 (t_{exp} = 2 h) to RF = 27.4 (t_{exp} = 8 h). Addition of 0.1 × IC_{50} M-16 to IC_{50} of KW-2149 did not affect the 50% survival levels as scored in CC531^{CDDP} cells with 8.1 μM KW-2149 alone. However, addition of 0.1 × IC_{50} M-18 (i.e. 0.7 μM) resulted in a marked decrease of the survival percentage from 50 to 32%. The theoretical survival calculated for 1.1 × IC_{50} KW-2149 (8.9 μM) and 1.1 × IC_{50} M-18 (7.8 μM) was 45 and 43%, respectively.

Table 4. IC_{50}[a] and RF of MMC, KW-2149, M-16 and M-18 in the CC531^{CDDP}/CC531[b] cell system (t_{exp} = 1 h)

	IC_{50} CC531^{CDDP} (nM)	IC_{50} CC531 (nM)	RF (–)
MMC	12.4	2.6	5.0
KW-2149	8.1	0.6	13.7
M-16	139.4	11.9	11.7
M-18	7.1	2.1	3.4

[a]The given concentration of the IC_{50} is the mean of three experiments.
[b]The RF for cisplatin is 7.1.

The mixture of IC_{50} of KW-2149 + 0.1 × IC_{50} of M-16 + 0.1 × IC_{50} of M-18 resulted in a 31% survival of CC531^{CDDP} cells. In CC531 control cells, addition of M-16 and/or M-18 to KW-2149 did not result in a marked increase in cytotoxicity in CC531 control cells; however, survival was in the range of 45–51%.

Activity in the CC531^{mdr+}/CC531 system. The tests in the CC531^{mdr+}/CC531 system have been performed separately from those with the CC531^{CDDP}/CC531 system, in contrast to the tests with the AOvC cells. Therefore data of CC531 in Tables 4 and 5 slightly differ. Cytotoxic activity of KW-2149, M-16 and MMC was hardly hindered by elevated expression of PgP 170 in the CC531^{mdr+}/CC531 system; RF values were in the range of 1.5 (M-16)–3.0 (KW-2149) (Table 5). Activity of M-18, however, was markedly lower in CC531^{mdr+} cells. At a t_{exp} = 1 h, the RF of M-18 was 7.4. Cytotoxic activity of all mytomycins tested increased with t_{exp} in both cells lines (Figure 10). This resulted in stable RF values for MMC, KW-2149 and M-16 (Figure 11). The RF of M-18, however, increased with exposure time from 7.4 at t_{exp} = 1 h to 153.3 at t_{exp} = 8 h. Nevertheless, IC_{50} of M-18 in CC531^{mdr+} cells decreased from 16.2 to 9.2 μM (1.8-fold) within the range of t_{exp} tested. The largest decrease of IC_{50} with t_{exp} was noted for M-18 in CC531 control cells (36.7-fold), which explains the sharp increase of RF with t_{exp} for M-18. Another large decrease in IC_{50} with t_{exp} was noted for KW-2149 in CC531^{mdr+} cells (35-fold). Addition of 0.1 × IC_{50} of M-16 and/or 0.1 × IC_{50} of M-18 did not result in a marked decrease of IC_{50} of KW-2149 in CC531 cells; 48% survival for KW-2149 + M-16 and KW-2149 + M-18; 46% for the triple combination. A similar pattern was also found in the control CC531 cells.

Discussion

In the present study we have examined the *in vitro* antitumor activity of a new MMC analog, i.e. KW-2149, and two of its metabolites against different human and murine cell lines with different types of resistance. We have compared these activity data with those obtained with MMC and have examined the impact of the presence or absence of the metabolites of KW-2149 on *in vitro* drug testing.

Overall, KW-2149 showed, in comparison with MMC, superior activity against all cell lines tested, which is consistent with the data of Morimoto *et al.*[11] On a molar base this superior activity of KW-2149 is impressive in our cell lines and in view of the

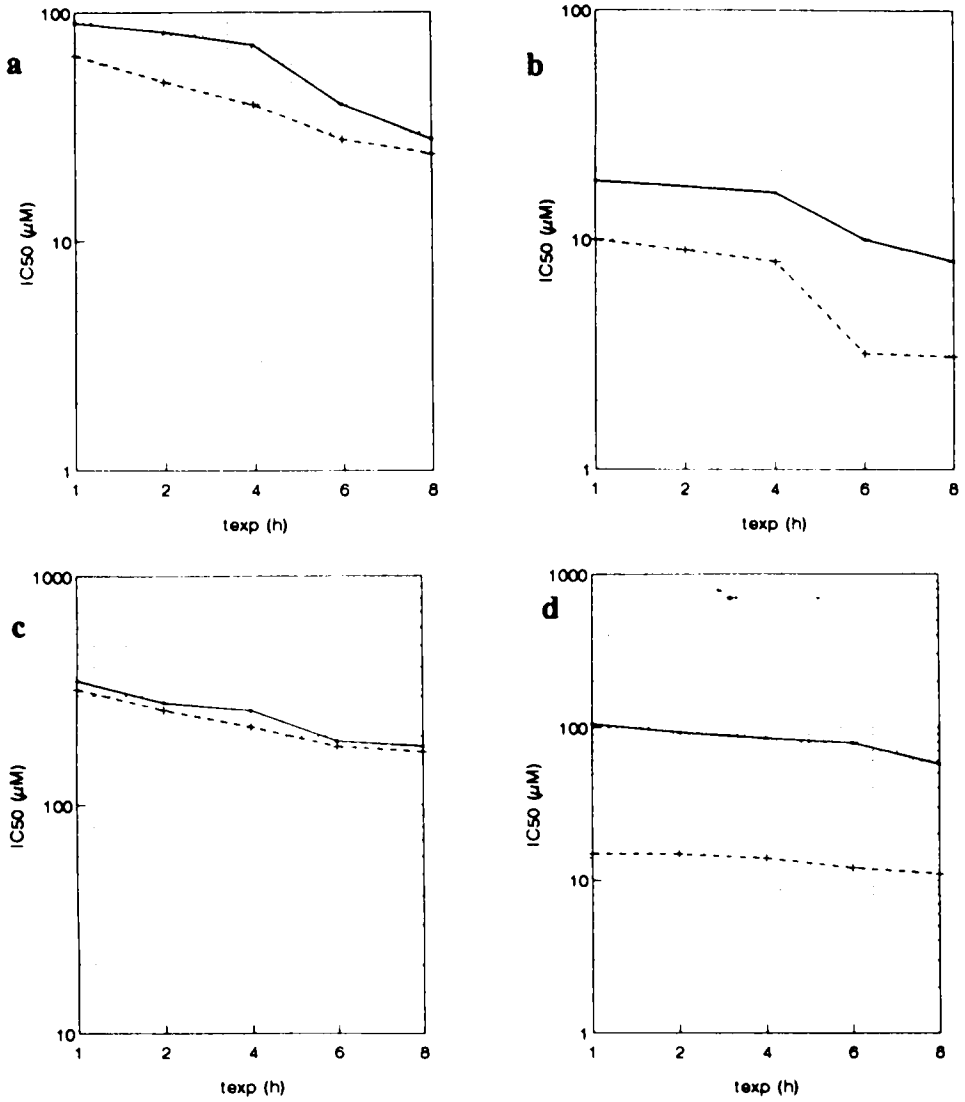

Figure 6. Activity of KW-2149, M-16, M-18 and MMC in the A2780^{mdr+}/A2780 system. Activity is expressed as the IC_{50} for each drug (μM) as a function of exposure time (h). (a) MMC, (b) KW-2149, (c) M-16 and (d) M-18 in the A2780 (+) and A2780^{mdr+} (●) cell line.

animal toxicology data this looks promising for clinical application of the new MMC analog.[12]

Separate testing of M-16 and M-18 showed significant activity of M-18, in general comparable with that of KW-2149. While the activity of M-18 is comparable with that of the parent compound on a quantitative basis, both their activities are affected differently by the resistance phenotype of the exposed cell line. In both the human ovarian cell line and in the rat colon carcinoma cell line, the activity of KW-2149 was hindered to a greater extent by cisplatin resistance. The activity of M-18 compared with that of KW-2149 was more affected by the expression of the MDR phenotype, both in the human ovarian and the rat colon carcinoma cell line. The activity of M-16 as a single agent was lowest. Taking the results of the human pharmacokinetic analysis into account, it might well be that M-18 contributes to a significant extent to the activity of KW-2149.[13] Within minutes of intravenous administration of KW-2149, blood levels of the parent drug decrease and both M-16 and M-18 become detectable, be it both at lower concentrations but both with much longer residence times. Contributive activities of metabolites to the parent drug are generally difficult to determine and even seem to be neglected in preclinical anticancer drug testing.

Here, the testing of mixtures has been proposed at the IC_{50} level of the parent compound with $10^{-1} \times IC_{50}$ level of the metabolites simulating the

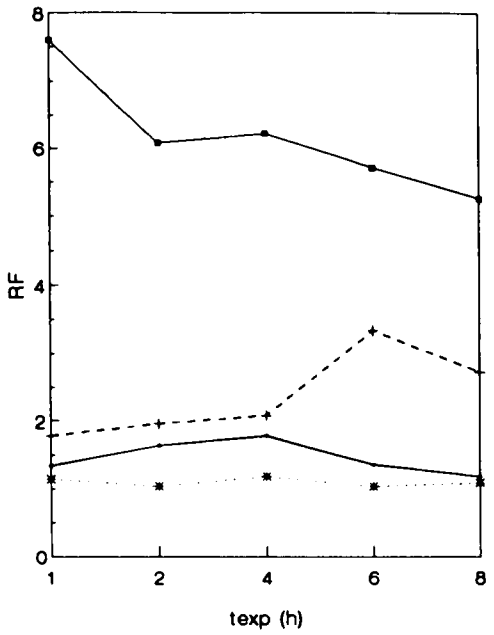

Figure 7. Influence of exposure time on RF in the A2780^{mdr+}/A2780 system for KW-2149 (+), M-16 (*), M-18 (■) and MMC (●).

Table 5. IC_{50}[a] and RF of MMC, KW-2149, M-16 and M-18 in the CC531^{mdr+}/CC531[b] cell system (t_{exp} = 1 h)

	IC_{50} CC531^{mdr+} (nM)	IC_{50} CC531 (nM)	RF (–)
MMC	5.1	2.3	2.2
KW-2149	2.1	0.6	3.0
M-16	19.3	13.2	1.5
M-18	16.2	2.2	7.4

[a]The given concentration of the IC_{50} is the mean of three experiments.
[b]The RF for adriamycin is 29 and for vinblastin is 90.

large differences between maximum concentrations determined in plasma. Additional effects of the metabolites have been observed in the cell lines we have used for assessing KW-2149 cytotoxic activity. Morimoto *et al.* found remarkable antitumor activity of KW-2149 in MMC-resistant murine leukemias.[11] KW-2149 significantly increased the survival of MMC-resistant P338 leukemia- and L1210 leukemia-bearing mice, indicating that KW-2149 is not subjected to MMC resistance in murine MMC-resistant leukemias. It has been suggested that the me-

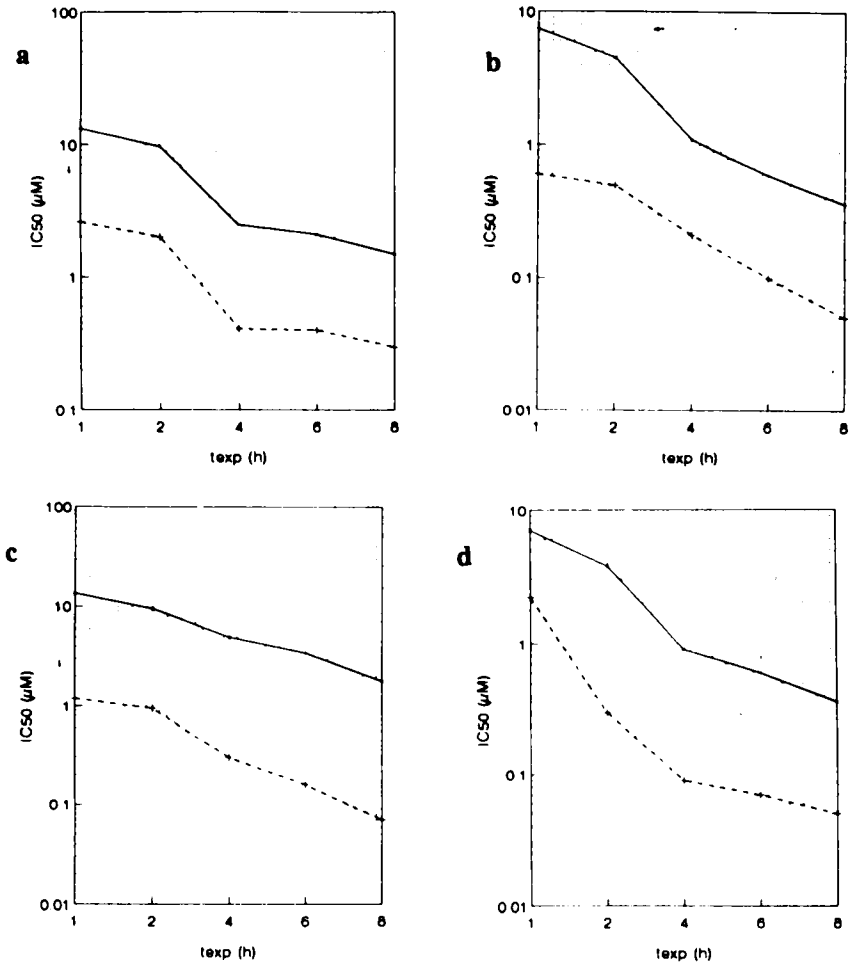

Figure 8. Activity of KW-2149, M-16, M-18 and MMC in the CC531^{CDDP}/CC531 system. Activity is expressed as the IC_{50} for each drug (μM) as a function of exposure time (h). (a) MMC, (b) KW-2149, (c) M-16 and (d) M-18 in the CC531^{CDDP} (●) and the CC531 (+) cell line.

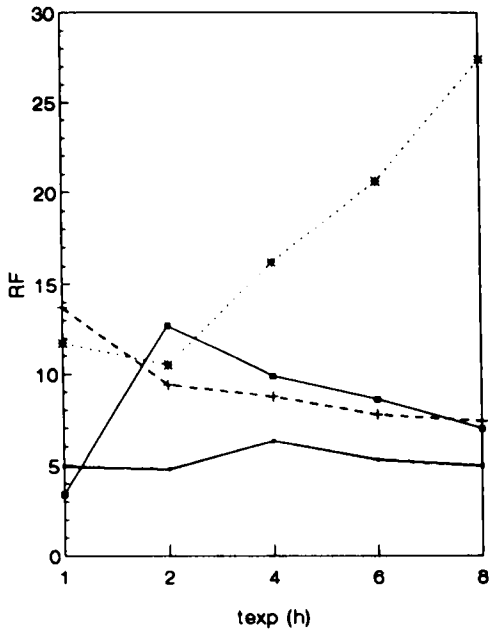

Figure 9. Influence of exposure time on RF in the $CC531^{CDDP}$/CC531 system for KW-2149 (+), M-16 (*), M-18 (■) and MMC (●).

chanisms of action of KW-2149 are different from those of MMC. Taking the present data into account, however, it can be assumed that *in vivo* KW-2149 activity is not limited to the parent compound alone, but also on the presence of its metabolites, especially M-18. This illustrates the importance of concomitant *in vitro* testing of parent drug and metabolites in order to reduce differences between *in vitro* and *in vivo* anticancer drug testing.

The exposure time dependency of the activity of MMC has been demonstrated previously, and has also been described for many other cytotoxic agents.[15,21,22] The influence of the exposure time on activity of KW-2149, M-16 and M-18 has been revealed presently. A new finding, however, is the present observation that increments in exposure time appeared as a means to overcome drug resistance (Figure 5, KW-2149 and M-18). It illustrates that resistance factors as they are measured during *in vitro* drug testing can be highly dependent on the exposure time applied. The *in vitro* anticellular spectrum of KW-2149 was comparable with that

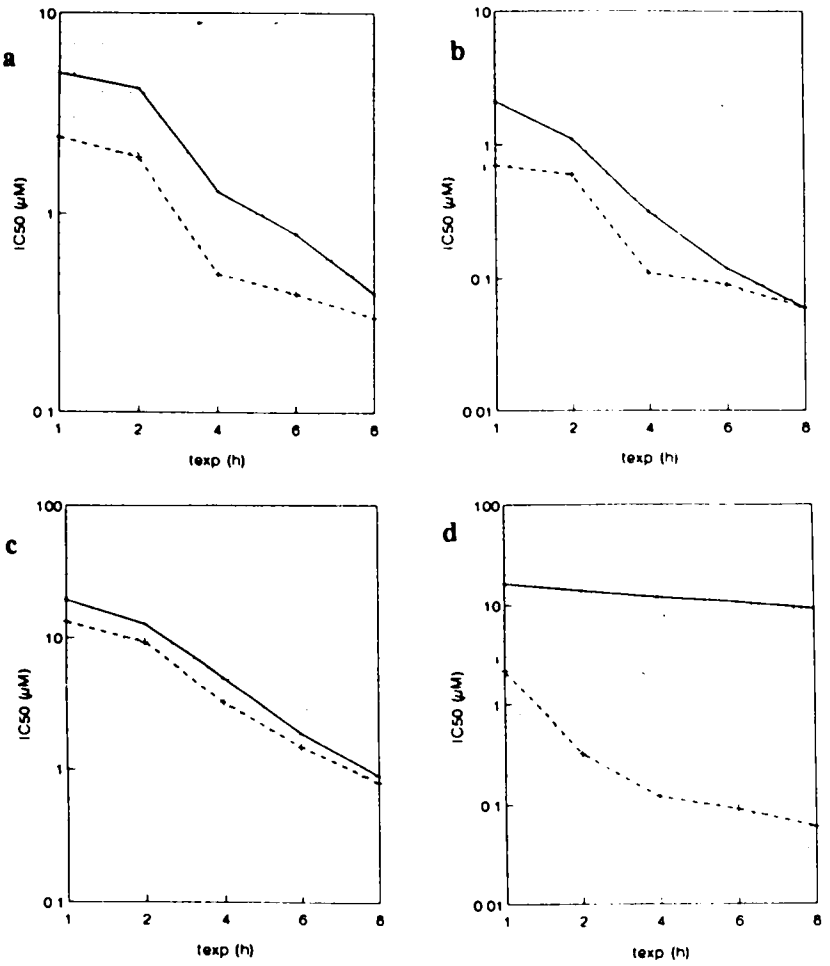

Figure 10. Activity of KW-2149, M-16, M-18 and MMC in the $CC531^{mdr+}$/CC531 system. Activity is expressed as the IC_{50} for each drug (μM) as a function of exposure time (h). (a) MMC, (b) KW-2149, (c) M-16 and (d) M-18 in the $CC531^{mdr+}$ (●) and the CC531 (+) cell line.

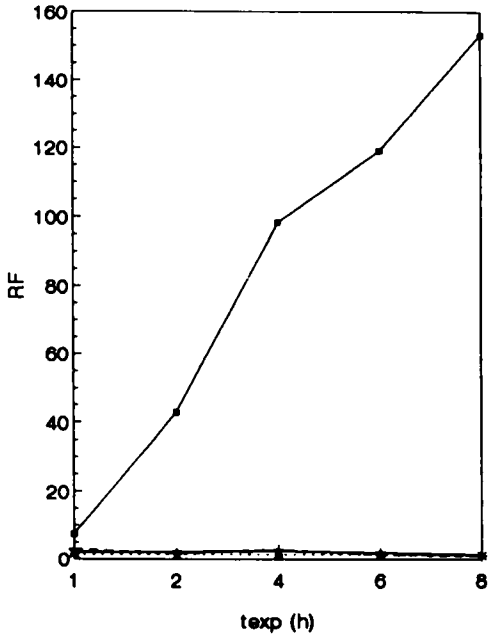

Figure 11. Influence of exposure time on RF in the CC531^{mdr+}/CC531 system for KW-2149 (+), M-16 (*), M-18 (■) and MMC (●).

of MMC; however, resistance found in MMC-resistant cells was clearly lower (Table 1, RF = 8.6 versus 3.1), whereas KW-2149 was found to be more hindered by cisplatin resistance than MMC (Table 2 and 4). Nevertheless, KW-2149 was still more active in cisplatin-resistant cells than MMC.

Efficacy of KW-2149 was hardly hindered by PgP 170 expression (Table 3 and 5). These data favor the application of KW-2149 against tumors with high PgP 170 expression and indicate its limitation against cisplatin pretreated tumors. Furthermore, it needs emphasizing that resistance can be overcome in the presence of the metabolites of KW-2149.

Conclusion

The present data confirm that KW-2149 is a promising MMC analog with a distinct activity and mode of action. We are currently examining the mechanistic differences between MMC KW-2149, M-16 and M-18 in these different cell lines in order to explain these differences in activity. The contribution of the activity of M-16 and M-18 to that of KW-2149 has major implications for forthcoming phase II studies. Its dependency on exposure time for efficacy and even as a tool to overcome resistance could be optimized in body cavities lacking intensive clearance such as the bladder cavity.[22]

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(Received 28 October 1993; received in revised form 8 February 1994; accepted 25 February 1994)