Intracellular Uptake of 7-con-O-Methylnogarol and Adriamycin by Cells in Culture and Its Relationship to Cell Survival

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ABSTRACT

7-con-O-Methylnogarol (7-OMEN) is a new anthracycline antitumor agent with significant activity in vivo against murine P388 and L1210 leukemia (140% increase in life span) and B16 melanoma (114% increase in life span). We report here the complex relationship that exists between intracellular drug concentration and killing of Chinese hamster ovary (CHO), B16, and L1210 cells in culture by 7-OMEN and Adriamycin.

The uptake of 7-OMEN and Adriamycin by CHO, B16, and L1210 cells was proportional to the extracellular concentration (µg/ml medium) of the drug. At similar concentrations, the CHO and B16 cells accumulated more 7-OMEN than Adriamycin. Based on intracellular concentration in CHO cells, Adriamycin was more lethal [lethal dose for 90% of the cells (LD90), 0.02 µg/10^6 cells] than was 7-OMEN (LD90, 0.1 µg/10^6 cells). However, based on concentration in CHO cells extracellular 7-OMEN was more lethal (LD90, 0.6 µg/ml) than was Adriamycin (LD90, 2.5 µg/ml). This was probably due to the very low level of uptake of Adriamycin by CHO cells.

For both 7-OMEN and Adriamycin, cell kill increased with increasing intracellular concentration. In contrast to the linear relationship between intracellular concentration and cell kill with Adriamycin, a biphasic relationship was seen with 7-OMEN. For 7-OMEN, cell kill was proportional to intracellular concentration up to approximately 0.04 µg/10^6 cells. Further increase in intracellular concentration did not result in proportional increase in cell kill.

Exponentially growing cells not only accumulated more 7-OMEN than did plateau-phase cells but were also inherently more sensitive to the drug. This latter conclusion was based on the observation that, even at similar intracellular concentration, more exponentially growing than plateau-phase cells were killed.

Fluorescence microscopy showed that 7-OMEN accumulated predominantly in the cytoplasm, whereas Adriamycin accumulated in the nucleus. The significance of this observation to the mode of action of these drugs is discussed.

INTRODUCTION

7-OMEN\(^2\) is an anthracycline antitumor agent prepared from nogalamycin (4) by Wiley et al. (16, 17) which had significant activity against murine P388 and L1210 leukemia (200 and 140% ILS, respectively) and B16 melanoma (114% ILS) (11). Since Adriamycin is probably the most clinically useful anthracycline antibiotic, the properties of 7-OMEN and Adriamycin were compared. In direct comparisons to 7-OMEN, Adriamycin was less active against L1210 leukemia (40% ILS) but was slightly more active against B16 melanoma (156% ILS) on a daily for 9 days dosing schedule (11). The lethality to CHO, B16, and L1210 cells in culture of 7-OMEN (LD\(_{50}\) range: 0.1 to 0.25 µg/ml) was similar to that of Adriamycin (LD\(_{50}\), 0.03 to 0.5 µg/ml) (2). In contrast to Adriamycin which interacts strongly with DNA, 7-OMEN was bound much less to DNA and inhibited DNA and RNA synthesis minimally at lethal doses (9). These results suggested that the lethality of 7-OMEN was mediated through some mechanism other than drug interaction with DNA (9). Structurally (Chart 1), 7-OMEN is different from Adriamycin in several respects. In view of the cytotoxicity of 7-OMEN, its antitumor effect in vivo, and a mode of action which is possibly different from other anthracyclines, we are interested in developing 7-OMEN as an antitumor drug for clinical use.

In this paper, we report the relationship between the intracellular concentrations of 7-OMEN and Adriamycin and the corresponding lethalties to cells. CHO, B16, and L1210 cells in different growth conditions (exponential, plateau phase, and synchronized) were used in these experiments. Parts of these results were reported previously (3).

MATERIALS AND METHODS

Cell Culture. CHO cells were maintained as a monolayer in exponential growth in Ham’s F-10 medium supplemented with 15% fetal calf serum. Cells grew exponentially up to a cell density of approximately 5 x 10^6 cells/75 sq cm. For plateau-phase populations, the cells were grown to confluency (10^7 to 2 x 10^7 cells/75 sq cm) and maintained without a medium change for 36 hr (14). Plateau-phase CHO cells accumulated predominantly (90 to 95%) in the G1 phase, with the remainder (5 to 10%) accumulating in G2 (14). The cloning efficiency of exponentially growing and plateau-phase cells ranged between 70 and 90%.

The B16 (clone F-10) melanoma cell line (7) was obtained from Dr. I. J. Fidler (Frederick Cancer Research Center, Frederick, Md.). The cells were grown as a monolayer in Eagle’s minimum essential medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, twice the recommended concentration of vitamins, 0.1 mM concentration of each nonessential amino acid, penicillin G (60 µg/ml), and streptomycin (10 µg/ml) (7).

L1210 cells were maintained in suspension culture in Rosewell Park Memorial Institute Medium 1634 supplemented with 5% fetal calf serum, NaHCO\(_3\) (750 µg/ml), penicillin G (100 µg/ml), and streptomycin (50 µg/ml).

Drugs. 7-OMEN was prepared by Wiley et al. (16) at The Upjohn Company. Adriamycin was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., and used as received. The structures of 7-OMEN and Adriamycin are shown in Chart 1.

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\(^2\) The abbreviations used are: 7-OMEN, 7-con-O-methylnogarol; ILS, increase in life span of tumor-bearing animal; CHO, Chinese hamster ovary; LD\(_{50}\), dose lethal to 50% of cells; LD\(_{90}\), dose lethal to 90% of cells.

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were planted in Linbro plates with 3.5-cm-diameter wells. For experiments, the standard curve was prepared with cell extract from the same number of cells used in the experiment. The results (Chart 2) show that the fluorescence depended on the cell line extracted. Thus, 7-OMEN in CHO extract was more highly fluorescent than that in L1210 extract. In the same cell extract, 7-OMEN was more fluorescent than Adriamycin was. The standard curve of 7-OMEN was linear up to a concentration of 1.5 μg/ml (data not shown), above which the fluorescence did not increase proportionally. Since the cells were detached from the plastic substrate with trypsin, the effects of detaching the cells with trypsin or with a rubber policeman were compared. Both methods gave the same intracellular concentration of 7-OMEN. In a material balance experiment, 95% of the 7-OMEN was recovered.

Since the molecular weights of 7-OMEN (M.W. 541) and Adriamycin (M.W. 543) are similar, the intracellular concentrations of the 2 drugs are expressed in μg instead of μmol.

Metabolism of 7-OMEN by Cells or in Medium. CHO and B16 cell monolayers (approximately 1.5 x 10⁷ cells) were incubated at 37° with 7-OMEN or Adriamycin (0.5 μg/ml) for 6 or 24 hr. The medium was removed and stored, and then the cell monolayer was harvested with 0.05% trypsin. The medium and the cells were extracted with methanol:HCl as described above. Acidic methanol extracts were diluted with water, neutralized with NaOH, buffered to pH 9, and extracted with ice-cold methylene chloride. The methylene chloride extract was kept on ice and evaporated to dryness with a stream of nitrogen; the extract was then reconstituted in a small volume of chloroform:methanol (9:1). Aliquots were chromatographed on thin-layer silica gel (E. Merck, Darmstadt, W. Germany) plates with the following solvent systems: for 7-OMEN, chloroform:methanol:water:acetic acid (78:20:2:0.02) and acetonitrile:chloroform:methanol:water:acetic acid (20:55:15:5:5); for Adriamycin, chloroform:methanol:acetic acid (93:5:2 and 76:20:4). Separated bands were visualized under 360 nm UV.

Condition for Drug Exposure and Determination of Cell Survival. B16 and CHO cells were planted as monolayers, and L1210 cells were in suspension culture, all prepared 24 hr before an experiment to ensure exponential growth during drug exposure. Unless otherwise mentioned, cells were exposed to drug in their respective growth medium for 2 hr. 7-OMEN and Adriamycin were dissolved in 0.1 M aqueous glucuronic acid at 1 mg/ml and were further diluted in medium prior to adding to the cells. Adequate vehicle controls were used in each experiment. For each drug concentration tested, 2 separate cell cultures were used.

After drug exposure, the B16 or CHO cell monolayer was harvested with 0.05% trypsin, and the cells were centrifuged and washed to remove drug. The cells were diluted in medium, and an aliquot was planted in Linbro plastic multiwell plates to determine cell survival was about 15%. In all cases, the cloning efficiency of the untreated (control) cells was normalized to 100%, and the cloning efficiency of the treated cells was expressed as a percentage of control survival. The coefficient of variation (S.D. expressed as a percentage of the mean) in cell survival was about 15%.

Determination of Intracellular Drug Concentration. The drug exposure protocol for these experiments was the same as described above. Uptake was quantitated by measuring fluorescence of cell extracts as described by Bachur et al. (1). In all cases, the cells were kept ice cold from the end of drug exposure until extraction with acidic methanol. After drug exposure, CHO and B16 cells were washed with ice-cold 0.9% NaCl solution and then harvested with 0.05% trypsin. The cells were then centrifuged and washed once with ice-cold medium. At the end of drug exposure, L1210 cells in suspension were centrifuged and washed in cold medium. In all cases, between 5 x 10⁶ and 10⁷ cells were suspended in 0.4 ml of cold 0.9% NaCl solution to which 4 ml of a 60% methanol:0.3 N HCl mixture were added. The cells were dispersed and then centrifuged at 10,000 rpm for 10 min at 4°. The supernatant was carefully removed, and its fluorescence was measured in a Hitachi Perkin-Elmer MPF-2A fluorometer. For 7-OMEN, the samples were excited at 468 nm, and emission was measured at 540 nm. For Adriamycin, the excitation and emission wavelengths were 468 and 550 nm, respectively. The fluorescence of the samples was compared to that of standards containing different amounts of 7-OMEN or Adriamycin added to drug-free cell extract. Representative standard curves are shown in Chart 2. The standard curves of the drugs in different cell extracts were quite reproducible from experiment to experiment. In all experiments, the standard curve was prepared with cell extract from the same number of cells used in the experiment. The results (Chart 2) show that the fluorescence depended on the cell line extracted. Thus, 7-OMEN in CHO extract was more highly fluorescent than that in L1210 extract. In the same cell extract, 7-OMEN was more fluorescent than Adriamycin was. The standard curve of 7-OMEN was linear up to a concentration of 1.5 μg/ml (data not shown), above which the fluorescence did not increase proportionally. Since the cells were detached from the plastic substrate with trypsin, the effects of detaching the cells with trypsin or with a rubber policeman were compared. Both methods gave the same intracellular concentration of 7-OMEN. In a material balance experiment, 95% of the 7-OMEN was recovered.

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RESULTS

Effect of Extracellular Drug Concentration and Exposure Time on Uptake by Cells. The time course of 7-OMEN uptake (Chart 3) showed that the intracellular drug concentration depended on both the drug concentration in the medium and the duration of cell-drug contact. At all concentrations tested, the cells contained the maximum amount of 7-OMEN after 4 hr of contact.

The effect of extracellular concentration on the uptake of 7-OMEN and Adriamycin by CHO, B16, and L1210 cells is shown in Chart 4. These results show that: (a) uptake of 7-OMEN was much greater than that of Adriamycin into both B16 and CHO cells. At the same extracellular concentration, B16 cells concentrated 2 to 5 times more 7-OMEN than Adriamycin. For CHO cells, the intracellular 7-OMEN concentration was 50 times that of Adriamycin at 1-µg/ml doses of either drug; (b) the intracellular concentration of 7-OMEN in B16 and L1210 cells was much greater than that in CHO cells. This was also true for Adriamycin. For example, at 1-µg/ml doses of extracellular Adriamycin, the intracellular concentration in B16 cells was about 170 times that in CHO cells.

Correlation between Intracellular Drug Concentration and Survival of Exponentially Growing Cells. Chart 5 shows the cell survival obtained at different drug concentrations in the medium. The results clearly show that, based on extracellular concentration, 7-OMEN was more lethal than Adriamycin for CHO cells (Chart 5B). For example, 90% of the CHO cells were killed at 0.6 µg 7-OMEN per ml medium as compared to 2.5 µg per ml medium required for Adriamycin. In contrast, for B16 cells, Adriamycin (LD90, 0.17 µg/ml) was more cytotoxic than was 7-OMEN (LD90, 0.5 µg/ml).

Charts 6 and 7 show the correlation between intracellular drug concentration and corresponding cell survival for B16 and CHO cells exposed to 7-OMEN and Adriamycin. The results show the following.

1. Based on intracellular concentration, Adriamycin was more lethal than 7-OMEN to both CHO and B16 cells. The 90% of the CHO cells were killed at intracellular concentration (per 10^6 cells) of 0.1 µg of 7-OMEN or 0.02 µg of Adriamycin. Similarly, at the same intracellular concentration (0.04 µg/1

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Chart 3. Intracellular concentration of 7-OMEN in CHO cells after different periods of exposure to drug.

Chart 4. Intracellular concentration of 7-OMEN and Adriamycin (ADR) in CHO, B16, and L1210 cells at different concentrations of drug in medium. Exponentially growing cells were exposed to drug for 2 hr. O, •, 2 experiments with B16 plus 7-OMEN; O, •, 2 experiments with B16 plus Adriamycin and, in CHO plus Adriamycin. ▲, ○, ■, ▼, 5 different experiments with CHO plus 7-OMEN. ▲, ○, •, 2 experiments with L1210 plus 7-OMEN.

Chart 5. CHO and B16 survival at different extracellular concentrations of OMEN and Adriamycin (ADR). The symbols are the same as in Chart 4; bars, S.D.
cells was correlated to percentage of cell survival (Chart 7).

3. In contrast to the results with 7-OMEN, a linear relationship was observed when the intracellular Adriamycin concentration in CHO and B16 cells was correlated to percentage of cell survival. This linear relationship between intracellular concentration and survival held to about the 0.5% survival level.

**7-OMEN Uptake and Cell Survival under Different Growth Conditions.** For these experiments, drug uptake into 2 cell populations that were markedly different in their sensitivity to the lethal effects of 7-OMEN was compared. These were: (a) exponentially growing versus plateau-phase cells; and (b) post-metaphase plus early G1 versus late G, cells. For both sets of all populations, 7-OMEN was much more lethal to the former population of cells than to the latter.

At the same concentration of extracellular drug, exponentially growing cells accumulated about twice as much 7-OMEN as did plateau-phase cells (Table 1). For example, at 1 µg/ml, the intracellular concentrations in exponentially growing and plateau-phase cells were 0.53 and 0.27 µg/10⁶ cells, respectively. However, when we correlated the intracellular drug concentration of exponentially growing and plateau-phase cells with their corresponding percentage of survival (Table 1), we found that exponentially growing cells not only accumulated more drug than did plateau-phase cells but also were intrinsically more sensitive to the lethal effects of the drug. Thus, at an intracellular concentration of 0.53 µg/10⁶ exponentially growing cells, only 0.35% of cells survived compared to 23% survival of plateau-phase cells at 0.66 µg/10⁶ cells.

The intracellular drug concentration and percentage of cell survival in postmetaphase plus early G, cells were compared to those of late G, cells, and results are shown in Table 2. The cells in the postmetaphase plus early G, group accumulated about twice as much drug (0.042 µg/10⁶ cells) as did the late G, cells, only 3% of the Adriamycin-treated cells survived as compared to 60% survival of 7-OMEN-treated cells.

2. A biphasic curve (Chart 6) was obtained when the intracellular 7-OMEN concentration in CHO cells was correlated to percentage of cell survival. Up to an intracellular concentration of about 0.04 µg/10⁶ cells, cell kill was proportional to uptake. Further increase in intracellular concentration did not result in proportionately increased cell kill. A biphasic curve was also suggested when the intracellular 7-OMEN concentration in B16 cells was correlated to percentage of cell survival (Chart 7).

**Drug uptake and survival of exponentially growing and plateau-phase CHO cells exposed to 7-OMEN**

<table>
<thead>
<tr>
<th>7-OMEN (µg/ml)</th>
<th>Exponentially growing cells</th>
<th>Plateau-phase cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.53 ± 0.04</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>3.0</td>
<td>1.53 ± 0.04 ± 0.004</td>
<td>0.66 ± 23 ± 3.6</td>
</tr>
</tbody>
</table>

* Average ± S.D. of 2 cultures planted in 4 Linbro plates each.

**Drug uptake and survival in postmetaphase plus early G, as compared to late G, cells exposed to 7-OMEN**

An exponentially growing culture of CHO cells was exposed to Colcemid for 2 hr to accumulate mitotic cells which were then selectively harvested as described previously (15). The mitotic cells (93% mitotic index) were planted and exposed to 7-OMEN from 0 to 2 hr (postmetaphase plus early G,) or from 5 to 7 hr (late G,). Cell survival was determined by cloning. The untreated control had survival of 32% which was normalized to 100% to calculate the survival of the drug-treated populations.

<table>
<thead>
<tr>
<th>7-OMEN (µg/ml)</th>
<th>Postmetaphase early G,</th>
<th>Late G,</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.042 ± 0.004± 0.04 ± 0.04</td>
<td>0.021 ± 0.001 ± 24.9 ± 1.2</td>
</tr>
</tbody>
</table>

* Average ± S.D. of 2 cultures planted in 4 Linbro plates each.

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G₁ cells (0.021 µg/10⁶ cells). However, at these intracellular concentrations, only 0.9% of the former population survived compared to 25% survival of the late G₁ cells. We do not know whether this difference in intracellular drug concentration could account for the marked difference in percentage of survival seen in these 2 cell populations (see “Discussion”).

**Efflux of 7-OMEN and Adriamycin from CHO and B16 Cells.** The efflux of the drugs from CHO and B16 cells after an initial exposure to the drugs for 2 hr is shown in Table 3. The results show that: (a) for 7-OMEN, the drug exited rapidly and at about the same rate from both CHO and B16 cells. For example, after 4 hr, both CHO and B16 cells contained about 20 to 30% of their initial (0 hr) intracellular concentrations of the drug; (b) for Adriamycin, the rate of efflux in CHO cells was much higher than in B16 cells. Thus, after 4 hr, CHO cells contained only about 19% of their initial (0 hr) intracellular concentration compared to 62% in the B16 cells. Although B16 cells were exposed to only 0.5 µg/ml compared to 5 µg/ml for CHO cells, the intracellular concentration at 0 hr in both cell types was similar. Therefore, it is valid to compare the efflux rates at these drug concentrations.

**Intracellular Localization of Drug Fluorescence.** Egorin et al. (5) reported that daunomycin localized in the cell nucleus, whereas the fluorescence of 7-OMEN was seen only in the cytoplasm, with no detectable nuclear fluorescence. We corroborate their findings. In order to use this information in elucidating the mechanism of action of the drug, the distribution of intracellular drug fluorescence was correlated to percentage of cell survival (Table 4). The fluorescence of 7-OMEN in B16 and CHO cells was visible in the cytoplasm at concentrations which killed 50 to 99% of the cells. This was probably due to the high intracellular uptake of this drug. In contrast, as a result of the low intracellular concentration of Adriamycin, we could not visualize Adriamycin fluorescence, even at concentrations that killed more than 99% of the cells.

**Metabolism of 7-OMEN in Cell or in Medium.** There was less than 5% metabolism of 7-OMEN over 6 hr and of Adriamycin over 24 hr, as evidenced by the thin-layer chromatographic search for metabolites.

**Discussion**

The intracellular concentration of 7-OMEN in L1210 cells reported by Egorin et al. (5) was similar to that reported here. Also in agreement with Egorin et al. (5), we found that cells accumulated more 7-OMEN than Adriamycin (this study) or daunomycin (5). If we assume that 10⁶ cells (diameter, 20 µm) occupy a volume of 0.005 ml, then we can calculate that, at a 7-OMEN concentration of 1 µg/ml medium, B16, CHO, and L1210 cells accumulated 240, 160, and 150 µg per ml of intracellular volume. For B16 cells, this is equivalent to 0.44 mM 7-OMEN within the cell. In comparison, at an Adriamycin concentration of 1 µg/ml in L1210 cells, we accumulated 99% of the B16 cells at 1 µg/ml, 0.05- to 1-µg/ml doses of drug were used in the uptake experiments. The low drug concentrations used in our experiments are in contrast to the supralethal concentrations of Adriamycin or daunomycin (about 10 µg/ml) often used by other workers (5, 8, 12, 13).

Our results clearly indicate the problems (outlined below) associated with trying to correlate the biological effect (e.g., cell kill or biochemical action) of a drug with its intracellular concentration.

1. There may be a deviation from a linear relationship between intracellular concentration and cell kill, as was seen with 7-OMEN. We do not know whether the biphasic curve (Charts 6 and 7) indicates a resistant subpopulation which accumulates drug without being killed or whether it is a characteristic of drug accumulation in the cytoplasm. The curve (Charts 6 and 7) became biphasic at about 10% cell survival, which would indicate that 10% of the cells were resistant. However, such a high proportion of resistant cells is unlikely.

2. Intracellular drug concentration may not be sufficient by itself to account for the sensitivity of different cell lines or of different cell populations. For example, even at the same intracellular concentration of 7-OMEN, exponentially growing B16 cells were exposed to only 0.5 µg/ml compared to 5 µg/ml for CHO cells. Since intracellular concentration at 0 hr in both cell types was similar. Therefore, it is valid to compare the efflux rates at these drug concentrations.
cells were killed to a greater extent and were, therefore, inherently more sensitive than were plateau-phase cells. Similar results were reported by Egorin et al. (5), who found that the resistance of P388/ADR cells to 7-OMEN could not be entirely explained by reduced drug accumulation. Even at the same intracellular concentration, P388/ADR cells were more resistant than were P388/S cells. Similarly, Meriweather and Bachur (10) found that the level of inhibition of DNA and RNA synthesis in L1210 cells could not be correlated with the levels of intracellular drug.

3. Information about the intracellular localization of a drug may not help in delineating its site of action. Fluorescent microscopy showed that most of the 7-OMEN and Adriamycin were localized in the cytoplasm and nucleus, respectively (our experiments and Ref. 5). However, this method is very insensitive (Table 4). Therefore, these results suggest, but do not prove, that Adriamycin mainly affects the nucleus, whereas 7-OMEN has its effects on the cytoplasm. In fact, it is possible that, although most of the 7-OMEN accumulates in the cytoplasm, only 10% of the intracellular concentration (which will be below the fluorescent level) may interact with the nucleus and thereby kill the cells. Noel et al. (12) used cell fractionation techniques to determine the subcellular localization of Adriamycin in rat embryo fibroblasts. They found that only 60% of the Adriamycin was localized in the nucleus, and the rest was in the lysosomes. Facchinetti et al. (6) found that, in macrophages, Adriamycin cytofluorescence accumulated slowly in the nucleus and later appeared in numerous cytoplasmic vesicles and vacuoles. These results indicate that other cellular structures attract Adriamycin and concentrate it within their boundaries.

REFERENCES

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