Comparison of the Cytotoxic Effects of the High- and Low-Molecular-Weight Anticancer Agents on Multidrug-resistant Chinese Hamster Ovary Cells *In Vitro*¹

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ABSTRACT

Neocarzinostatin (NCS), styrene-maleic acid copolymer-conjugated neocarzinostatin (SMANCS), and ricin exhibited cytotoxicity against two different types of Chinese hamster ovary cells, parental AUXB1 cells and the multidrug-resistant (MDR) subline CH₄5 cells at the nanomolar range. These doses were much lower than those of the other anticancer drugs tested (micromolar range), even after a short incubation. MDR CH₄5 cells were 20 to 900 times more resistant to Adriamycin, aclacinomycin, vinblastine, and mitomycin C than were AUXB1 cells. However, the resistance of CH₄5 cells to NCS, SMANCS, or ricin was relatively low: the 50% colony inhibitory concentration was only 5 to 10 times higher than that for parental AUXB1 cells. CH₄5 cells were not resistant to 5-fluorouracil and cis-diaminedichloroplatinum(II), but the effective doses of these agents to them were 10³ to 10⁴ times higher, and longer incubation times were required to produce the same cytotoxicity as NCS and SMANCS. Furthermore, cell-bound NCS, SMANCS, and ricin were not released from AUXB1 or CH₄5 cells during a 120-min incubation, although Adriamycin was excreted very rapidly from CH₄5 cells after binding and internalization. These results strongly suggest that NCS, SMANCS, and ricin, which are internalized into cells by endocytosis, were not excreted from the cells by active efflux and exhibited a pronounced anticancer effect against MDR cells.

INTRODUCTION

One of the most serious problems in cancer chemotherapy is MDR² of cancer cells. The active efflux of anticancer drugs mediated by the P-glycoprotein of MDR cells is now well known to be a basic mechanism of MDR (1). One tactic to overcome MDR is the inhibition of active efflux by suitable agents such as calcium channel blockers (2–5), calmodulin inhibitors (4–8), synthetic isoprenoids (9), bisbocaurine alkaloids (10, 11), and lysomotropic agents (12), which directly or indirectly act on P-glycoprotein. However, there are problems with many of the techniques involving the use of these agents, especially, maintenance of effective concentrations of such agents in tumor tissue without side effects (1, 2).

Whether or not anticancer drugs can be excreted by P-glycoprotein may depend on the cell’s uptake mechanism and intracellular localization of the drug. It may be possible to overcome MDR by using anticancer drugs that are internalized into the target cells by endocytosis. Recently, we found that the proteinaceous anticancer drug NCS and its styrene-maleic acid copolymer conjugate, SMANCS, were internalized into cells by endocytosis via a specific receptor (13–16). Endocytotic internalization is also observed for ricin (17). Thus, we decided to investigate whether NCS, SMANCS, and ricin might not be subject to active efflux by P-glycoprotein. Furthermore, it is difficult to maintain the effective concentration of most low-molecular-weight anticancer drugs in tumor tissues for a long period because of their rapid clearance, whereas we previously found that macromolecular drugs, including SMANCS, accumulated in tumor tissue to a greater degree than low-molecular-weight anticancer drugs (18, 19). In addition, several pharmacological properties of SMANCS were found much improved compared with NCS *in vitro* and *in vivo*: increased stability, targeting efficiency to tumor, cell surface affinity, a rapid internalization into cells, and cell killing rate (19–22).

In the experiment described here, we evaluated whether NCS and SMANCS, which are internalized into tumor cells differently from low-molecular-weight agents, were effective anticancer drugs against MDR cancer cells.

MATERIALS AND METHODS

Drugs and Chemicals. NCS (M, 12,000) and SMANCS (M, 16,000) were obtained from Kayaku Antibiotics Research Ltd., Tokyo, and Kuraray Ltd., Osaka, Japan, respectively. The activity of SMANCS resides in the NCS portion, which primarily affects DNA metabolism. The chemical entity of SMANCS is well established and the reproducible product is available (20). Ricin (M, 64,000) was from Seikagaku Kogyo Ltd., Tokyo. ADM, MMC, and 5-FU were from Kyowa Hakko Kogyo Ltd., Tokyo. ACM and CDDP were from Yamanouchi Pharmaceutical and Nippon Kayaku Ltd., Tokyo, respectively. Stanners’ modified minimal essential medium was purchased from Flow Laboratories, McLean, VA. FITC-labeled NCS, FITC-labeled SMANCS, and FITC-labeled ricin were prepared by essentially the same method as described (23). All other chemicals were from commercial sources. Free FITC moiety was not liberated from F-NCS or F-SMANCS during binding experiments as confirmed previously (13–16).

Cell Lines. An auxotrophic mutant of Chinese hamster ovary cells, AUXB1, and its MDR subline containing a high content of P-glycoprotein, CH₄5CS, were generous gifts from Dr. Victor Ling, Ontario Cancer Institute, Toronto, Ontario, Canada (24, 25). The cells were cultured in Stanners’ modified minimal essential medium containing 10% heat-inactivated fetal calf serum (growth medium) at 37°C as usual.

Cytotoxicity Studies. Cytotoxicity was measured by inhibition of colony formation (15, 16). The cells were plated in 96-well flat-bottom plates (Falcon no. 3072, 6.4-mm diameter) at a density of 2 x 10⁴ cells/well and were cultured overnight in the growth medium in the bottom of the wells. After removal of the medium, cells were exposed to various concentrations of drugs in the growth medium for 1 or 24 h at 37°C. Then, drug-containing medium was removed by aspiration, the cells in each well were trypsinized and diluted with growth medium, and about 500 cells were seeded into 35-mm diameter dishes (Falcon no. 3001). After incubation for 5–6 days, the numbers of colonies formed were stained and counted.

To study the time dependency of drug-induced cytotoxicity, 500 cells were placed in 35-mm diameter dishes and were cultured overnight in growth medium. Then, the cells were exposed to a fixed concentration of the drug that would completely inhibit colony formation in 24 h (ID₅₀), followed by incubation with the drug at 37°C for periods ranging from 5 min to 24 h. The assay was repeated at least twice to confirm the results.

Received 4/24/89; revised 11/22/89; accepted 12/4/89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*¹This work was supported by a Cancer Research Grant from Japanese Monbusho.

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*The abbreviations used are: MDR, multidrug resistance; KRP, Krebs-Ringer phosphate buffer; ID₅₀, 50% of inhibitory dose; ID₁₀₀, 100% inhibitory minimal dose; NCS, neocarzinostatin; SMANCS, styrene-maleic acid copolymer-conjugated neocarzinostatin; ADM, Adriamycin; ACM, aclacinomycin; VBL, vinblastine; MMC, mitomycin C; 5-FU, 5-fluorouracil; CDDP, cis-diaminedichloroplatinum(II); FITC, fluorescein isothiocyanate; F-NCS, FITC-labeled neocarzinostatin; F-SMANCS, FITC-labeled SMANCS; F-ricin, FITC-labeled ricin; GSH, glutathione.
from 0 to 240 min. At the end of each incubation time, the supernatant was removed and the cells were rinsed once with 0.01 M phosphate-buffered 0.15 M saline, pH 7.4, followed by incubation in fresh, drug-free growth medium at 37°C for 5–6 days. Drug-induced inhibition of colony formation was determined as described above.

Binding Studies (15, 16). AUXB1 or CH*C5 cells were plated at a density of 1 x 10^6 cells/well in 24-well plates (Falcon; 16-mm diameter, no. 3047) and were cultured for 2 days at 37°C. The medium was replaced with 500 μl of the growth medium, which contained a constant amount of ADM, F-NCS, F-SMANCS, or F-ricin at a pH of 7.4 (adjusted with 20 mM NaOH) to remove unbound drug. For quantification of cell-associated FITC conjugates, cells were lysed in 1 ml of 0.1% Triton X-100, after which 750 μl of 4 N HCl in 67% ethanol was added to extract ADM. The fluorescence intensity of the lysate was measured with the fluorescence spectrophotometer with an excitation at 490 nm and the emission at 585 nm. Efflux Studies. AUXB1 cells and CH*C5 cells were exposed to a constant amount of ADM, F-NCS, F-SMANCS, or F-ricin for 1 h at 37°C in the same way as described above (see Fig. 3). Cells were rinsed three times with cold KRP and were further incubated at 37°C in drug-free growth medium. At various times, the medium was discarded, cells were washed with cold KRP three times, and cell-associated drugs and cellular protein were quantified after lysing the cells as described above.

RESULTS

Cytotoxicity of Drugs to AUXB1 and CH*C5 Cells. Cytotoxicity of ADM to the parental cell line AUXB1 and to the resistant cell line CH*C5 after a 1-h drug exposure is shown in Fig. 1. The ID₉₀ of ADM for CH*C5 cells was 720-fold greater than that for AUXB1 cells. The ID₉₀ values of both cell lines in colony-forming assays after 1- and 24-h incubations with ADM and the other anticancer drugs are summarized in Table 1.

NCS, SMANCS, and ricin effectively inhibited the colony formation of the two cell lines at very low concentrations compared with the other drugs (Table 1). The ID₉₀ of NCS or SMANCS for AUXB1 cells was 3–4 nM and that of ricin was 1.5 nM for the 1-h treatment; ID₉₀ values for the other drugs were 1 μM to 25 μM. ID₉₀ values for 1-h exposure of CH*C5 cells to NCS or SMANCS and to ricin were about 30 nM and 5.5 nM, respectively, whereas those of the other drugs were 12 μM to 15 μM, that is, 400 to 500,000 times higher.

CH*C5 cells were 20 to 900 times more resistant to ADM, ACM, MMC, and VBL than were the AUXB1 cells in our test system. The cytotoxicity of these agents also depended on incubation time. For instance, for the 1-h exposure, the ID₉₀ of ADM for CH*C5 cells was 25 times as high as the ID₉₀ for the 24-h exposure. CH*C5 cells showed no resistance to 5-FU or CDDP. These agents also inhibited the colony formation of both cell lines in a time-dependent manner. Although CH*C5 cells were resistant to NCS, SMANCS, and ricin, their degree of resistance was relatively small; only 5- to 10-fold higher than AUXB1 cells. Activity of both NCS and SMANCS in either cell type was not affected by incubation time (Table 1).

Time Course of Cytotoxic Action of Drugs. According to the ID₉₀ of the 1- and 24-h exposures of the drugs shown in Table 1, the effect of all drugs except NCS and SMANCS seemed to depend greatly on the incubation time. As shown in Fig. 2, the fractions surviving after exposure to NCS and SMANCS were immediately reduced. About 90% of AUXB1 and CH*C5 cells were killed after only 6 and 10 min, respectively, by these drugs. In contrast, ADM required 100 and 140 min to kill 90% of AUXB1 and CH*C5 cells, respectively. In the case of 5-FU or CDDP, more than 60% of the cells survived after the 240-min incubation period.

Accumulation of Drugs in AUXB1 and CH*C5 Cells. We compared the accumulation of NCS, SMANCS, and ricin with that of ADM in both cell lines. Fig. 3 shows the uptake profiles of ADM, F-NCS, F-SMANCS, and F-ricin by both AUXB1 and CH*C5 cells at 37°C or 4°C. ADM accumulated progressively in AUXB1 cells at 37°C even after 3 h, whereas CH*C5 cells reached a plateau after 30 min. After 3 h of treatment, the uptake of ADM by AUXB1 cells was about 2.2 times higher than that by CH*C5 cells. The uptake of F-ricin by AUXB1 cells was also about 2 times more than that of CH*C5 cells at 37°C (Fig. 3). No significant differences in the uptake of F-NCS or F-SMANCS were observed between AUXB1 and CH*C5 cells.

Efflux of Drugs from AUXB1 and CH*C5 Cells. Many investigators have reported that the decreased accumulation of anticancer drugs in MDR cell lines was due to increased efflux activity in these cell lines (1). Therefore, we examined the efflux of the drugs from AUXB1 and CH*C5 cells. The efflux of cell-associated ADM from CH*C5 cells was markedly increased compared with the parental AUXB1 cells (Fig. 4A). After a 30-min incubation, about 90% of cell-associated ADM was lost from CH*C5 cells, whereas more than 80% of the drug was retained in AUXB1 cells. In contrast, there was no significant difference in the efflux of F-NCS, F-SMANCS, or F-ricin in the two different cell lines (Fig. 4, B–D). Almost all F-NCS and F-SMANCS associated with the cells were retained in both cell lines even after 120 min of incubation at 37°C.

DISCUSSION

The macromolecular agents NCS, SMANCS, and ricin effectively killed the MDR subline of Chinese hamster ovary cells (CH*C5) similarly to the sensitive line (AUXB1) in vitro. To obtain 50% inhibition of the colony formation of AUXB1 cells, 2–4 nM NCS and SMANCS and 1.5 nM ricin, with 1 h of incubation, were required, whereas for CH*C5 cells, about 30 nM of NCS and SMANCS and 5.5 nM of ricin were needed.
Table 1  Drug cross-resistance: ID₅₀ values in clonogenic assays after 1- and 24-h incubations of AUXBI and CH₆C₅ cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>AUXBI 1 h</th>
<th>AUXBI 24 h</th>
<th>CH₆C₅ 1 h</th>
<th>CH₆C₅ 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADM</td>
<td>1.0</td>
<td>0.27</td>
<td>3.7</td>
<td>720.0</td>
</tr>
<tr>
<td>ACM</td>
<td>6.0</td>
<td>0.022</td>
<td>270</td>
<td>&gt;210</td>
</tr>
<tr>
<td>VBL</td>
<td>&gt;140</td>
<td>0.001</td>
<td>&gt;70,000</td>
<td>&gt;140</td>
</tr>
<tr>
<td>MMC</td>
<td>4.4</td>
<td>0.090</td>
<td>49</td>
<td>300.0</td>
</tr>
<tr>
<td>5-FU</td>
<td>25,000</td>
<td>33.0</td>
<td>760</td>
<td>15,000.0</td>
</tr>
<tr>
<td>CDDP</td>
<td>7.8</td>
<td>0.87</td>
<td>9.0</td>
<td>12.0</td>
</tr>
<tr>
<td>NCS</td>
<td>0.0039</td>
<td>0.0024</td>
<td>1.6</td>
<td>0.031</td>
</tr>
<tr>
<td>SMANCS</td>
<td>0.0032</td>
<td>0.0026</td>
<td>1.2</td>
<td>0.028</td>
</tr>
<tr>
<td>Ricin</td>
<td>0.00156</td>
<td>0.000042</td>
<td>37.1</td>
<td>0.00549</td>
</tr>
</tbody>
</table>

*TD, time dependency, which means time-dependent augmentation of cytotoxicity in ID₅₀. TD = ID₅₀ 1 h / ID₅₀ 24 h.

Fig. 2. Time-dependent cytotoxicity of CDDP (●), 5-FU (■), ADM (○), NCS (▲), and SMANCS (△) in AUXBI (A) and CH₆C₅ cells (B). AUXBI and CH₆C₅ cells were exposed to a drug concentration that completely inhibited colony formation in 24 h (based on Fig. 1) for various times at 37°C. At the end of each incubation period, the cells were washed with phosphate-buffered saline and growth medium was added to the culture for 5–6 days.

These ID₅₀ values were very low compared with those of the other low-molecular-weight anticancer drugs, which were between 1 μM and 25 mM. For NCS, SMANCS, and ricin, the ratios of the ID₅₀ values for resistant cells to values for parental cells were relatively small (5- to 10-fold) compared with the ratios of the anthracyclines (ADM and ACM), Vinca alkaloid (VBL), and MMC, which varied greatly (20- to 889-fold) (Table 1). The sensitivity of CH₆C₅ cells to 5-FU and CDDP was not very different from that of AUXBI cells, and molar ID₅₀ values for these two drugs were 2 × 10⁴ to 6 × 10⁶ times higher than those for NCS and SMANCS (Table 1). In addition, the cytotoxic action of ADM, 5-FU, and CDDP required a much longer exposure time than that of NCS and SMANCS: more than 2 h versus less than 10 min (Fig. 2). As seen in Fig. 3, however, the cellular accumulation of ADM in both cell lines seemed to be much greater than that of NCS or SMANCS on a molar basis. Thus, these results indicate that the cells were killed by a much smaller amount of NCS or SMANCS, which was internalized into the cells very rapidly (13–16).

Fig. 3. Binding kinetics of ADM (A), F-NCS (B), F-SMANCS (C), and F-radin (D) for AUXBI cells (■) and CH₆C₅ cells (○). Cell monolayers were incubated with 3.3 μM ADM, 10 μM F-NCS, 10 μM F-SMANCS, or 15.6 μM F-radin for various times at 4°C (●) and at 37°C (○). After incubation, the amounts of cell-bound drugs were determined as described in the text. Points, mean values of duplicate samples.

whereas almost all F-NCS, F-SMANCS, and F-radin remained within the cells in both resistant and sensitive cell lines, even after 120 min of incubation (Fig. 4). Ling’s group (24, 29) and Luk et al. (28) also demonstrated that the content of P-glycoprotein correlated directly with both a decrease in intracellular drug concentration and the degree of drug resistance of the cells. In their proposed mechanism of MDR, P-glycoprotein functions as an energy-dependent export pump to reduce intracellular levels of anticancer drugs (30). It was found previously that NCS, SMANCS, and ricin were taken up into cells by endocytosis (13–16, 17). Therefore, the intracellular site of...
localization of NCS, SMANCS, or ricin (phagolysosome) is different from that of the other low-molecular-weight anticancer drugs, which incorporated into cytosol by passive transport (31–33). A rapid efflux of anticancer drugs by P-glycoprotein may depend on appropriate intracellular localization (accessibility to P-glycoprotein) of the drugs which is not the case with endocytosis-dependent macromolecules. Thus, a method to overcome MDR may be possible by using macromolecular drugs.

CH<sup>C5</sup> cells were 5- to 10-fold more resistant than AUXB1 cells to NCS, SMANCS, or ricin. Cytotoxicity of these macromolecular drugs is known to depend on their incorporation into the cells (13–16, 34). The present study revealed that little difference in the uptake of F-NCS or F-SMANCS by sensitive and resistant cells existed (Fig. 3, B and C). However, the uptake of F-ricin by CH<sup>C5</sup> cells was about one-half of that by AUXB1 cells (Fig. 3D); therefore, a relatively low resistance to ricin in CH<sup>C5</sup> cells may be attributed to the reduced uptake of the toxin. Contrary to the present finding, Sehested et al. (35, 36) showed that fluid-phase endocytosis was increased in multidrug-resistant cells compared to the sensitive parents. We have, however, not observed any significant increase in the uptake velocity in CH<sup>C5</sup> cells (Fig. 3).

Hamilton et al. (37) reported that ADM-resistant cells became high in cytoplasmic GSH content. It was reported previously that the presence of GSH or other thiols in cell or cell-free systems augment the ability of NCS and SMANCS (38–40). We determined the content of GSH and nonprotein sulfhydryls. However, their contents were almost the same in AUXB1 and CH<sup>C5</sup> cells (data not shown).

Therefore, a small difference (less than 10-fold) in cytotoxicity described above of NCS or SMANCS against these sensitive and resistant cell lines needs to be explained by other factors, such as DNA topoisomerase II (41) and/or DNA repair enzymes (42), for which further studies are needed.

The results presented here show that NCS and SMANCS were still effective at the 30 nm range against the MDR cancer cells because of their potent cytotoxic action and independence from the P-glycoprotein-dependent efflux process in the resistant cells. It is suggested that the use of macromolecular anticancer agents is a new approach to circumvent MDR in addition to their other advantages such as higher tumoritropism and improved pharmacological properties (18–22).

ACKNOWLEDGMENTS

We thank Dr. Victor Ling for his generous supply of two cell lines, AUXB1 and CH<sup>C5</sup>.

REFERENCES


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