Hypersensitizing Effect of Pluronic L61 on Cytotoxic Activity, Transport, and Subcellular Distribution of Doxorubicin in Multiple Drug-resistant Cells

Annie Venne, Shengmin Li, Rosemonde Mandeville, Alexander Kabanov, and Valery Alakhov

Abstract

The present study demonstrated that poly(oxypolyethylene) and poly(oxypolypropylene) block copolymer pluronic L61 (L61)-hypersensitized multidrug-resistant CH51 Chinese hamster ovary cells and MCF-7/ADR human breast carcinoma cells to the cytotoxic action of doxorubicin. Dox/L61 and MCF-7/ADR cells manifested 290- and 700-fold increases, respectively, in their sensitivity to Dox/L61 formulation compared with free Dox. Their sensitive counterparts Aux-B1 and MCF-7 displayed only marginal or no increase at all in their response to Dox/L61. The study of the drug transport performed by flow cytometry showed that L61 enhanced the drug uptake and reduced the P-glycoprotein-mediated drug efflux. Visualization of Dox subcellular distribution in CH51 cells by fluorescent microscopy revealed that Dox was sequestered in cytoplasmic vesicles, whereas incubation of the cells with Dox/L61 altered the drug compartmentalization by releasing the drug from these vesicles and shifting it to the nucleus. These findings suggested that the hypersensitive response of multidrug-resistant cells to the action of Dox/L61 was caused by an increase in the drug accumulation and changes in its subcellular distribution.

Introduction

Multidrug resistance is often associated with overexpression of P-gp and lowered accumulation and retention of drugs in tumor cells (1, 2). We have recently reported that poly(oxypolyethylene) and poly(oxypolypropylene) block copolymer pluronic P85 sensitizes P-gp-expressing MDR SKVLB human ovarian carcinoma cells with respect to various MDR type drugs, such as anthracyclines, vincristine, and others (3). In the presence of pluronic P85, the cytotoxic activity of the drugs against MDR cells increased up to 1000 times, whereas only marginal changes in their activity were observed in the case of sensitive cells. This made MDR cells hypersensitive to the drug-copolymer formulation compared with their sensitive counterparts.

The in vivo efficacy studies of Dox formulated with pluronic L61 demonstrated that the composition of Dox with the most lipophilic of all the copolymers studied (Dox/L61) was the most resistant tumors demonstrated that the composition of Dox with the most lipophilic of all the copolymers studied (Dox/L61) was the most active. To analyze the mechanism of hypersensitive response of MDR cells to Dox/L61, we studied its transmembrane transport and the effect of the copolymer on the subcellular distribution of the drug.

Materials and Methods

Drugs. Dox was purchased from Sigma Chemical Co. (St. Louis, MO), and L61 was kindly provided by BASF (Passipany, NJ). Dox/L61 was in the laboratories of Supratek Pharma, Inc. (Montreal, Quebec, Canada).

Cell Lines. Aux-B1 Chinese hamster ovary cells and their MDR derivatives MCF-7/ADR were kindly provided by Dr. Y. L. Lee (William Beaumont Hospital, Royal Oak, MI). The cells were maintained in culture in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. MCF-7 and their MDR derivatives MCF-7/ADR were kindly provided by Dr. Y. L. Lee (William Beaumont Hospital, Royal Oak, MI). The cells were maintained in culture in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum.

Cytotoxicity Assay. The cells were plated (3000 cells/well) in a 96-well plate and allowed to reattach overnight. Dox or Dox/L61 was incubated with the cells for 2 h at 37°C with 5% CO2 (L61 final concentration, 0.1%/w/v). The cells were washed three times and cultured for 4 days. The drug cytotoxicity was determined by a standard 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-carboxanilide inner salt assay (4). The absorbance at 540 nm was determined using a microplate reader. All the experiments were carried out in triplicate. SEM values were less than 10% (P < 0.05).

Drug Uptake Studies. The cells in suspension (2.5 x 105 cells) were preincubated for 10 min at 37°C prior to drug addition. Free Dox (10 mg/ml) or 0.5 mM free Rhod 123, 10 mg/ml Dox/0.1% (w/v) L61 or 0.5 mM Rhod 123/0.1% (w/v) L61, and 10 mg/ml Dox plus 50 mg/ml Vrp or 0.5 mM Rhod 123 plus 50 mg/ml Vrp were added to the cells and incubated for various time intervals at 37°C. After incubation, the cells were placed on ice and washed twice. The cell fluorescence was analyzed by flow cytometry on a Coulter Epics XL cytometer, excitation 488 nm (argon laser), using a 620-nm filter. A 525-nm filter was used for Rhod 123 analysis. A minimum of 10,000 events was analyzed for each histogram generation. The experimental values of cell fluorescence were normalized according to the cell size by dividing the mean channel fluorescence value by the forward light scatter value (5). The experiments were conducted three times. SEM values were less than 10% (P < 0.05).

Rho123 and Rho123/L61 Efflux. For the drug efflux study, 2 x 105 cells were used for each kinetic curve. Initially, the cells were loaded with 0.5 mM Rhod 123, 0.5 mM Rhod 123/0.1% (w/v) L61, or 0.5 mM Rhod 123 plus 50 mg/ml Vrp for 45 min at 37°C. The cells were then placed on ice, washed twice, and resuspended in Rhod 123-free media. In Rhod 123/L61 and Rhod 123/Vrp series, the drug-free media contained L61 or Vrp at the same concentrations that were used for the loading. The cells were incubated at 37°C in the Rhod 123-free medium, and the samples were collected after various time intervals and analyzed by flow cytometry. A 525-nm filter was used for Rhod 123 analysis. A minimum of 10,000 events was analyzed for each histogram generation. The experimental values of cell fluorescence were normalized according to the cell size by dividing the mean channel fluorescence value by the forward light scatter value (5). The experiments were conducted three times. SEM values were less than 10% (P < 0.05).


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3. The abbreviations used are: P-gp, P-glycoprotein; Dox, doxorubicin; L61, pluronic L61; MDR, multidrug-resistant; Rhod 123, rhodamine 123; Vrp, verapamil.


5. The experiment was repeated three times. SEM values were less than 10% (P < 0.05).

6. The experiment was repeated three times. SEM values were less than 10% (P < 0.05).

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Fluorescent Microscopy. Cells (1 × 10^6) were incubated with 50 μg/ml Dox, 50 μg/ml Dox/0.1% (w/v) L61, or 50 μg/ml Dox plus 50 μM Vrp for 90 min at 37°C. The cells were then washed with PBS three times, placed on slides, and analyzed immediately after that using a fluorescent microscope at ×500 magnification.

Results and Discussion

The cytotoxic activity of Dox/L61 formulation was evaluated using two MDR cell sublines, namely, CH<sup>®</sup>C5 Chinese hamster ovary cells and MCF-7/ADR human breast carcinoma cells. These cells are highly resistant to Dox and are characterized by resistance factors of 70 and 110, respectively, compared with their parental sublines. Both

<table>
<thead>
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<th>Cell line</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (ng/ml)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Increase in sensitivity by L61&lt;sup&gt;b&lt;/sup&gt; (fold)</th>
<th>Resistance factor&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td>MCF-7</td>
<td>2,000 ± 58</td>
<td>2,000 ± 65</td>
<td>1</td>
</tr>
<tr>
<td>MCF-7/ADR</td>
<td>222,000 ± 12,000</td>
<td>300 ± 15</td>
<td>1.3</td>
</tr>
<tr>
<td>Aux-B1</td>
<td>1,000 ± 29</td>
<td>700 ± 21</td>
<td>1</td>
</tr>
<tr>
<td>CH&lt;sup&gt;®&lt;/sup&gt;C5</td>
<td>70,000 ± 2,470</td>
<td>250 ± 9</td>
<td>3.5</td>
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<sup>a</sup> IC<sub>50</sub> values were determined by standard 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-carboxanilide inner salt assay. Each value is mean ± SEM of the two experiments in which triplicates were assayed.

<sup>b</sup> Ratio of IC<sub>50</sub> of Dox alone/IC<sub>50</sub> of Dox/L61.

<sup>c</sup> Relative resistance was calculated as IC<sub>50</sub> of each MDR cell line divided by IC<sub>50</sub> of the drug-sensitive parent line for both Dox and Dox/L61.

Table 1 Comparison of Dox and Dox/L61 cytotoxicity in two different MDR cell lines and their sensitive parental lines

CH<sup>®</sup>C5 and MCF-7/ADR cells overexpress the mdr<sub>1</sub> gene product P-gp (6, 7). Table 1 shows that in the presence of L61, the IC<sub>50</sub> values of Dox decreased 280- and 740-fold in CH<sup>®</sup>C5 and MCF-7/ADR cell lines, respectively. At the same time, the activity of Dox/L61 against parental sensitive Aux-B1 and MCF-7 cells did not differ much from that of Dox alone. The last column in Table 1 illustrates that the resistance factors of CH<sup>®</sup>C5 and MCF-7/ADR cells to Dox/L61 were 0.35 and 0.15, respectively, which was indicative of their hypersensitivity to this formulation. L61 used as a control was not cytotoxic at the concentrations used in the experiments (data not shown).

The flow cytometry study of Dox uptake into Aux-B1 and CH<sup>®</sup>C5 cells revealed that L61 induced a substantial increase (7.2-fold) in the drug accumulation in the MDR subline (Fig. 1a). A smaller but significant rise (1.6-fold) in the drug uptake was also observed in the sensitive cells. The drug uptake increased 2.4- and 1.1-fold in MCF-7/ADR and MCF-7 cells, respectively (Fig. 1b). The efficacy of Dox uptake could not be estimated precisely because of the quenching of the drug fluorescence during its intercalation with DNA (8). To exclude the quenching effect, the same experiment was performed with Rho123, which is often used as a fluorescent probe for the transport assay in P-gp-expressing MDR cells. This compound preferably accumulates in mitochondria without any substantial loss in its fluorescence intensity (9) and has characteristics of interaction with L61 similar to Dox (10). Fig. 1a shows that the copolymer dramatically (124-fold) enhanced accumulation of Rho123 in CH<sup>®</sup>C5 cells and, to a lesser extent (23-fold), in Aux-B1 cells. In the case of MCF-7/ADR and MCF-7 cells, the copolymer increased Rho123 uptake considerably in mitochondria without any substantial loss in its fluorescence intensity (9) and has characteristics of interaction with L61 similar to Dox (10).
HYPERSENSITIZATION OF MDR CELLS WITH PLURONIC L61

Fig. 3. Intracellular distribution of 50 μg/ml Dox in resistant CHRC5 cells visualized by fluorescence microscopy (×500) after a 60-min incubation. A, free Dox; B, Dox in the presence of Vrp (50 μM); C, Dox in the presence of L61 (0.1%, w/v).

uptake by 30- and 2.8-fold, respectively (Fig. 1b). It can also be seen that Vrp, which reverses MDR by inhibiting P-gp in a competitive manner (11), affected the drug uptake by MDR cells at a much lesser degree than L61, suggesting that a substantial part of Dox/L61 gets into MDR cells via a P-gp-independent pathway. It has been reported that endocytosis, secretion, and some other processes related to phase transitions in the plasma membrane are more active in MDR cells than in sensitive ones, due to higher membrane fluidity of the former (12). This suggested that the increase in the drug uptake observed in MDR cells in the presence of L61 was conditioned by higher flexibility of their membranes.

To estimate the effect of the copolymer on P-gp function, we analyzed the kinetics of Rho123 efflux in CHRC5 cells (Fig. 2, a and b). The fast component in the efflux kinetic rate was observed for free Rho123, which was characteristic of a P-gp-mediated process (13). This component was missing when Rho123/L61 formulation was used. Instead, only low-rate efflux was registered in this case, suggesting that some P-gp-independent mechanism was responsible for the decrease in the intracellular level of the drug. Fig. 2b shows the results of a more detailed study of the effect of L61 on Rho123 efflux by CHRC5 cells. In these experiments, the copolymer was added to the cells in three different ways: (a) the cells were loaded with Rho123/L61 and then washed with Rho123- and L61-free medium; (b) the cells were loaded with Rho123/L61, and the replacing medium contained L61 at the same concentration as in Rho123/L61 formulation; and (c) the cells were first loaded with free Rho123 and then transferred into L61-containing medium. The initial fluorescence values measured immediately after a 60-min incubation of the cells with the drugs were 10.74 ± 1.12, 11.50 ± 1.23, 0.22 ± 0.02, and 0.20 ± 0.01 in procedures a, b, c, and control (free Rho123), respectively. The results obtained clearly demonstrated that L61 produced the highest efflux inhibition when it was present in the external medium. When the cells were loaded with Rho123/L61 and then both components were removed from the medium, a very weak effect on the drug efflux was observed. An intermediate response was registered when L61 was present in the medium both during and after the loading of the cells with Rho123. We have previously reported that pluronic copolymers are effectively uptaken by cells (14). Therefore, it can be assumed that in the above-described experiments, the copolymer was preferably present: (a) inside the cells; (b) both inside and outside the cells; and (c) outside the cells. The results represented in Fig. 2b suggest that L61 inhibited the rapid drug efflux by interacting with the external side of the cell membrane.

The specific feature of MDR cells is that Dox is sequestered in the cytoplasmic vesicles (15-17), which apparently diminishes the amount of Dox associated with the nucleus (18). Using fluorescent microscopy, we analyzed the effect of L61 on subcellular distribution of Dox in CHRC5 cells and compared it with that of Vrp. As can be seen in Fig. 3a, after a 1-h incubation with free Dox, the drug was concentrated in the cytoplasmic vesicles, and a very low level of fluorescence was associated with the nucleus. When Dox was added to the cells in the presence of Vrp, an increase in fluorescence was observed in the nucleus, whereas the vesiculated form of the drug remained virtually unaffected (Fig. 3b). When Dox was incubated with L61, fluorescence was mainly located in the nucleus, and practically no drug was sequestered in the vesicles (Fig. 3c).

Sequestration of Dox and other positively charged compounds in acidic cytoplasmic vesicles is attributed to high pH gradients between the vesicles and cytosol in MDR cells. High retention of the drugs in these compartments can be explained by protonation of the drug molecules, which reduces their ability to diffuse through the vesicular membrane. The fact that ionophoric compounds, such as nigericin and
monensin, reverse MDR supports this hypothesis (17). We have previ-
ously observed that pluronic copolymers are capable of inducing ionic
currents through the liposomal membrane. Thus, it can be assumed that
L61 reduces accumulation of Dox in the vesicles by altering pH gradients
in the intracellular compartments. Further speculation can be made that
the reduction of pH gradients between the acidic vesicles and cytoplasm
might lead to acidification of the latter, which might enhance the drug
uptake, as was observed in this study (Fig. 1).

Accumulation of cytotoxic drugs in the intracellular vesicles has
been reported for different types of MDR cells, both P-gp expressing
and P-gp nonexpressing. Furthermore, overexpression of the lung
resistance-associated protein (19), which is associated with drug-
sequestrating vesicles (20), has been shown to have a very high
correlation index for clinically developed drug resistance (21). The
ability of L61 to enhance the drug uptake and to reduce its compart-
mentalization in MDR cells makes this copolymer a potent candidate
for a delivery vehicle for chemotherapeutic agents.

It can be suggested that there are at least three major events
involved in the hypersensitive response of MDR cells to Dox/L61: (a)
an increase in the drug uptake by these cells under the action of the
copolymer; (b) inhibition of the drug efflux by the copolymer, most
likely by making it capable of bypassing P-gp; and (c) release of the
drug from the cytoplasmic vesicles by L61, which increases its avail-
bility to DNA. As observed previously, the amount of Dox required
to bind with DNA to produce the same DNA damage is smaller in
MDR cells than in sensitive ones (3). Therefore, high susceptibility of
MDR cells to the action of Dox/L61 can be explained by an increased
accumulation of this drug and its altered subcellular distribution,
which, together with higher sensitivity of their DNA to the damaging
action of Dox, makes these cells hypersensitive.

The ability of L61 to hypersensitize MDR cells distinguishes this
copolymer from other known multidrug resistance-modulating agents,
such as P-gp and multidrug resistance-associated protein inhibitors,
cyclosporines, liposomes, low molecular weight surfactants, and an-
tisense oligonucleotides. Due to this specific property, Dox/L61 has
the potential to be used not only for treatment of MDR tumors, but
also for prevention of multidrug resistance development, especially
in the case of relapsed tumors that are often drug resistant due to positive
selection of MDR cells during initial chemotherapy.

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