The Retention or Efflux of Phthalanilide (NSC 60339)–Lipid Complexes by Sensitive or Resistant Murine Tumor Cells and Escherichia coli B

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SUMMARY

The retention and efflux of lipid-bound 2-chloro-4',4''-di(2-imidazolin-2-yl)terephthalanilide (NSC 60339) has been correlated with the sensitivity, resistance, or cross-resistance of 7 tumor lines to phthalanilide treatment in vivo. The sensitive tumors (L1210, L1210/MTX, L1210/ara-C, and P815) rapidly took up the drug and retained it primarily as lipid-bound drug for the 24-hr experimental period. The resistant tumor, L1210/NSC 60339, and 2 cross-resistant tumors, P388/VCR and P815/VLB, took up as much drug as the sensitive tumors did by 0.5 hr, but there was an efflux of lipid-bound drug from these resistant tumors by 24 hr. Two other drug fractions, hydrophilic-bound and "free," generally decreased in both sensitive and resistant tumors. All 3 drug fractions are extracted from Escherichia coli B, P388, and P388/NSC 60339 that were treated in vitro, and the efflux of drug from these tumor cells in vitro are described. The drug-lipid complexes which were extracted from phthalanilide-resistant and cross-resistant tumors after treatment in vivo were more hydrophobic than those from sensitive tumors. The relative hydrophobicity of the crude drug complexes from P388 and P888/60339 cells was similar to the relative hydrophobicity of highly purified complexes. The drug-lipid complexes from the 2 phthalanilide-sensitive tumors, which were resistant to Methotrexate (MTX) and cytosine arabinoside (ara-C), did not show such hydrophobic character. Therefore, it is possible that the mechanism of resistance to Vinca alkaloids, which are cross-resistant to the phthalanilides and are also large cationic drugs, may involve the formation and mobilization of drug-lipid complexes.

INTRODUCTION

The substituted phthalanilides, designed as "phosphatide blockers" (6), are extracted as drug-lipid complexes from a variety of tissues (14) and from phthalanilide-sensitive and -resistant P388 leukemia cells (19). The lipids which complex with 2-chloro-4',4''-di(2-imidazolin-2-yl)terephthalanilide (NSC 60339)2 in P388 cells and with 4',4''-bis(1,4,5,6-tetrahydro-2-pyrimidinyl)terephthalanilide (NSC 57153)2 in dog brain are new phospholipids characterized by their unusual glycerol: fatty acid:phosphorus:nitrogen:sulfur ratios and by their unidentified ninhydrin positive components (18, 21).3 The concentrations of extractable lipid-bound and hydrophilic-bound NSC 60339 are significantly related to the chemotherapeutic response of P388 cells that were grown and treated in suspension-culture and assayed in BDF1 mice, whereas the intracellular concentration of "free" NSC 60339 is not (20). Following treatment in vivo, sensitive P388 cells retain the drug up to 24 hr and it is extracted primarily as lipid-complexes; there is a substantial efflux of drug from resistant P388 cells under the same conditions (19). These drug-lipid complexes from the resistant P388 cells are more hydrophobic than are the corresponding complexes extracted from sensitive cells; this increased hydrophobicity of the complexes parallels the magnitude of efflux of NSC 60339 from P388 cells (19).3

The drug, NSC 60339, has a high chemotherapeutic activity against a wide range of experimental murine leukemias and lymphomas in animals (4, 8-10, 12) and bacteria (11, 13, 16), but development of resistance and cross-resistance to the phthalanilide has been found (2, 3, 7, 8). The present study quantitates and characterizes the phthalanilide complexes in several sensitive and resistant tumors as well as E. coli B and evaluates the possibility that this mechanism of resistance—increased hydrophobicity and loss of drug-lipid complexes—may also apply to the Vinea alkaloids, which are cross-resistant to the phthalanilides (7). Preliminary reports have been made by Yesair et al. (18).

MATERIALS AND METHODS

Prior to drug treatment an inoculum of 10⁶ tumor cells was grown i.p. in BDF1 hybrid mice (C57B1/6¢ and DBA/24) for 7 days. The following phthalanilide-sensitive tumors4 were used: P388, L1210, cytosine arabinoside-resistant L1210 (L1210/ara-C), Methotrexate-resistant L1210 (L1210/MTX)...

1 Supported by the Cancer Chemotherapy National Service Center, Contract No. PH 43-65-61, National Cancer Institute, NIH, USPHS.

2 The phthalanilides were synthesized by the Research Institute of Dr. A. Wander, Berne, Switzerland, and obtained through the Clinical Branch, Collaborative Research, National Cancer Institute, NIH, USPHS.

3 D. W. Yesair, unpublished data.
and P815. The resistant tumors included the phthalanilide-resistant P388 (P388/60339) and L1210 (L1210/60339), vincristine-resistant P388 (P388/VCR) and vinblastine-resistant P815 (P815/VLB).

NSC 60339 was administered i.p. at 32 mg/kg as a single dose either 0.5 or 24 hr before harvesting the treated cells on Day 7. Freshly harvested cells from 10 mice were pooled and centrifuged at 700 g for 15 min. The ascites fluid was decanted and the cells were washed twice with cold 0.9% NaCl. The drug content of washed cells was extracted into chloroform:methanol (2:1, v/v) and partitioned between a water:chloroform:methanol biphasic (14). Total drug in the aqueous phase was determined and differentiated into hydrophilic-bound and “free” drug by chromatography (15). Drug associated with lipid in the nonaqueous phase was determined by the acid-displacement method (14, 20).

For drug treatment in vitro, control P388 and P388/NSC 60339 cells were harvested from BDF1 mice on Day 7, washed twice with saline, and suspended in Fischer’s medium (5) with or without 10% horse serum. Drug (NSC 60339) was added to 15-ml aliquots of cell suspension (3-5 × 107 cells/ml) and incubated with stirring at 37°C for 4 hr. At the end of the incubation period, the cells were centrifuged and washed twice with 0.9% saline. The drug content of cells was determined as described in the preceding paragraph. To evaluate the efflux of drug from these treated cells, the cells were removed from the drug-containing medium by centrifugation, washed with saline, and resuspended in Fischer’s medium with or without 10% horse serum, depending upon the particular experiment. Aliquots of cells were taken at various times and analyzed for drug content.

Cultures of E. coli B were grown overnight at 37°C in medium containing salts and 2% glucose or trypticase-soy broth, and treated with NSC 60339 at concentrations of 3, 10, and 30 μg of drug/ml for 90 min. The total number of cells before and 90 min after the addition of drug was obtained by plate counts after diluting the sample with fresh growth medium and plating on eosin methylene blue (EMB) agar. After washing the cells twice with saline, drug was extracted into chloroform:methanol and fractionated into 3 components as described previously.

The method to evaluate the relative hydrophobicity of drug-lipid complexes with Freon 113 solvent systems has been described previously (19). Briefly, the solvent systems contained 25 parts of methanol, 6 parts of water, and the solvent ratios of Freon 113 and chloroform shown in Chart 1. The volumes used for the partitioning were 10 ml each of the aqueous and nonaqueous phases. Approximately 50 μg of drug as a lipid complex was partitioned for each experimental point.

In order to define more precisely the relative hydrophobicity of drug-lipid complexes, they have been extracted from 0.5- and 24-hr-treated sensitive or resistant P388 cells, purified by partitioning in different solvent systems (21), and subjected to isocratic distribution. The solvent system for isocratic distribution was H2O:methanol:Freon 113:CHCl3 (6:25:30:38, v/v/v/v, 60 ml of each phase/tube).

RESULTS

Drug Complexes in Tumor Lines Treated in Vivo. Sensitive or resistant P388 leukemia cells take up equivalent amounts of NSC 60339 in 0.5 hr and within 24 hr, sensitive cells retain the complex but resistant cells lose most of the complex (19). We now have evaluated several other tumor lines for content of drug complexes to determine if this phenomenon is general (Table 1).

In L1210, total intracellular drug concentration was the same at 0.5 and 24 hr; the aqueous drug fractions (hydrophilic-bound and “free” drug) decreased and the lipid-bound drug increased. In the phthalanilide-resistant L1210/60339 the content of total drug and all fractions decreased from 0.5 to 24 hr.

The concentration of drug components in the vincristine-resistant lymphocytic leukemia P388 (P388/VCR) and in the vinblastine-resistant mast cell leukemia P815 (P815/VLB) are also shown in Table 1. The amount of lipid-bound phthalanilide in P388, P388/60339, and P388/VCR was equivalent at 0.5 hr, and there was somewhat more aqueous phase drug in the P388/VCR. By 24 hr all drug fractions had decreased significantly in the vincristine-resistant cells; this was similar to the NSC 60339-resistant cell lines but unlike the sensitive cells. Similarly, in vinblastine-resistant cells (P815/VLB), all drug fractions decreased significantly between 0.5 and 24 hr; in P815, the drug-lipid concentration doubled and the aqueous-phase fractions remained constant or decreased slightly. Interestingly, P815 was the most sensitive tumor line studied and showed the greatest intracellular drug-lipid concentration.

In contrast to the phthalanilide-resistant and cross-resistant tumors which showed an efflux of drug between 0.5 and 24 hr, methotrexate- and cytosine arabinoside-resistant lines, which are sensitive to phthalanilide treatment, retained lipid-bound and hydrophilic-bound drug, whereas, “free” drug was the only fraction which decreased (Table 1). The intracellular concentrations of lipid-bound and hydrophilic-bound drug in L1210/MTX and L1210/ara-C (Table 1) were somewhat higher than comparable fractions extracted from L1210 (Table 1), and each of these resistant lines is somewhat more sensitive to phthalanilide treatment.3

Relative Hydrophobicity of Drug-lipid Complexes from Cells Treated in Vivo. The phthalanilide-lipid complexes from phthalanilide-resistant P388 leukemia cells are more hydrophobic than corresponding complexes from sensitive cells (19). Since cross-resistant tumors paralleled the phthalanilide-resistant P388 (P388/60339) cells in efflux of intracellular drug-lipid complexes, it was of interest to evaluate the relative hydrophobicity of drug-lipid complexes from these tumor lines by our method of sequential partitioning of complexes in a homologous series of solvents. The data are summarized in Chart 1.

By increasing Freon 113 at the expense of chloroform, complexes from both sensitive and resistant tumors are displaced into the aqueous phase. A greater percentage of complex in the nonaqueous phase indicates that the complexes are more hydrophobic. This is attributed to an increased hydrophobicity of

4 All tumor-bearing animals were supplied by Mr. I. Wodinsky of Arthur D. Little, Inc.

5 I. Wodinsky, unpublished data.
Table 1

<table>
<thead>
<tr>
<th>Tumors</th>
<th>No. of expts.</th>
<th>Total µg NSC 60339 per 10⁸ Cells</th>
<th>Lipid-bound drug</th>
<th>Hydrophilic-bound drug</th>
<th>&quot;Free&quot; drug</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
<td>24</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Sensitive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1210</td>
<td>4</td>
<td>15.9 ± 1.6</td>
<td>13.0 ± 2.0</td>
<td>6.0 ± 0.6</td>
<td>7.6 ± 0.6</td>
</tr>
<tr>
<td>L1210/MTX</td>
<td>6</td>
<td>31.9 ± 0.7</td>
<td>10.8 ± 0.2</td>
<td>10.8 ± 0.2</td>
<td>8.8 ± 0.3</td>
</tr>
<tr>
<td>L1210/ara-C</td>
<td>3</td>
<td>29.9 ± 2.6</td>
<td>21.9 ± 1.5</td>
<td>12.1 ± 1.5</td>
<td>12.4 ± 0.3</td>
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<tr>
<td>P388</td>
<td>7-10</td>
<td>108 ± 23</td>
<td>9.4 ± 2.2</td>
<td>8.0 ± 0.6</td>
<td>7.2 ± 0.6</td>
</tr>
<tr>
<td>P815</td>
<td>5-6</td>
<td>19.0 ± 1.0</td>
<td>22.9 ± 0.9</td>
<td>7.5 ± 0.3</td>
<td>13.8 ± 0.4</td>
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<tr>
<td><strong>Resistant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1210/60339</td>
<td>4</td>
<td>12.9 ± 0.8</td>
<td>4.4 ± 0.3</td>
<td>4.8 ± 0.2</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>P388/60339</td>
<td>7-15</td>
<td>9.6 ± 3.4</td>
<td>2.1 ± 1.1</td>
<td>8.4 ± 1.0</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>P388/VCR</td>
<td>3</td>
<td>15.7 ± 3.1</td>
<td>2.5 ± 0.7</td>
<td>9.4 ± 2.4</td>
<td>2.2 ± 0.9</td>
</tr>
<tr>
<td>P388/VELB</td>
<td>6-7</td>
<td>15.2 ± 0.9</td>
<td>4.6 ± 0.1</td>
<td>6.5 ± 0.5</td>
<td>2.1 ± 0.1</td>
</tr>
</tbody>
</table>

NSC 60339 concentrations extracted from tumors after drug exposure in vivo.

a Mean ± S.D.
b Data published in Cancer Res., 20: 202, 1966, Table 1.
c Hydrophilic-bound drug was not determined separately but included in "free" drug fraction. Therefore, "free" drug represents all the drug in the aqueous phase and includes the hydrophilic-bound fraction.
d Hours after treatment.

Chart 1. Partition of NSC 60339-lipid complexes obtained from several sensitive or resistant tumors. The composite curves are the averages for all tumors studied at 0.5 and 24 hr. Two to four separate experiments were done for each tumor. Experimental details are in Material and Methods. MTX, Methotrexate; ara-C, 1-B-D-arabinofuranosylcytosine; VLB, vinblastine; VCR, vincristine.

the lipid since the drug does not change. From regression analyses on these data, it is concluded that the slopes were not significantly different. Therefore, the 24-hr complexes for all tumor lines were more hydrophobic than was the 0.5-hr complexes. On comparing complexes from resistant and sensitive tumors, it was found that complexes from the phthalanilide-resistant and cross-resistant tumor lines were more hydrophobic than those from the sensitive lines. The methotrexate- and cytosine arabinoside-resistant lines, which are not cross-resistant to this phthalanilide, did not contain such hydrophobic complexes.

The difference in hydrophobicity seen for the crude drug complexes was further demonstrated by the countercurrent distribution of highly purified complexes that were derived from 0.5- or 24-hr-treated sensitive or resistant P388 cells (Chart 2). The complexes from 0.5-hr-treated cells were the most hydrophobic and occurred mostly in Tube 0 for sensitive P388 cells and in Tubes 1 and 2 for the resistant cells. The drug-lipid complexes from 24-hr-treated cells were more hydrophobic and peaked in Tubes 2 and 3 for sensitive cells and Tube 4 for resistant cells.

Drug Complexes in Sensitive and Resistant P388 Cells Treated in Vitro. The uptake and efflux of NSC 60339 by P388 and P388/60339 in vitro has been characterized to determine the feasibility of studying the synthesis and the increased hydrophobicity of the lipid components in a more defined system in vitro rather than in vivo. Both P388 and P388/60339 cells that were treated for 4 hr in suspended culture took up drug as a function of extracellular drug concentration (Table 2). The intracellular concentration of each drug component increased and was nearly equivalent in the 2 tumor lines.

The efflux of drug from sensitive P388 and resistant P388/60339 cells, which contained approximately 30-60 µg NSC 60339 per 10⁸ cells, was determined. After washing treated cells and resuspending them in Fischer's medium that contained 10% horse serum, both the sensitive and resistant cells formed aggregates (clumps) of cells. The resistant cells were nearly all aggregated, as judged from the "clearing" of the cell suspension, whereas sensitive cells seemed to be somewhat less aggregated by the same criterion. The nonaggregated sensitive cells that were resuspended in fresh medium for 8 hr contained approximately 50% of the initial drug content (Chart 3). This
Retention or Efflux of Phthalanilide-Lipid Complexes

Table 2

<table>
<thead>
<tr>
<th>Tumors</th>
<th>Extracellular concentration of NSC 60339 (µg NSC 60339 per 10⁸ Cells (Av. of 4 exp.)</th>
<th>Total drug</th>
<th>Lipid-bound drug</th>
<th>Hydrophilic-bound drug</th>
<th>&quot;Free&quot; drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>P388</td>
<td>20 15.2 3.0 4.5 7.6</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40 31.0 6.3 5.7 19.0</td>
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<tr>
<td></td>
<td>60 48.1 8.0 6.9 33.2</td>
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<tr>
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<td>80 73.7 11.4 9.5 52.9</td>
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<tr>
<td>P388/60339</td>
<td>10 7.3 0.8 2.0 4.5</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>20 13.2 2.2 3.5 7.5</td>
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<td>40 27.8 3.2 6.8 17.8</td>
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<td></td>
<td>60 50.0 6.2 13.5 30.3</td>
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<tr>
<td></td>
<td>80 77.1 9.0 15.3 52.8</td>
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</tbody>
</table>

Uptake of NSC 60339 by P388 and P388/60339 cells suspended in Fischer's medium with serum for 4 hours in vitro. P388 and P388/60339 cells were harvested from BDF₁ mice, washed twice with saline and suspended in Fischer’s medium containing 10% horse serum for 4 hr at 37°C. Other experimental details are in Materials and Methods.

Chart 2. Countercurrent distribution of purified NSC 60339-lipid complexes from phthalanilide-sensitive and -resistant P388 cells. Conventional numbering system was employed; the total number of transfers equaled the number of the last tube. The nonaqueous (lower) phases were transferred. The drug content of each tube was the total quantity of drug in both phases. ●—●, 3.8 mg of drug as a lipid complex that was purified from 0.5-hr-treated sensitive P388 cells; ○—○, 5.4 mg from 24-hr-treated sensitive P388 cells; ▲—▲, 6.5 mg from 0.5-hr-treated resistant P388 cells; and △—△, 6.5 mg from 24-hr-treated resistant P388 cells. Experimental details are in Materials and Methods.

decrease was reflected in all fractions. A similar trend was also found for the more aggregated resistant cells, but spurious cell counts hindered us from obtaining quantitative data. On the other hand, when sensitive or resistant cells were treated and resuspended in Fischer's medium without 10% horse serum, no aggregation of cells occurred. In addition, little or no (<10%) efflux of drug from the cells of either line occurred during a 5-hr incubation at 37°C, and the distribution of intracellular drug was equivalent throughout the experimental period.

Formation of a Drug-lipid Complex in Escherichia coli B.

The quantity of extractable phthalanilide-lipid complexes was related to chemotherapy for P388 lymphocytic leukemia cells (20) and the present studies support such a conclusion. Therefore, it was of interest to determine if such complexes could be extracted from microbial cells which are also susceptible to phthalanilide treatment (11, 13, 16).

Chart 3. The efflux of NSC 60339 from treated P388 cells that were resuspended in Fischer's medium with serum. In this representative experiment, cells were pretreated with NSC 60339 for 4 hr at 74 µg of NSC 60339/ml of Fischer's medium containing 10% horse serum. Experimental details are in Materials and Methods.

Drug-lipid complexes were extracted from E. coli B after phthalanilide treatment which caused the cessation of growth (Table 3). The intracellular distribution of drug among the 3 fractions was similar to that observed under comparable conditions with P388 leukemia cells treated in vitro (20). However, when the treated microbial cells were diluted and plated onto EMB agar, colonies appeared (48–96 hr); at the highest drug treatment (90 min) the number of colonies was nearly equivalent to the initial concentration of cells. This suggests that these concentrations of NSC 60339 are bacteriostatic.
DISCUSSION

It has been shown previously that the concentration of extracted phthalanilide (NSC 60339) complexes relate significantly to the chemotherapeutic response of P388 cells and that the efflux of drug-lipid complexes is characteristic of NSC 60339-resistant P388 cells (19, 20). In this study, the retention of drug which is extracted as NSC 60339-lipid complexes are also characteristic of 4 tumors that are sensitive to phthalanilide treatment, whereas the efflux of NSC 60339 is characteristic of 3 phthalanilide-resistant or cross-resistant tumors. The other drug fractions, hydrophilic-bound and "free," generally decreased in both sensitive and resistant tumors. All drug components can be extracted from treated E. coli B cells which are susceptible to NSC 60339 treatment (11, 16) and from P388 and P388/60339 which are treated in vitro.

Studies to evaluate efflux of drug from both sensitive and resistant P388 that were treated in vitro demonstrated that horse serum enhanced the efflux of drug from both cell lines. Similarly, serum has been found to be an important factor in the efflux of cholesterol from MB 111 mouse lymphoblasts and L-strain mouse fibroblasts in tissue culture (1). Although extracellular components may be important for the efflux of drug from cells, the efflux specificity is probably associated with the nature of the drug complexes.

The substantial efflux of phthalanilide from resistant tumors coincided with an increased hydrophobicity of the extracted drug-lipid complexes. The similarity in these partition curves (Chart 1) to those from sensitive and resistant P388 cells (19), and the fact that relative hydrophobicity for crude P388 complexes were similar to highly purified complexes (Chart 2), suggests that the same type of new phospholipids were involved, especially since the phospholipids from such different sources (P388 cells and dog brain) had similar chemical composition (18, 21). However, differences in chemical composition and relative stability of the extracted P388 and P388/60339 lipids (Chart 2), and the similarity in the percent distribution (which can be calculated from the data in Table 1) of the drug components from sensitive and resistant tumors, are consistent with the possibility that all 3 drug fractions—lipid-bound, hydrophilic-bound and some of the "free"—may be derived from a drug complex that is fragmented by the extraction procedure. For these reasons, we have emphasized the relationship between the concentration of extractable lipid complexes and the chemotherapeutic responsiveness of the various tumors.

The efflux and the increased hydrophobicity of the phthalanilide-lipid complexes from the Vinca alkaloid-resistant tumors, which are cross-resistant to the phthalanilides, is particularly interesting because these tumors had not been previously exposed to the phthalanilides. These characteristics were not unique features of all drug-resistant cells. Methotrexate- and cytosine arabinoside-resistant tumors, which are sensitive to phthalanilide treatment, retained the drug-lipid complex, and these complexes were not as hydrophobic as those from phthalanilide-resistant or phthalanilide-cross-resistant cells. This may indicate that the resistance to the Vinca alkaloids involves the formation and mobilization of Vinca alkaloid-lipid complexes. Furthermore, this mechanism for resistance should be considered for other large cationic drugs, e.g., methylglyoxal-bisguanilyhydrzone (NSC 22946, MeGAG) which are also cross-resistant to phthalanilide treatment (7).5

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