In Vitro Evaluation of the New Anticancer Agents KT6149, MX-2, SM5887, Menogaril, and Liblomycin Using Cisplatin- or Adriamycin-resistant Human Cancer Cell Lines

Yuichiro Ohe, Kazuhiko Nakagawa, Yasuhiro Fujiwara, Yasutsuna Sasaki, Koichi Minato, Masami Bungo, Shigeki Niimi, Naoya Horichi, Masaaki Fukuda, and Nagahiro Saijo

Department of Internal Medicine, National Cancer Center Hospital [Y. O., Y. F., Y. S., M. F.] and Pharmacology Division, National Cancer Center Research Institute [K. N., K. M., M. B., S. N., N. H., N. S.J., 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104, and Second Department of Internal Medicine, The Jikei University School of Medicine [Y. O.], 25-8, Nishinshinbashii 3-chome, Minato-ku, Tokyo 105, Japan

ABSTRACT

A new model to predict antitumor activity of new analogues was developed, and the cross-resistance against cisplatin (CDDP) and Adriamycin (ADM) (ADM) was examined. A preclinical evaluation of various new analogues using this new model was performed. The antitumor activities of KT6149, MX-2 (KRN8602), SM5887, menogaril (TUT-7), and liblomycin (NK313) were evaluated against four non-small cell lung cancer cell lines, PC-7, -9, -13, and -14; two small cell lung cancer cell lines, H69 and N231; four CDDP-resistant cell lines, PC-7/1.0, PC-9/0.5, PC-14/1.5, and H69/0.4; a human myelogenous leukemia cell line, K562; and its ADM-resistant subline, K562/ADM by clonogenic assay. The relative antitumor activities of these new analogues were compared with those of parental agents, mitomycin C, ADM, bleomycin, and several anticancer drugs, CDDP, daunomycin, vinodesine, and etoposide.

KT6149 was more active than mitomycin C against all lung cancer cell lines and the human myelogenous leukemia cell line. Menogaril showed greater activity than ADM, and MX-2 showed activity similar to ADM. However, the antitumor activity of SM5887 was lower than that of ADM. SM5887 and menogaril showed cross-resistance to K562/ADM. Nevertheless, the antitumor activity against K562/ADM of MX-2 was similar to that of the parental cell lines. The activity of liblomycin was similar to that of bleomycin.

Thus, KT6149 appears to be the best analogue for use in a clinical trial against lung cancer. MX-2 was active even against ADM-resistant cancer cells.

The values of relative resistance to CDDP or ADM were 4.7, 8.1, 7.5, 20.0, and 13.6 for PC-7/1.0, PC-9/0.5, PC-14/1.5, H69/0.4, and K562/ADM, respectively. CDDP-resistant cell lines showed no cross-resistance with other drugs in this study. K562/ADM showed cross-resistance against daunomycin, etoposide, and vinodesine. In contrast, mitomycin C and bleomycin had nearly equal activity against K562 and K562/ADM. However, K562/ADM was 2.4-fold more sensitive to CDDP than its parent K562 (P < 0.001). These results suggested that the mechanism of CDDP resistance is different from that of multidrug resistance.

INTRODUCTION

Although SCLC is responsive to initial chemotherapy, relapses are common, and only 5 to 10% of patients have long-term survival of greater than 3 yr (1). In advanced NSCLC patients, chemotherapy has little impact on the survival rate (2). It is essential to identify new effective anticancer drugs in order to improve the survival rate in lung cancer.

Although CDDP and ADM have been demonstrated to be active drugs against SCLC, the acquired resistance to CDDP and/or ADM is a common cause of chemotherapy failure in SCLC. It is important to develop new anticancer drugs which are active against CDDP- and/or ADM-resistant tumor cells for the treatment of patients in whom CDDP and/or ADM treatment was not effective.

Many new anticancer agents used in Phase II studies are ineffective, and the patients who enter these studies with inactive agents sometimes have the disadvantage of a shortened survival time. However, it is important not to overlook the possible new active anticancer agents. Therefore, critical preclinical evaluation of new anticancer agents is important. We have developed a new model to predict the antitumor activity of new analogues using relative antitumor activity. This model is proposed for the prediction of the cross-resistance of CDDP and ADM, using their resistant cell lines.

KT6149 is a new derivative of MMC and has been demonstrated to be more active against mouse tumors and human xenografts than MMC. The LD50 value of KT6149 in mice is higher than that of MMC. MX-2, SM5887, and menogaril are new anthracyclines with greater antitumor activity and lower cardiac toxicity than ADM. Liblomycin is a new derivative of BLM, and its pulmonary toxicity has been predicted to be lower than that of BLM (3, 4).

In order to accurately determine the preclinical evaluation of the new analogues, KT6149, MX-2, SM5887, menogaril, and liblomycin, the antitumor activity of these drugs against several human cancer cell lines and their CDDP- or ADM-resistant cell lines was evaluated by clonogenic assay and compared to that of their parent compounds, MMC, ADM, and BLM.

MATERIALS AND METHODS

Tumor Cell Lines and Culture. Four NSCLC-, two SCLC-, four CDDP-resistant lung cancer cell lines, a human myelogenous leukemia cell line, and the derived ADM-resistant sublines were used in a clonogenic assay. The NSCLC cell lines, PC-7, -9, -13, and -14, were kindly donated by Professor Y. Hayata of the Tokyo Medical College. PC-7, -9, and -14 were derived from an adenoscarcinoma, and PC-13 was from a large cell carcinoma (5, 6). The SCLC cell lines, H69 and N231, were established at the National Cancer Institute, USA (7). CDDP-resistant cell lines, PC-7/1.0, PC-9/0.5, PC-14/1.5, and H69/0.4, were established in our laboratory (8), and they were maintained at concentrations of 1.0, 0.5, 1.5, and 0.4 µg/ml of CDDP, respectively. The human myelogenous leukemia cell line, K562 (9), and its ADM-resistant subline, K562/ADM (10), were kindly donated by Dr. T. Tsuruo of the Japanese Foundation for Cancer Research. This cell line was maintained at the concentration of 0.3 µg/ml of ADM. The characteristics of these cell lines are shown in Table 1. Prior to use in the clonogenic assay, the cell lines were propagated by culturing in

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2 To whom requests for reprints should be addressed, at Department of Internal Medicine, National Cancer Center Hospital, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan.

3 The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; CDDP, cisplatin; ADM, Adriamycin; MMC, mitomycin C; L<sub>D<sub>50</sub> 50% lethal dose; BLM, bleomycin; FBS, fetal bovine serum; VP-16, etoposide; VDS, vinodesine; DM, daunomycin; HS, horse serum; IC<sub>50</sub> 50% inhibitory concentration; PPC, peak plasma concentration; CBDCA, carboplatin.

* Personal communication.
Table 1 Characteristics and plating cell numbers of human cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Histology</th>
<th>Prior chemotherapy</th>
<th>Cell no.</th>
<th>Plating efficiency (%)</th>
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<tr>
<td>PC-7</td>
<td>Adeno*</td>
<td>No</td>
<td>3 x 10^4</td>
<td>9</td>
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<tr>
<td>PC-7/1.0</td>
<td>Adeno*</td>
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<td>8</td>
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<td>Adeno</td>
<td>No</td>
<td>1 x 10^4</td>
<td>20</td>
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<tr>
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<td>Adeno</td>
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<td>1 x 10^4</td>
<td>9</td>
</tr>
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<td>3 x 10^4</td>
<td>5</td>
</tr>
<tr>
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</tr>
<tr>
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<td>2</td>
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<tr>
<td>N231</td>
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<td>5</td>
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<tr>
<td>K562</td>
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<td>20</td>
</tr>
<tr>
<td>K562/ADM</td>
<td>CML</td>
<td>Yes*</td>
<td>1 x 10^4</td>
<td>18</td>
</tr>
</tbody>
</table>

* Adeno, adenocarcinoma of the lung; large, large cell carcinoma of the lung; small, small cell carcinoma of the lung; CML, chronic myelogenous leukemia.

RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS (Gibco) plus penicillin (100 units/ml) and streptomycin (100 µg/ml). The CDDP-resistant sublines and ADM-resistant subline were cultured for 2 to 3 wk prior to the analyses in drug-free medium.

Anticancer Drugs. The chemical structures of the new anticancer agents, 7-N-[2-[2-(R-L-glutamylamino)ethyl]dithio][ethyl]] mitomycin C (KT6149), 3'-deamino-3'-morpholino-13-deoxo-10-hydroxycarmi-noycin hydrochloride (MX-2, KRN8602), (7S,9S)-9-acetyl-9-amino-7-[2-deoxy-D-erythro-pentopyranosyl]oxy]-7,8,9,10-tetrahydro-6,11-dihydroxy-5,12-naphthacendione hydrochloride (SM5887), 7-con-0-methylloganor (menogaril, TUT-7), and 3-[(N-methyl-N-[3'-bis-(3',4'-dibenzylxoybenzyl]amino)propyl]amino| propylaminobleomycin hydrochloride (liblomycin, NK313) are shown in Fig. 1.

All drugs except CDDP were dissolved in distilled water and stored at -20°C. The drugs were diluted with RPMI 1640 plus 10% FBS just prior to each experiment.

Clonogenic Assay. The clonogenic assay used in this study was a modification of the double agar method developed by Hamburger and Salmon (11–13). Tumor cells were harvested from the cell culture and counted. Viable cells were determined by the trypan blue dye exclusion method. The number of cells plated was determined according to the growth rate and the plating efficiency of each cell line (Table 1). All assays were performed using 6-well plate dishes (Linbro, VA; No. 76-058-05). Tumor cells plated in the top layer consisted of RPMI 1640 plus 10% FBS, 0.3% agar, and the appropriate concentration of each drug. All experiments were performed with continuous drug exposure. One ml per well was layered onto the previously prepared underlayer. The underlayer contained 0.5% agar in enriched McCoy's Medium 5A (Gibco). Enriched McCoy's Medium 5A consisted of 50 ml of heat-inactivated HS, 25 ml of heat-inactivated HS, 4 ml of 2.2% sodium pyruvate, 4 ml of 200 mM glutamine, 0.8 ml of 2.1% serine, 4 ml of 100 units/ml of penicillin, and 100 µg/ml of streptomycin, mixed with 400 ml of McCoy's Medium 5A. All assays were performed in triplicate, and the value obtained from one experiment represents the mean of the triplicate assay. Plates were incubated in a humidified atmosphere of 5% CO₂ at 37°C. After 7 to 21 days, the number of colonies was counted once very 2 to 3 days with a CP-2000 automated colony counter (Shiraimatsu Instrument, Osaka, Japan). Colonies larger than 50 µm in diameter were regarded as positive. The percentage of colony inhibition was determined by the following formula.

\[
\% \text{ of colony inhibition} = 100 - \frac{\text{no. of maximal colonies/test dish}}{\text{no. of maximal colonies/control dish}} \times 100
\]

Analysis. The IC₅₀ value was determined graphically from the dose-response curve at three or four drug concentration points. The experiments were repeated 3 to 6 times for each drug and each cell line. The relative antitumor activity, calculated by the following formula (14), was used to compare the antitumor effect of each drug.

Relative antitumor activity = peak plasma concentration (µg/ml) / IC₅₀ values (µg/ml)

The PPC of MMC, CDDP, VP-16, VDS, ADM, DM, menogaril,
and BLM has been reported in previous Phase I studies (Table 2) (15). However, accurate pharmacokinetic data of KT6149, MX-2, SM5887, and liblomycin have not been reported in humans. Therefore, the PPC of KT6149, MX-2, SM5887, and liblomycin was obtained by the following formula (16).

\[
\log(\text{PPC}) = 0.755 \times \log(\text{mice LD}_{50}) - 0.788
\]

Statistical Analysis. All data were analyzed for significance by the two-tailed Student t test. P values were calculated by comparison of experimental groups.

RESULTS

Comparison of the IC_{50} Values of Several Anticancer Drugs

Used Clinically. The IC_{50} values for MMC, CDDP, VP-16, VDS, BLM, DM, and ADM against six human lung cancer cell lines and four cell lines selected for CDDP resistance and against the human myelogenous leukemia cell line and its ADM-resistant subline were determined by clonogenic assay (Tables 3 to 6).

PC-7/1.0, PC-9-0.5, PC-14/1.5, and H69/0.4 were more resistant to CDDP than the parental cell lines, with the relative resistance (IC_{50} of resistant cell line/IC_{50} of parental cell line) of 4.7, 8.1, 7.5, and 20.0, respectively. As shown in Table 7, no cross-resistance was observed between the parental and the CDDP-resistant cell line to other drugs in this study. K562/ADM was 2.4-fold more sensitive to CDDP than its parental cell line, K562 (P < 0.001) (Fig. 2).

Comparison of the Antitumor Activity of KT6149 and MMC. As shown in Table 4, KT6149 and MMC had nearly the same IC_{50} values. In a previous Phase I study, PPC of MMC was reported to be 0.52 μg/ml (15). However, PPC of KT6149 in humans has not been reported yet, and therefore the predicted PPC was calculated from the LD_{50} value of mice using the above-mentioned formula (16). The predicted PPC of KT6149 was 1.71 μg/ml (Table 2). As shown in Table 4, KT6149 showed a higher relative antitumor activity than MMC in all cell lines.

Comparison of the Antitumor Activity of New Anthracyclines and ADM. MX-2, SM5887, menogaril, and ADM were also effective against CDDP-resistant sublines as the parental cell lines. MX-2 and menogaril had similar IC_{50} values to ADM in human lung cancer cell lines and its CDDP-resistant sublines. SM5887 showed higher IC_{50} values than that of ADM in all lung cancer cell lines in this study (Table 5). In a previous Phase I study, the PPC values of menogaril and ADM were reported to be 0.85 and 0.60 μg/ml, respectively (15). The predicted PPC values MX-2 and SM5887, calculated from the LD_{50} values of mice (16), were 0.54 and 2.73 μg/ml, respectively. Menogaril had a higher relative antitumor activity than ADM, while MX-2 had activity similar to ADM. However, the antitumor activity of SM5887 was lower than that of ADM in lung cancer cell lines (Table 5). ADM, SM5887, and menogaril were less effective against K562/ADM than its parental cell line, K562. Nevertheless, MX-2 had the same antitumor activity against K562/ADM to its parental cell line (Tables 4 and 7).

Comparison of the Antitumor Activity of Liblomycin and BLM. IC_{50} values of liblomycin against human lung cancer cell lines and human myelogenous leukemia cell lines were slightly higher than those of BLM (Table 6). The reported PPC of BLM is 3.00 μg/ml (15), and the predicted PPC of liblomycin, from LD_{50} values of mice, is 5.78 μg/ml. Liblomycin had a relative antitumor activity similar to BLM.

DISCUSSION

MMC is considered to be one of the active anticancer agents against NSCLC. KT6149 is a derivative of MMC with less toxicity than MMC in mice. KT6149 showed no cross-resistance and no collateral sensitivity against CDDP- or ADM-resistant sublines. KT6149 was more active than MMC against all lung cancer cell lines, and thus it can be concluded by this

<table>
<thead>
<tr>
<th>IC_{50} values (μg/ml)</th>
<th>Relative antitumor activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMC</td>
<td>KT6149</td>
</tr>
<tr>
<td>PC-7</td>
<td>0.024 ± 0.005</td>
</tr>
<tr>
<td>PC-7/1.0</td>
<td>0.040 ± 0.018</td>
</tr>
<tr>
<td>PC-9</td>
<td>0.040 ± 0.012</td>
</tr>
<tr>
<td>PC-9/0.5</td>
<td>0.039 ± 0.010</td>
</tr>
<tr>
<td>PC-13</td>
<td>0.044 ± 0.015</td>
</tr>
<tr>
<td>PC-14</td>
<td>0.25 ± 0.06</td>
</tr>
<tr>
<td>PC-14/1.5</td>
<td>0.21 ± 0.07</td>
</tr>
<tr>
<td>H69</td>
<td>0.032 ± 0.018</td>
</tr>
<tr>
<td>H69/0.4</td>
<td>0.06 ± 0.04</td>
</tr>
<tr>
<td>N231</td>
<td>0.049 ± 0.023</td>
</tr>
<tr>
<td>K562</td>
<td>0.18 ± 0.09</td>
</tr>
<tr>
<td>K562/ADM</td>
<td>0.28 ± 0.09</td>
</tr>
</tbody>
</table>

*a Mean ± SD.

Table 4 Comparison of IC_{50} values and relative antitumor activity (peak plasma concentration/IC_{50}) between MMC and KT6149 of human cancer cell lines and the CDDP- or ADM-resistant sublines.
liblomycin had slightly higher IC\textsubscript{50} values than BLM in this study and a relative resistance to BLM-inactivating enzymes (17). Liblomycin had activity of BLM in animal testing data (3, 4). In addition, liblomycin is effective against lung cancer than ADM, and MX-2 is effective against K562/ADM similar to the parental cell lines. From these findings, it can be proposed that menogaril is more effective against lung cancer than ADM, while MX-2 had an activity similar to ADM.

Menogaril had a higher relative antitumor activity than ADM, while MX-2 had an activity similar to ADM. The antitumor activity of SM5887 was lower than that of ADM. SM5887 is transformed into its active form in vivo, so the antitumor activity of SM5887 was lower than that of ADM, while MX-2 had an activity similar to ADM. The evaluation of the antitumor activity of new compounds using the relative antitumor activity values is still a controversial method that KT6149 may be effective for clinical use.

Menogaril had a higher relative antitumor activity than ADM, while MX-2 had an activity similar to ADM. The antitumor activity of SM5887 was lower than that of ADM. SM5887 is transformed into its active form in vivo, so the antitumor activity of SM5887 is most likely underestimated in this study. SM5887 and menogaril were less effective against K562/ADM than the parental cell line, K562. MX-2 had antitumor activity against K562/ADM similar to the parental cell lines. From these findings, it can be proposed that menogaril is more effective against lung cancer than ADM, and MX-2 is effective even against ADM-resistant cancer cells. Liblomycin is a derivative of BLM with a pulmonary toxicity which is less than that of BLM in animal testing data (3, 4). In addition, liblomycin is resistant to BLM-inactivating enzymes (17). Liblomycin had slightly higher IC\textsubscript{50} values than BLM in this study and a relative antitumor activity similar to BLM.

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PRECLINICAL EVALUATION OF NEW AGENTS ON RESISTANT CELLS

ADM-resistant cancer cells have shown cross-resistance to not only other anthracyclines and Vinca alkaloids but also other anticancer agents. It is also important to identify effective agents against ADM-resistant sublines in the screening of new anticancer anthracyclines. Therefore, we used the ADM-resistant human myelogenous leukemia subline, K562/ADM, in this study. K562/ADM was 13.6-fold more resistant to ADM than its parental cell line and showed cross-resistance with DM, VP-16, and VDS. In contrast, MMC and BLM had almost the same efficacy against K562 as K562/ADM. Moreover, K562/ADM was 2.4-fold more sensitive to CDDP than its parental cell line, K562. These results suggest that the mechanism of CDDP resistance is different from that of multidrug resistance.

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