Specific Distribution of TOP-53 to the Lung and Lung-Localized Tumor Is Determined by its Interaction with Phospholipids

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ABSTRACT

We have investigated the mechanism of TOP-53 distribution to the lung and lung-localized tumor. In contrast to etoposide (VP-16), TOP-53 contains a basic aminoalkyl group that may predispose it to interact specifically with phospholipids, consequently leading to an increase of drug accumulation in the tissues. Therefore, we have studied its interaction with phospholipids in vitro using an organic solvent-water partition system. TOP-53 appeared to have the most potent binding affinity (K_a = 563 x 10^{-2} μM) to phosphatidylserine (PhS), whereas VP-16 showed no interaction with any phospholipid tested. PhS content determined after HPLC separation varied among tested tissues; however, large quantities were found in normal lung and lung cancer tissues far exceeding those present in the liver and kidney. The predicted tissue-to-plasma partition coefficient values, estimated based on PhS content and its binding affinity, resembled those experimentally determined. We concluded that tissue distribution of TOP-53 is determined by PhS content in the tissues and by binding affinity. As a result of specific accumulation in the lung, TOP-53 appeared to show a strong antitumor activity (increase of life span = 171%) against cancer metastasizing to the lung, whereas VP-16 was less effective (increase of life span = 78%). These results suggest that TOP-53 may have an advantage over VP-16 in the treatment of lung cancers in patients.

INTRODUCTION

Several attempts have been made to develop anticancer agents by chemical modification of podophyllotoxin, a compound known to interact with the microtubular system of the cell (Fig. 1; Ref. 1). As a result, VP-16 and VM-26 have been developed and introduced for the treatment of cancer patients. However, their mechanism of action appeared to differ from that of podophyllotoxin, though both were found to induce DNA fragmentation that later was found to be the result of the inhibition of DNA topoisomerase II (2).

Recently, VP-16 has been widely used in cancer chemotherapy of small cell lung cancer and malignant lymphoma (3–5), but its efficacy against a NSCLC is low because of insufficient inhibition of DNA topoisomerase II and, among other factors, a poor tissue distribution, especially to the lung, which may also be considered a disadvantage. To exert a desired pharmacological effect, a sufficient amount of an active compound must be present in the “effector” tissue(s), which in the case of cancer disease involves an organ invaded by a primary tumor and the other tissues being targeted during the process of its metastasizing.

During the search for new and potent topoisomerase II-inhibitors active against NSCLCs, we have focused on the development of new chemical entities related to the structure of podophyllotoxin (6–8). Keeping in mind the penetration of a drug in the effector organ, the glucopyranoside-like function was omitted in the drug design process and replaced with a basic aminoalkyl function to fortify an interaction of the compounds with the intracellular molecules, consequently leading to an increase of intracellular drug accumulation.

As a result of such an approach, we have found a novel podophyllotoxin derivative, TOP-53, that is a non-glucoside derivative with an aminoalkyl residue directly connected to position 4-β of podophyllotoxin through the C-C bond instead of the ether (O-C) bond. TOP-53 exerted significant antitumor activity against murine solid tumors and human cancer xenografts; its antitumor activity was especially well manifested in lung metastatic tumors, supposedly as a result of its specific accumulation in the lung tissue (9). On the basis of these results, Phase I clinical study of TOP-53 has been initiated in Japan.

Recent trends in the development of clinically active compounds indicate the necessity of using experimental systems closely reflecting clinical conditions. For instance, the evaluation of an anticancer agent against pancreatic cancer requires a model of a tumor localized in the pancreas. Furthermore, the accumulation of a drug in the organ in question will determine to some extent its final pharmacological effect. Very good
response of cancers localized in the lung to TOP-53 treatment raised the question of the mechanism(s) involved in its tissue distribution, particularly in the lung.

There are few reports indicating the presence of drug-macromolecule interactions determining tissue distribution of a drug. Specific binding of DNA intercalators determines their tissue affinity (10). The intercalators prefer DNA-rich tissues. On the other hand, Vinca alkaloids having a high binding affinity to tubulin tend to accumulate in the tissues rich in tubulin (11, 12). Many basic drugs, such as imipramine, quinidine, and propanolol, were shown to preferentially accumulate in the lung because of specific interaction with phosphatidylserine, a phospholipid widely distributed in that organ.

In this study, we tried to identify a mechanism of specific distribution of TOP-53 to the lung. Because TOP-53 interacts with neither DNA nor with tubulin, but possesses a basic aminoalkyl chain, it prompted us: (a) to investigate its interaction with anionic phospholipids; (b) to identify the most specific phospholipid; (c) to determine its content in the lung and other tissues; and (d) to correlate its tissue distribution in selected organs and in tumor tissue.

MATERIALS AND METHODS

Drugs and Reagents. TOP-53 was synthesized by Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan) as described previously (6). VP-16 was obtained from Nippon Kayaku (Tokyo, Japan). Quinidine sulfate dihydrate of analytical grade was purchased from Wako (Osaka, Japan). The following standard phospholipids were obtained from Sigma (St. Louis, MO) and used without further purification: PhS (No. P-7769, bovine brain), PhG (No. P-5650), Phl (No. P-0639, soybean), saturated PhC (No. P-6267), PhA (No. P-9511, egg yolk lecithin), and PhE (No. P-3273). All other reagents were of an analytical grade. For in vivo experiments, TOP-53 and VP-16 were dissolved in saline or in saline containing 6.5% Tween 80 and 3.5% DMSO, respectively.

Animals. Specific pathogen-free male C57BL/6 mice, were injected i.v. into male BDF1 mice as a cell suspension prepared in saline (3.0 \times 10^5 cells/0.2 ml/mouse). The survival of drug-treated and control animals was observed, and the antitumor activity was evaluated as the percentage of ILS relative to control animals. The observation was terminated on day 60 and the live mice were considered long-term survivors in this experimental model. Control animals were able to survive approximately 21 days, and their death was caused by the extensive metastatic foci developed in the lung.

Measurement of Tissue Distribution of TOP-53 and VP-16. TOP-53 or VP-16 were injected i.v. into BDF1 mice bearing experimental lung metastasis at equal doses of 12 mg/kg. The main tissues, such as normal lung tissue, lung metastatic nodules, liver, kidney, spleen, heart, and blood, were collected at 0-min, 5-min, 15-min, 1-h, 2-h, 4-h, 8-h, 12-h, 24-h, and 48-h intervals after i.v. injection. Four animals were used per one experimental point. The blood samples were centrifuged at 3000 rpm for 15 min to obtain plasma samples. An aliquot of ~100–500 mg of a tissue sample was homogenized with 4 volumes of acetonitrile, and then the organic phase was separated by centrifugation at 3000 rpm for 15 min. The resulting supernatant was transferred to a conical test tube and evaporated to dryness under a stream of nitrogen. The dried residue was dissolved in 200 μl of a mobile phase (20 mM KH2PO4/methanol, 60:40) and analyzed by the HPLC. The analysis was performed on octadecyl silane II column (4.6 × 150 mm, 5 μm; GL Science, Inc., Tokyo Japan), with a mobile phase consisting of 20 mM KH2PO4/methanol (60:40, v/v) at a flow rate of 1 ml/min, and the elution was monitored at the wavelength of 254 nm using a SPD-6V UV-detector (Shimadzu, Kyoto, Japan). Tissue:drug concentrations were calculated by the external standard method. The plasma samples were extracted with 4 volumes of chloroform, the organic phase being separated by centrifugation and evaporated to dryness. The dried residue was dissolved in 200 μl of mobile phase (20 mM KH2PO4/methanol, 60:40 containing 66 μM EDTA) and analyzed by reverse-phase HPLC as indicated above, except for detection, which was performed with an E-110 electrochemical detector (Eicom, Tokyo Japan). The above extraction procedure was associated with a satisfactory recovery rate of ~80–95%, depending on the type of tissue used.

Pharmacokinetic Analysis. The blood plasma and tissue concentration time courses were subjected to a noncompartmental pharmacokinetic analysis using the WINNONLIN program, version 1.1 (Scientific Consulting, Inc.).

Binding of TOP-53, VP-16, and Quinidine to Phospholipids. Binding of TOP-53, VP-16, and quinidine to phospholipids was determined by using an organic solvent-water partition system according to the slightly modified method of Nishiura et al. (13). Briefly, 2 ml of buffer solution [0.25 M sucrose, 0.05 M Tris-HCl buffer (pH 7.4)] containing a drug at concentrations ranging from 1–100 μM was vortexed with 2 ml of the heptane solution containing an individual phospholipid (8 μg as an inorganic phosphorus/ml) in a screw-capped glass tube at room temperature for 30 s. The mixture was then centrifuged at 2000 rpm for 10 min, and the separated aqueous phase was analyzed by HPLC. The binding parameters of quinidine to an
individual phospholipid were calculated from Scatchard plots as described by Weber et al. (14) and by Sastrasinh et al. (15).

Measurement of Tissue Phospholipids Concentrations in Mice. Phospholipids were extracted from tissue samples according to the method of Folch et al. (16). A tissue sample (0.15–0.5 g) was homogenized with 9 volumes of a chloroform/methanol (2:1 v/v) mixture, and the homogenate was centrifuged at 2500 rpm for 10 min. The separated organic layer was washed out with 0.5 ml of saline. After centrifugation, the organic layer was transferred into a test tube and evaporated to dryness under a stream of nitrogen. The individual phospholipids (PhC, PhE, PhA, PhI, PhG, and PhS) were separated by the HPLC method. The HPLC fractionation was performed according to the method reported by Sasaki (17). The concentration of each individual phospholipid was determined after its conversion to inorganic phosphorus as described by Ames and Dubin (18), and the inorganic phosphorus was assayed by the procedure of Chen et al. (19). The final concentration of phospholipid was calculated by multiplying the concentration of inorganic phosphorus by 25, as reported in the literature (19, 20).

Observed and Predicted Kp for TOP-53. The observed Kp values were calculated based on plasma and tissue concentrations of TOP-53 determined at 2 h after drug administration. The predicted Kp values were calculated based on the concentration of PhS in a tissue and the binding constant of TOP-53 to PhS. The calculations were performed according to Yata et al. (21), using the following formula:

$$K_p = 14.3 \times \log (nK) \times C_{PhS} - 8.09$$

where, nK is the binding capacity and C_{PhS} is the tissue concentration of PhS. The observed Kp values of TOP-53 were initially correlated with PhS concentrations found in rat tissues, which were collected from the publications of Yata et al. (21) and Nishura et al. (13). The correctness of using PhS concentration data derived from different species is justified by the fact that there is a small variation in lipid composition, particularly PhS concentration, among diverse animal species, as reported by Baxter et al. (22).

**Statistical Evaluation.** Bonferroni t test was used for statistical evaluation.

**RESULTS**

**Tissue Distribution of TOP-53 and VP-16 in Mice Bearing LLC Lung Metastasis.** TOP-53 rapidly disappeared from mouse plasma, and its concentration declined biphasically; however, its high concentrations were retained for a long period of time, especially in the lung and lung metastasis, spleen, and also in the organs of elimination (kidney and liver; Fig. 2A). The plasma and tissue concentrations of VP-16 rapidly declined with time, and there was no significant retention in tested mouse tissues. The maximum plasma concentration of TOP-53 reached the level of 3.2 μg/ml, whereas those in the lung and lung metastasis were 41.4 and 39.2 μg/g of tissue, respectively. The respective AUCs were 2.9, 259.7, 292.4 μg/hr/ml or g of tissue. On the other hand, the maximum concentration of VP-16 in plasma was 21.6 μg/ml, and those in the lung and lung metastasis were 11.9 and 11.2 μg/g of tissue, respectively. The AUCs were 11.4, 16.1, 27.8 μg/hr/ml or g of tissue, respectively.

VP-16 concentrations in the lung and lung metastasis were much lower than those in the plasma (Fig. 2B).

**Binding of TOP-53, VP-16, and Quinidine to Phospholipids.** TOP-53 showed the highest binding affinity to phosphatidylserine as manifested by the association constant of $K_a = 563 \times 10^{-2} \mu M$, whereas the binding to other phospholipids was of low affinity. Also, quinidine showed a similar binding pattern, with the highest affinity to PhS as expressed by the association constant of $K_a = 242 \times 10^{-2} \mu M$. The binding to other phospholipids was of very low affinity. In contrast to TOP-53, VP-16 exerted only a weak interaction with either phospholipids tested (Table 1).
The increased survival time of tumor-bearing animals after treatment either with TOP-53 or VP-16 is shown in Fig. 5. The treatment with TOP-53 at its optimum dosing schedule resulted in significant prolongation of the survival of tumor-bearing animals, their survival having been increased by 171%. On the other hand, the treatment with an optimal dose of VP-16 was associated with a significantly lower survival, only 78%. Moreover, four of six mice treated with TOP-53 were able to survive due to the curative effect of TOP-53. No long-term

Table 1  Binding affinity of TOP-53, quinidine, and VP-14 to phospholipids

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>TOP-53 (µM)</th>
<th>Quinidine (µM)</th>
<th>VP-14 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhS</td>
<td>2.97 ± 0.30</td>
<td>3.14 ± 0.24</td>
<td>0.65 ± 0.14</td>
</tr>
<tr>
<td>PhG</td>
<td>0.88 ± 0.19</td>
<td>1.06 ± 0.15</td>
<td>ND</td>
</tr>
<tr>
<td>PhI</td>
<td>0.23 ± 0.13</td>
<td>0.46 ± 0.07</td>
<td>2.52 ± 0.17</td>
</tr>
<tr>
<td>PhA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PhE</td>
<td>4.96 ± 0.46</td>
<td>4.87 ± 0.34</td>
<td>6.44 ± 0.62</td>
</tr>
<tr>
<td>PhC</td>
<td>6.86 ± 0.49</td>
<td>7.03 ± 0.60</td>
<td>10.55 ± 0.47</td>
</tr>
<tr>
<td>Total</td>
<td>15.91 ± 0.99</td>
<td>16.56 ± 1.03</td>
<td>20.17 ± 0.46</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD of three mice. The value of phospholipid concentration in each tissue and tumor (mg/g of tissue).

Table 2  Concentration of phospholipid components in each tissue and tumor

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Lung</th>
<th>Lung tumor</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhS</td>
<td>2.97 ± 0.30</td>
<td>3.14 ± 0.24</td>
<td>0.65 ± 0.14</td>
<td>1.58 ± 0.16</td>
</tr>
<tr>
<td>PhG</td>
<td>0.88 ± 0.19</td>
<td>1.06 ± 0.15</td>
<td>ND</td>
<td>0.48 ± 0.08</td>
</tr>
<tr>
<td>PhI</td>
<td>0.23 ± 0.13</td>
<td>0.46 ± 0.07</td>
<td>2.52 ± 0.17</td>
<td>1.67 ± 0.25</td>
</tr>
<tr>
<td>PhA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PhE</td>
<td>4.96 ± 0.46</td>
<td>4.87 ± 0.34</td>
<td>6.44 ± 0.62</td>
<td>7.57 ± 0.93</td>
</tr>
<tr>
<td>PhC</td>
<td>6.86 ± 0.49</td>
<td>7.03 ± 0.60</td>
<td>10.55 ± 0.47</td>
<td>7.86 ± 1.05</td>
</tr>
<tr>
<td>Total</td>
<td>15.91 ± 0.99</td>
<td>16.56 ± 1.03</td>
<td>20.17 ± 0.46</td>
<td>19.16 ± 0.60</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD of three mice. The value of phospholipid concentration in each tissue and tumor (mg/g of tissue).

Tissue Phospholipid Concentration in Mice. Tissue composition of phospholipids and their respective concentrations are shown in Table 2. The liver and kidney contained higher concentrations of total phospholipids than did lung and lung cancer tissue. However, there is a tissue-related distribution pattern, namely, the lung tissue contains the increased amount of PhS (2.97 ± 0.30 mg/g of tissue) when compared with those of liver and kidney (0.65 ± 0.62 and 1.58 ± 0.16 mg/g of tissue, respectively), whereas the content of PhI shows an opposite trend. The concentrations of PhS in the lung and lung cancer are comparable, indicating that both tissues contain a similar binding capacity for the drugs specifically interacting with PhS.

Relationship between Kp Values and Tissue Phospholipid Concentration. On the basis of the assumption that the concentrations of phospholipids are similar in different animal species at the initial stage, we have compared the Kp values of TOP-53 with the reported PhS concentrations for rats (21, 13). As shown in Fig. 3A, there was significant correlation between the Kp values of TOP-53 observed in mice and PhS tissue concentrations derived from rats. Also, significant relationship ($r = 0.81$) was found between the observed Kp value of TOP-53 and the tissue concentration of PhS measured in a limited number of mouse tissues (Fig. 3B). However, no correlation has been found between total tissue phospholipid concentrations and tissue distribution of TOP-53 (data not shown). Therefore, PhS may be considered as a determinant of tissue distribution of TOP-53. As a matter of fact, the additional line of evidence is derived from the binding properties of TOP-53 to PhS. On the basis of the binding parameters of TOP-53 to PhS, the Kp value was calculated (predicted Kp value) and then correlated with the observed Kp values in mice, resulting in significant correlation ($r = 0.81$) as shown in Fig. 4.

Antitumor Activity of TOP-53 against Metastasizing Lung Cancer. The increased survival time of tumor-bearing animals after treatment either with TOP-53 or VP-16 is shown in Fig. 5. The treatment with TOP-53 at its optimum dosing schedule resulted in significant prolongation of the survival of tumor-bearing animals, their survival having been increased by 171%. On the other hand, the treatment with an optimal dose of VP-16 was associated with a significantly lower survival, only 78%. Moreover, four of six mice treated with TOP-53 were able to survive due to the curative effect of TOP-53. No long-term
surviving animals were observed in the case of treatment with VP-16.

**DISCUSSION**

TOP-53 was shown to exert excellent antitumor activity against lung cancers, especially against NSCLC, exceeding the antitumor activity of VP-16 (9). The cell-killing kinetics of TOP-53 were both concentration and time-dependent, therefore the AUC determines its antitumor effect (23). TOP-53 was found to be active against lung-localized cancers because of its pronounced accumulation in that organ as revealed by the experiments on its biodisposition. In this study, we have tried to clarify the mechanism(s) of specific tissue distribution of TOP-53, assuming the presence of specific intracellular molecules that have an ability to bind TOP-53 and to concentrate in it.

Tissue distribution of several drugs depends on the presence of specific binding molecules present in an organ; e.g., DNA intercalating agents distribute well to the tissues rich in DNA content (10), whereas Vinca alkaloids tend to accumulate in the tissues rich in tubulin protein (11). The drugs containing basic amino groups, but not having the above-mentioned properties, also can be well distributed even in tissues of a nontarget organ (13, 21, 24). Yata et al. (21) have reported that such drugs can be specifically bound to PhS, a phospholipid present at different concentrations in the tissues that may serve as a determinant of tissue distribution. Because TOP-53 also contains a basic side chain that may predispose it to a specific interaction with the phospholipids, it was of great interest to investigate whether TOP-53 may specifically interact with phospholipids, and if so, what kind of phospholipid is responsible for that specific interaction.

We have found that TOP-53 was able to interact with phospholipids, and the most specific interaction was observed with PhS, a phospholipid reported to determine the distribution of other drugs (21, 24). On the basis of the binding affinity of TOP-53 to PhS, and knowing the tissue concentration of PhS, we were able to predict the Kp (21) that appeared to correlate well with the observed values. This finding indicated that TOP-53 is predominantly bound to the intracellular PhS. Taking into account tissue distribution of endogenous PhS, the lung tissue was shown to be rich in PhS (21, 22) in comparison with other organs. This finding may explain a significant accumulation of TOP-53 in the lung. On the other hand, PhS concentration was also found to be high in the tumor originating from the lung, indicating that TOP-53 can penetrate well not only lung tissue but also tumor tissue localized in the lung, which is important for antitumor activity of TOP-53. In our experiments we have confirmed that TOP-53 was specifically distributed to the lung tissue, a well-penetrated tumor mass, resulting in good antitumor activity. In the other experiment (data not shown), TOP-53 showed a weak antitumor activity against cancers localized in the liver, that organ containing lower concentration of PhS and thus showing a lower accumulation of TOP-53, indicating that specific tissue distribution may determine the activity of a compound. Also it is important to keep in mind that the liver is an eliminating organ, thus additionally lowering the distribution of a drug and its antitumor activity. However, one may also expect some tumors to contain a large amount of PhS; therefore, they may accumulate TOP-53 specifically despite its lower accumulation tendency in normal tissue of the invaded organ. We did not examine the concentration of PhS in the tumors of other origin, but this point seems to be important for additional consideration.

The distribution pattern of VP-16 and, in consequence, its
biological properties, differed significantly from those of TOP-53. There was no interaction between phospholipids and VP-16, therefore its tissue penetration, accumulation, and retention were significantly lower than those of TOP-53. The lack of specific intracellular binding sites for VP-16 determined its low cellular uptake as revealed in the intracellular uptake studies (data not shown). Tissue distribution of VP-16 is characterized by low Kp values, thus contrasting the properties of TOP-53. Moreover, the retention of VP-16 in mouse tissues is far shorter than that of TOP-53. One may expect that these properties, to some extent, are responsible for weaker antitumor activity of VP-16, especially against lung-localized cancers.

Because TOP-53 is an AUC-dependent drug, its activity depends on both concentration and exposure time (25–28). Our results indicate that TOP-53 provides a good pharmacokinetic profile in vivo to fulfill the above-mentioned requirement of AUC-dependency. As shown in our experiments, the AUC of TOP-53 in the lung, and especially in the tumor localized in the lung, may reflect its potent antitumor activity. Taking into account the antitumor activity of TOP-53 against lung cancers, not only in small cell lung cancer but also in NSCLC as determined in previous experiments (9), and its biodisposition properties revealed in recent study, there is a high expectation of antitumor activity of TOP-53 superior to that of VP-16 in the treatment of patients suffering from lung cancers.

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