Podophyllotoxin-resistant Mutants of Chinese Hamster Ovary Cells: Cross-Resistance Studies with Various Microtubule Inhibitors and Podophyllotoxin Analogues

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ABSTRACT

The cross-resistances of several mutants of Chinese hamster ovary cells which have been obtained after one and two selection steps in the presence of the microtubule inhibitor podophyllotoxin (PodR and PodR' mutants, respectively) towards various other inhibitors of microtubule assembly (e.g., colchicine, Colcemid, vinblastine, griseofulvin, maytansine, steganacin, nocodazole, and taxol) have been examined. Based upon their specific patterns of cross-resistance/sensitivity to various microtubule inhibitors, both the PodR and PodR' classes of mutants appear to be of more than one kind. Studies on the binding of [3H]podophyllotoxin to cytoplasmic extracts indicate that one of the PodR mutants which has been shown previously to be affected in a Mr 66,000 to 68,000 microtubule-associated protein shows reduced binding of the drug in comparison to the parental PodR and PodR' cells. The different PodR' and PodR' mutants exhibited proportionally increased cross-resistances to various podophyllotoxin analogues (e.g., deoxypodophyllotoxin, epipodophyllotoxin, β-peltatin, 4'-demethylpodophyllotoxin, α-peltatin, podophyllotoxin-β-D-glucoside, β-peltatin-β-D-glucoside, picropodophyllotoxin, and podophyllaic acid) which possess microtubule-inhibitory activity. However, with the exception of one PodR class of mutant, none of the mutants exhibited any cross-resistance to 4'-demethylpipopodophyllotoxin thienyldiene-β-D-glucoside and 4'-demethylpipodophyllotoxin ethylidene-β-D-glucoside, the 2 podophyllotoxin analogues which lack microtubule-inhibitory activity. The cross-resistance studies with these mutants, which, based upon the biochemical studies and their highly specific patterns of cross-resistance, are presumably affected in microtubules, provide some very novel insights into the mechanisms of action of various microtubule inhibitors. The results presented in this paper also show that the cross-resistance studies with the set of podophyllotoxin-resistant mutants provide a sensitive and highly specific screening procedure for identifying compounds which possess podophyllotoxin-like activity and for investigating the structure-activity relationship among them. The results of structure-activity relationship studies for the various podophyllotoxin analogues examined are discussed.

INTRODUCTION

The plant alkaloid podophyllotoxin is a potent inhibitor of cell division which acts by interfering with the assembly of microtubules required for spindle formation during mitosis (1, 6, 26). Although podophyllotoxin is a competitive inhibitor of colchicine binding to tubulin, important differences in the binding characteristics of these drugs (namely, in comparison to colchicine, binding of podophyllotoxin to tubulin occurs much more rapidly, is easily dissociable, shows less temperature dependence, and is not competed by tropolone) indicate that their binding sites and the mechanisms of interaction with microtubules are not identical (4, 6, 14, 26, 28). This inference is further strengthened by our studies of cellular mutants which have been selected for resistance to podophyllotoxin (8, 11). Many of these mutants, while developing increased resistance to podophyllotoxin, became more sensitive to colchicine, and very interestingly the biochemical alteration in some of these mutants has been localized in a protein (Mr 66,000 to 68,000) which appears to comprise a microtubule-associated protein (11).

In earlier work, we have observed that cross-resistance studies with mutant cells which are affected in the target site of a drug can provide valuable information regarding the similarities and differences in the modes of action of various inhibitors of the same process (9, 10, 12, 13). Therefore, in an attempt to gain insight into the mechanisms of action of microtubule inhibitors, cross-resistances of several independently isolated PodR and PodR' (podophyllotoxin-resistant mutants obtained after one and two steps of selection, respectively) mutants to various inhibitors of microtubule functions, e.g., colchicine, Colcemid, vinblastine, steganacin, griseofulvin, maytansine, nucodazole, and taxol (Chart 1; Refs. 1 and 6), and to a number of derivatives of podophyllotoxin have been examined. Results of these studies, which are presented here, provide novel information regarding the mechanisms of action of various microtubule inhibitors and also show that both the PodR' and PodR' classes of mutants are of more than one kind. The binding of [3H]podophyllotoxin to cytoplasmic extracts from some of the PodR' and PodR' mutants has also been investigated.

MATERIALS AND METHODS

Cell Culture and Cell Lines. The different CHO2 lines used in these studies have been described earlier (8) and are listed in Table 1. Podophyllotoxin-resistant (PodR) mutants which have been obtained from sensitive cells after a single selection step are referred to as PodR, whereas those for which selection involved 2 sequential steps are denoted as PodR. The cells were routinely grown in monolayer culture at 37°C in α-minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% fetal calf serum by procedures described earlier (7, 8, 11–13).

Measurement of the Degree of Drug Resistance. The degree of
resistance of mutant cell lines towards various drugs was determined by seeding about 100 and 200 cells (in 0.5 ml of growth medium) into the wells of 24-well tissue culture dishes containing 0.5 ml of the different dilutions of the drugs (including control, which contained no drug) made twice the final concentrations desired in the growth medium (8, 9, 11). The dishes were incubated for 6 to 7 days at 37 °C, after which they were stained for about 30 min with 0.5% methylene blue in 50% methanol; subsequently, the number of colonies was scored. The relative plating efficiencies (same as cloning efficiencies) in the presence of different concentrations of the drugs were determined as the relative plating efficiencies (same as cloning efficiencies) in the presence of different concentrations of the drugs.

**Drugs and Chemicals.** The sources of various drugs and chemicals were: colchicine, Colcemid, vinblastine sulfate, and puromycin from Sigma Chemical Co., St. Louis, Mo.; griseofulvin and nocodazole from Aldrich Chemical Co., Milwaukee, Wis.; _(-)-steganacin_ from Dr. J. P. Robin (27), Laboratoire de Synthese Organique, Lémans, France; maytansine (NSC 153858), taxol (NSC 125973), deoxypodophyllotoxin (NSC 403148), _β_-peltatin (NSC 24819), _α_-peltatin (NSC 24817), podophyllinic acid (NSC 35475), and VM-26 (NSC 122819) from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.; other podophyllotoxin derivatives such as podophyllotoxin_4'-demethylpodophyllotoxin, _β_-peltatin_β_-glucoside, 4'-demethylepipodophyllotoxin, and VP16-213 were kindly provided by Dr. R. S. Gupta.

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**Binding of [3H]Podophyllotoxin and [3H]Colchicine to Cytoplasmic Extracts.** For preparation of cell extracts suitable for binding studies, cells growing exponentially in suspension cultures were harvested by low-speed centrifugation and washed twice by resuspension in ice-cold (0-4 °C) phosphate-buffered saline (containing, per liter, 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na2HPO4, and 0.085 g of KH2PO4) and centrifugation. The washed cells were then resuspended at a concentration of about 5 × 10^5 cells/ml in cold buffer containing 0.02 M sodium phosphate buffer (pH 6.8), 1% Triton X-100, 1 × 10^-2 M MgCl2, 5 × 10^-4 M GTP, and 0.02 M NaCl, and the suspension was homogenized with 30 to 40 strokes of a tightly fitting glass Dounce homogenizer. The cell homogenate was centrifuged at 15,000 × g for 15 min at 4 °C, and the supernatant obtained from this run (n = 3 to 4 mg protein per ml) was used within a short time (0 to 2 hr) in binding studies.

The binding of [3H]podophyllotoxin and [3H]colchicine to cytoplasmic extracts was studied by procedures similar to those described by various investigators (2, 4, 14, 17, 20). In these experiments, 5 to 10 μl of [3H]-labeled drugs of known specific activity ([3H]podophyllotoxin, 36.2 Ci/mmol (final concentration, 2 × 10^-3 μl), [3H]colchicine, 3.4 Ci/mmol (final concentration, 5 × 10^-7 M) were added to 80 μl of cell extracts. After 60 min of incubation at 37 °C, the samples were filtered through a prewetted Whatman DE81 (DEAE-cellulose paper) filter paper disc using mild suction. The filters were rinsed 4 times with 4 to 5 ml of cold buffer containing 0.02 M sodium phosphate buffer (pH 6.8), 1 × 10^-3 M MgCl2, 5 × 10^-4 M GTP, and 0.02 M NaCl. After air drying for 10 to 15 min, the discs were counted in 4 ml of Aquasol (New England Nuclear). The binding of [3H]podophyllotoxin and [3H]colchicine was found to be proportional to the amount of cell extracts (i.e., protein) in the range which was examined (40 to 90 μl of extracts). The parallel blanks lacking cell extracts were included in all experiments, and the amount of radioactivity bound in such experiments (usually not more than 2% of the bound) was subtracted in each case. Since podophyllotoxin-colchicine-binding activity of cell extracts is known to be unstable (4, 26), to avoid the problem of variable losses in the activities of different cell extracts during preparation, the amount of [3H]podophyllotoxin bound to each extract was normalized with respect to the amount of [3H]colchicine bound to the same extract in a parallel experiment. The podophyllotoxin-colchicine-binding activity of various cell extracts was calculated with respect to a constant amount of [3H]colchicine bound to each extract.

**RESULTS**

**Cross-Resistance Patterns of PodR Mutants toward Other Microtubule Inhibitors.** The dose-response curves toward podophyllotoxin of several single-step podophyllotoxin-resistant mutants which have been studied are shown in Chart 2. To find out whether the genetic lesions responsible for all of these mutants were similar or different, cross-resistances of the above cell lines towards various other inhibitors of microtubule functions were determined (Chart 3). As seen in Chart 3 (A to C), all of the PodR mutants examined exhibited increased resistance toward colchicine, Colcemid, and steganacin, and a slight cross-resistance was also observed for nocodazole (Chart 3G). However, interestingly, all of the PodR mutants examined showed increased sensitivity towards taxol (Chart 3F).
Cross-Resistance Studies with PodR Mutants

3H), which in contrast to the other microtubule inhibitors acts by stabilizing the microtubule structure (22, 23). In contrast to the above inhibitors, interesting differences were observed in the sensitivity of the mutant lines towards other microtubule inhibitors such as vinblastine, griseofulvin, and maytansine. For example, of the mutants examined, only PodR² and PodR⁵ showed increased resistance to vinblastine (Chart 3D). Both of these mutants also showed increased resistance to maytansine, although PodR² was more resistant in comparison to the PodR⁵. However, of these 2 mutants, only the PodR² mutant exhibited increased resistance to griseofulvin (Chart 3E). The remaining 2 mutants, PodR² and PodR¹⁶, did not exhibit any increased resistance to either vinblastine or griseofulvin and showed slight sensitivity toward maytansine (Chart 3F).

To find out whether the cross-resistance of the PodR² mutants to various microtubule inhibitors was specific or whether it was the result of a nonspecific membrane alteration affecting cellular permeability of various drugs, cross-resistance of the mutant cells to a number of other compounds which act by other mechanisms was also examined. The compounds which were investigated in this regard included VM-26 and VP16-213, the 2 semisynthetic derivatives of podophyllotoxin which lack microtubule-inhibitory activity (18–20, 24), and puromycin, to which many of the mutants of CHO cells which are affected in membrane permeability exhibit cross-resistance (16). The results of these studies with VM-26 are shown in Chart 3I. As can be seen, of the mutants examined, only the PodR⁵ line exhibits slightly increased resistance towards VM-26. In comparison to the WT cells and the other mutant cell lines, PodR⁵ mutant also exhibits slight cross-resistance to VP16-213 (D₁₀ WT cells, 5 x 10⁻⁷ M; D₁₀ PodR⁵ line, 6 x 10⁻⁷ M) and puromycin (D₁₀ WT cells, 4 ug/ml; D₁₀ PodR⁵ line, 5 ug/ml) (results not shown).

We have reported earlier that mutants exhibiting higher degrees of resistance to podophyllotoxin (PodR² mutants) can be obtained from PodR² cells by carrying out a second step selection in the presence of higher concentrations of podophyllotoxin (8). Based upon their cross-resistance patterns toward various microtubule inhibitors, the PodR² mutants were again of more than one kind (11). For example, in comparison to the parental PodR¹⁶ cells, one type of PodR² mutant (e.g., PodR² and PodR¹⁶) showed increased cross-resistance to vinblastine, nocodazole, maytansine, and taxol, but these mutants at the

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**Chart 2.** Survival curves of various PodR mutants in the presence of increasing concentrations of podophyllotoxin. •, WT (PodR) cells; ○, EOT (PodR) cell line; △, PodR²; ▲, PodR⁵; □, PodR⁸; ■, PodR¹⁶.

**Chart 3.** Cross-resistance patterns of the PodR² mutants toward various inhibitors of microtubule assembly and VM-26. The relative plating efficiencies of various cell lines in the presence of different drug concentrations were determined as described in "Materials and Methods." •, WT (PodR) cells; ○, EOT (PodR) cell line; △, PodR²; ▲, PodR⁵; □, PodR⁸; ■, PodR¹⁶.
same time had also become much more sensitive to colchicine, Colcemid, and steganacin (11). In contrast to these mutants, the second type of Pod\(^{6}\) mutant (e.g., Pod\(^{6}\)-3) showed increased resistance to all of the microtubule inhibitors, including colchicine, Colcemid, steganacin, and griseofulvin. These studies also showed that some of the Pod\(^{6}\) mutants of the first kind (e.g., Pod\(^{6}\)-6 and Pod\(^{6}\)-7) were affected in a protein with a molecular weight of 66,000 to 68,000 which appeared to comprise a microtubule-associated protein (11).

**Binding of \(^{[3}\text{H}\)Podophyllotoxin to Cytoplasmic Extracts from Pod\(^{6}\) and Pod\(^{6}\) Cells.** To gain further insight into the mechanism of podophyllotoxin resistance of the mutant cells, the binding of \(^{[3}\text{H}\)podophyllotoxin to cytoplasmic extracts derived from EOT (Pod\(^{6}\)) cells and a set of Pod\(^{6}\) (Pod\(^{6}\)-16) and Pod\(^{6}\) (Pod\(^{6}\)-6) mutants was investigated. In these experiments, cell extracts from all 3 of these cell lines were prepared at the same time, and the binding of both \(^{[3}\text{H}\)podophyllotoxin and \(^{[3}\text{H}\)colchicine to such extracts was studied in parallel. Since it is known from earlier work that the colchicine-podophyllotoxin-binding activity of cell extracts is unstable and that variable losses may occur in it during preparation of cell extracts (4, 26), the amount of \(^{[3}\text{H}\)podophyllotoxin bound to various cell extracts was normalized with respect to a constant amount of \(^{[3}\text{H}\)colchicine bound. The results of our binding studies with the above set of cell lines in 4 independent experiments are shown in Table 2.

As can be seen, in comparison to the Pod\(^{6}\) cells, extracts from both Pod\(^{6}\)-16 and Pod\(^{6}\)-6 showed reduced binding of \(^{[3}\text{H}\)podophyllotoxin. Although the observed differences in the binding of \(^{[3}\text{H}\)podophyllotoxin between the Pod\(^{6}\) and Pod\(^{6}\)-16 cell lines were rather small, similar differences were observed in all 3 experiments. In the case of Pod\(^{6}\)-6 cell line, the binding of \(^{[3}\text{H}\)podophyllotoxin in comparison to the Pod\(^{6}\) cells was reduced to approximately one-half (0.51 ± 0.1 1 (S.D.)), which indicates strongly that the genetic lesion in this mutant affects the binding of podophyllotoxin to microtubules.

**Cross-Resistance to Mutants to Various Podophyllotoxin Derivatives and the Structure-Activity Relationship Studies.** In earlier studies, we have shown that the cross-resistance of mutant cell lines to various derivatives of a drug could provide information regarding the specificity of the genetic lesion and that such studies are also very useful in establishing the structure-activity relationships between related compounds (9, 10, 12, 13). Because of the clinical usefulness of some of the derivatives of podophyllotoxin (5, 15, 21), a large number of its analogues have been synthesized, and it was of much interest to examine the cross-resistance of the Pod\(^{6}\) mutants to such compounds. The chemical structures of some of the analogues which have been used in the present study are shown in Chart 4 and, with the exception of VM-26 and VP16-213, all of these analogues possess some degree of antimitotic activity (3, 15, 16, 18, 28). In view of their close structural similarity, all of these compounds are expected to enter the cells by a similar mechanism, and those analogues which possess antimitotic activity are expected to act at the same sites as does podophyllotoxin. If the genetic alterations in the Pod\(^{6}\) mutants specifically affect the cellular site which is responsible for the action of this drug on microtubules, then these mutants should exhibit cross-resistance to all of the analogues except VM-26 and VP16-213, which act by other mechanisms (19, 20, 24). Furthermore, one should expect that the Pod\(^{6}\) mutants which are resistant to higher concentrations of podophyllotoxin should exhibit higher degrees of resistance to various active analogues in comparison to the Pod\(^{6}\) cells.

**Table 2**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pod(^{6})</th>
<th>Pod(^{6})-16</th>
<th>Pod(^{6})-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5,500</td>
<td>4,980 (0.91)</td>
<td>3,320 (0.61)</td>
</tr>
<tr>
<td>2</td>
<td>13,300</td>
<td>11,260 (0.85)</td>
<td>6,960 (0.52)</td>
</tr>
<tr>
<td>3</td>
<td>10,020</td>
<td>9,830 (0.98)</td>
<td>3,570 (0.36)</td>
</tr>
<tr>
<td>4</td>
<td>7,515</td>
<td>6,570 (0.87)</td>
<td>4,160 (0.55)</td>
</tr>
</tbody>
</table>

\(^{a}\)The amount of \(^{[3}\text{H}\)podophyllotoxin bound to various cell extracts has been normalized to a constant amount (10\(^{6}\) cpm) of \(^{[3}\text{H}\)colchicine bound which was measured in parallel in all of the experiments.

\(^{b}\)Numbers in parentheses, ratios of \(^{[3}\text{H}\)podophyllotoxin bound to mutant cell extracts as compared to the extracts from Pod\(^{6}\) cells.

\(^{c}\)Not determined.
similar results. In Table 3, the D_{10} values of the above cell lines toward the various podophyllotoxin analogues as well as the degrees of resistance of the mutant cell lines as compared to the parental Pod^s^ cells are indicated. As can be seen, both the Pod^m^ and Pod^m^6 mutants exhibited increased cross-resistance to all of the podophyllotoxin analogues shown in Chart 4, except VM-26 and VP16-213, which do not possess microtubule-inhibitory activity (14, 18, 24). Furthermore, as expected, for all of the analogues, the Pod^m^6 line showed a higher degree of resistance as compared to the Pod^m^16 cells.

The proportionate increase in the levels of cross-resistance of the Pod^m^ and Pod^m^6 mutants to only those podophyllotoxin analogues which possess antimitotic activity strongly indicates that the genetic lesions in these mutants are affecting the binding site of these compounds which is involved in their effects on microtubules. This result in turn indicates that the cellular toxicity of these analogues is due to their effects on microtubules. In such cases, the concentrations of various analogues which produce equivalent cellular toxicity provide a good measure of their relative activity (10, 12). The D_{10} values of various podophyllotoxin analogues in molar concentrations are shown in Table 4. Assuming the D_{10} value of podophyllotoxin to be 1, the relative toxicities of various podophyllotoxin analogues have been calculated. Table 4 also shows the relative tubulin-binding affinities of various podophyllotoxin analogues (as compared to podophyllotoxin) which have been calculated from the microtubule-inhibitory-tubulin binding affinities of these compounds as have been reported in literature.

**Table 3**

**Cross-resistance of Pod^m^ and Pod^m^6 mutants to various podophyllotoxin analogues**

The D_{10} values for various cell lines towards different analogues were determined from survival curves similar to those shown in Chart 3. The Pod^s^ cells refer to both the WT and EOT cell lines which showed very similar sensitivities towards all of these compounds. The degrees of resistance of cell lines were calculated as the ratios of the D_{10} values for the cell line as compared to that of the Pod^s^ cells. In these studies, a compound was considered to possess podophyllotoxin-like activity if the Pod^m^ and Pod^m^6 mutants exhibited increasing degrees of resistance towards it.

<table>
<thead>
<tr>
<th>Podophyllotoxin analogue</th>
<th>Pod^s^ (per ml) for cell lines</th>
<th>Pod^m^6 (per ml) for cell lines</th>
<th>Podophyllotoxin-like activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Podophyllotoxin</td>
<td>6 ng (1)^a</td>
<td>30 ng (5)</td>
<td>67 ng (11.2)</td>
</tr>
<tr>
<td>Epipodophyllotoxin</td>
<td>45 ng (1)</td>
<td>80 ng (1.8)</td>
<td>250 ng (5.6)</td>
</tr>
<tr>
<td>Deoxyepipodophyllotoxin</td>
<td>3 ng (1)</td>
<td>8 ng (2.7)</td>
<td>20 ng (6.7)</td>
</tr>
<tr>
<td>β-Peltatin</td>
<td>1 ng (1)</td>
<td>2.5 ng (2.5)</td>
<td>8 ng (8)</td>
</tr>
<tr>
<td>4'-Demethylpodophyllotoxin</td>
<td>15 ng (1)</td>
<td>40 ng (2.7)</td>
<td>125 ng (8.3)</td>
</tr>
<tr>
<td>4'-Demethylepipodophyllotoxin</td>
<td>120 ng (1)</td>
<td>200 ng (1.7)</td>
<td>400 ng (3.3)</td>
</tr>
<tr>
<td>α-Peltatin</td>
<td>1.5 ng (1)</td>
<td>4.5 ng (2.8)</td>
<td>12 ng (7.5)</td>
</tr>
<tr>
<td>Podophyllotoxin-β-D-glucoside</td>
<td>4.5 μg (1)</td>
<td>12.5 μg (2.8)</td>
<td>30 μg (6.7)</td>
</tr>
<tr>
<td>β-Peltatin-β-D-glucoside</td>
<td>0.4 μg (1)</td>
<td>0.7 μg (1.7)</td>
<td>1.5 μg (3.8)</td>
</tr>
<tr>
<td>Picropodophyllotoxin</td>
<td>100 ng (1)</td>
<td>180 ng (1.8)</td>
<td>400 ng (4)</td>
</tr>
<tr>
<td>Podophyllinc acid</td>
<td>5 ng (1)</td>
<td>16 ng (3.2)</td>
<td>45 ng (9)</td>
</tr>
<tr>
<td>VM-26</td>
<td>30 ng (1)</td>
<td>30 ng (1)</td>
<td>30 ng (1)</td>
</tr>
<tr>
<td>VP16-213</td>
<td>0.30 μg (1)</td>
<td>0.30 μg (1)</td>
<td>0.30 μg (1)</td>
</tr>
</tbody>
</table>

^a Numbers in parentheses, relative degree of resistance.

**Table 4**

**Relative toxicity and Tubulin-binding affinities of various podophyllotoxin analogues**

The molar D_{10} values for Pod^s^ cells were calculated from the data given in Table 3. Assuming the molar D_{10} values for podophyllotoxin to be 1, the relative toxicities of other analogues were obtained as the ratios of the D_{10} values for podophyllotoxin as compared to the other analogues. The tubulin-binding affinities of various podophyllotoxin analogues were taken from the data of either Kelleher (14) or Loike et al. (18). While in the work of Kelleher (14) these values were obtained as the K_i values for the inhibition of [3H]colchicine to tubulin by various analogues, in the latter study (18) these values indicate the concentrations of various compounds which are required to inhibit the assembly of microtubules in vitro by 50%. Assuming the value for podophyllotoxin to be 1, the relative tubulin-binding affinities of other analogues were calculated from the ratio of the concentrations of podophyllotoxin as compared to the other analogues in the same study.

<table>
<thead>
<tr>
<th>Podophyllotoxin analogue</th>
<th>D_{10} for Pod^s^ cells (μM)</th>
<th>Toxicity of analogue relative to podophyllotoxin (μM)</th>
<th>Tubulin-binding affinity of analogue (μM)</th>
<th>Tubulin-binding affinity of analogue relative to podophyllotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Podophyllotoxin</td>
<td>1.45 x 10^{-5}</td>
<td>1</td>
<td>0.51^a 0.6^b</td>
<td>1</td>
</tr>
<tr>
<td>Epipodophyllotoxin</td>
<td>1.1 x 10^{-7}</td>
<td>0.13</td>
<td>5.2^a</td>
<td>0.12</td>
</tr>
<tr>
<td>Deoxyepipodophyllotoxin</td>
<td>7.5 x 10^{-8}</td>
<td>1.9</td>
<td>0.54^a</td>
<td>0.94</td>
</tr>
<tr>
<td>β-Peltatin</td>
<td>2.5 x 10^{-8}</td>
<td>5.8</td>
<td>0.12^a</td>
<td>4.25</td>
</tr>
<tr>
<td>4'-Demethylpodophyllotoxin</td>
<td>3.8 x 10^{-8}</td>
<td>0.38</td>
<td>0.65^a</td>
<td>0.78</td>
</tr>
<tr>
<td>4'-Demethylepipodophyllotoxin</td>
<td>3 x 10^{-7}</td>
<td>0.05</td>
<td>2.0^a</td>
<td>0.3</td>
</tr>
<tr>
<td>α-Peltatin</td>
<td>4 x 10^{-8}</td>
<td>3.6</td>
<td>0.6^b</td>
<td>1.2</td>
</tr>
<tr>
<td>Podophyllotoxin-β-D-glucoside</td>
<td>7.8 x 10^{-6}</td>
<td>0.0019</td>
<td>180^c</td>
<td>0.0028</td>
</tr>
<tr>
<td>β-Peltatin-β-D-glucoside</td>
<td>7 x 10^{-7}</td>
<td>0.02</td>
<td>310^c</td>
<td>0.0016</td>
</tr>
<tr>
<td>Picropodophyllotoxin</td>
<td>2.4 x 10^{-7}</td>
<td>0.06</td>
<td>10^c</td>
<td>0.05</td>
</tr>
<tr>
<td>Podophyllinc acid</td>
<td>1.2 x 10^{-8}</td>
<td>1.2</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>VM-26</td>
<td>4.6 x 10^{-8}</td>
<td>Inactive</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>VP16-213</td>
<td>5 x 10^{-7}</td>
<td>Inactive</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
</tbody>
</table>

^a Kelleher (14).

^b Loike et al. (18).
R. S. Gupta

than one kind. In one type of the cytoplasmic extracts from this mutant as compared to the protein (11). The greatly reduced binding of [3H]podophyllotoxin (Refs. 14 and 18; Table 4). The relationship between the relative binding of various podophyllotoxin analogues. Data for this plot have been taken from Table 4.

(Refs. 14 and 18; Table 4). The relationship between the relative cellular toxicity of these analogues and their relative binding affinity is illustrated in Chart 5. As can be seen for the various podophyllotoxin analogues that were examined, these 2 values show an excellent correlation (correlation coefficient, 0.87). These results provide strong support to the view that the cellular toxicity of these analogues is primarily due to their effect on microtubules.

DISCUSSION

Results presented in this paper show that the podophyllotoxin-resistant mutants of CHO cells which are obtained after a single selection step are of more than one kind. The different types of mutants exhibit specific patterns of cross-resistance to various antimitotic drugs but, with the possible exception of PodR5, these do not show any cross-resistance to either the 2 podophyllotoxin analogues VM-26 and VP16-213, which lack microtubule inhibitory activity, or other unrelated compounds such as puromycin. Although the biochemical functions which are affected in different types of mutants have not yet been identified, their highly specific cross-resistance to only various microtubule inhibitors strongly indicates that the lesions in all of these mutants (with the possible exception of PodR5) most probably directly affect microtubules, such that their interactions with podophyllotoxin and other antimitotic drugs are altered. Further studies on the identification of the affected component(s) in these mutants should prove very useful in understanding the mode(s) of interaction of these drugs with microtubules.

We have shown earlier that, based upon their cross-resistance to various microtubule inhibitors, the PodRmutants which are obtained after a second-step selection are also of more than one kind. In one type of PodRmutant (e.g., PodR6), the genetic lesion has been shown to affect a protein P (M, 66,000 to 68,000) which appears to be a microtubule-associated protein (11). The greatly reduced binding of [3H]podophyllotoxin to the cytoplasmic extracts from this mutant as compared to the parental Pod and PodR cells, as observed in the present studies, provides strong evidence that the genetic lesion in this mutant directly affects the microtubule structure. However, how an alteration in the protein P affects the interaction of podophyllotoxin with microtubules is not clear at present, and further studies on the binding of [3H]podophyllotoxin using purified tubulin and the protein P are required to understand this aspect.

In earlier studies, we have shown that cross-resistance with mutants which are affected at the target site of a drug to other inhibitors of the same process provides valuable information regarding the similarities or dissimilarities in their modes of action (9, 10, 12, 13). The rationale of cross-resistance studies in understanding the mechanism of action of drugs may be briefly stated as follows. If any 2 given compounds act at the same common site in an identical manner, then all of the cellular mutants which have been selected for resistance to one of these drugs and bear alterations in its target site should also exhibit increased cross-resistance to the other compound. However, if 2 compounds act at nonidentical or overlapping sites but on the same cellular target, then cellular mutants resistant to one drug may or may not exhibit altered resistance to the other compound, depending upon how the former alteration has affected the other site (10, 12). Based upon the above premise, the cross-resistance studies with mutants have led to establishing that several structurally unrelated compounds, e.g., emetine, cryptopleurine, etc., acted in an identical manner and possessed common structural determinants responsible for their activities as inhibitors of protein synthesis (10, 12, 13). In the present investigation, cross-resistance of the PodR and the PodRmutants to only those podophyllotoxin derivatives which possess antimitotic activity and the observed proportional increase in the levels of resistance of the mutants provide strong suggestive evidence that the genetic lesions in these mutants are highly specific and involve the cellular target site(s) of these compounds. The cross-resistance patterns of these PodRmutants to other microtubule inhibitors could then provide novel and valuable information regarding the modes of action of these other inhibitors. Some of the possible inferences that can be drawn from these studies are: (a) while all the PodRmutants show proportional increase in their degree of resistance to various podophyllotoxin analogues, similar behavior was not observed for any of the other microtubule inhibitors examined (nocodazole may be one possible exception, since in this case, increasing degree of resistance was observed for the various PodR and PodRmutants, but the level of resistance of PodRmutants was only very slight, i.e., 1.1-1.2-fold). These results indicate that none of the other inhibitors, e.g., colchicine, Colcemid, vinblastine, steganacin, maytansine, griseofulvin, and taxol, act in exactly the same manner as podophyllotoxin; (b) the sensitivity pattern of various PodR and PodRmutants towards colchicine, Colcemid, and steganacin was always affected in a very similar manner. Although colchicine and Colcemid are analogues, structure of steganacin shows partial resemblance to both colchicine and podophyllotoxin (Chart 1). However, the behavior of our mutants strongly suggests that the mechanism of action of steganacin should be very similar to that of colchicine and not like podophyllotoxin. Steganacin is also a competitive inhibitor of colchicine binding to tubulin, which is in accordance with the above inference (25); and (c) all of the PodRmutants, while developing increased resistance to podophyllotoxin, colchicine, Col-

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cemicid, and steganacin, become much more sensitive to taxol. In other studies, we have observed that CHO cell mutants which have been selected for resistance to taxol show increased sensitivity towards colchicine, Colcemid, steganacin, and podophyllotoxin. The antagonistic behavior of various Pod and taxol-resistant mutants towards these drugs is of much interest because taxol, in contrast to colchicine, steganacin, and podophyllotoxin, which depolymerize cellular microtubules, acts in an opposite way by binding to tubulin and stabilizing the microtubule structure (22, 23). Although the mechanism of action of taxol is not well understood at present the antagonistic effects of the above genetic lesions suggest that these drugs bind to a common target the affinity for these drugs of which is altered in an opposite manner by the above types of genetic lesions.

The cross-resistance studies with a set of Pod, Pod, and Pod mutants to various compounds also provide a very simple and highly specific procedure for screening compounds which may possess podophyllotoxin-like activity. The relative activities of various analogues in this system correlate very well with their known microtubule-inhibitory or -binding activities (14, 18). Kelleher (14) has earlier suggested a screening procedure for such compounds which is based on their tubulin-binding affinities; however, this assay procedure cannot distinguish podophyllotoxin-like compounds from other microtubule inhibitors, such as colchicine, steganacin and nocodazole (which competes for binding to tubulin), that do not act in the same manner as podophyllotoxin.

From the data on the relative cellular toxicity of various podophyllotoxin analogues, the effects of various structural modifications on the biological activity of podophyllotoxin can be inferred. The structure-activity relationships between various podophyllotoxin analogues have also been investigated earlier, and our results are in general in good agreement with the earlier work (3, 14, 18, 28). Some inferences that can be drawn from these data are: (a) replacement of an OH group at C-4 with an H, i.e., conversion of podophyllotoxin to deoxy-podophyllotoxin, results in a slight (1.8-fold) increase in activity; (b) substitution of an OH group at C-5 in deoxypodophyllotoxin (i.e., its conversion to /β-peltatin) results in a further 3-fold increase in activity. In fact, as has also been noted by Kelleher (14), /β-peltatin is the most active of all podophyllotoxin analogues which have been examined; (c) removal of the methyl moiety from the 4'-methoxy group (cf. podophyllotoxin versus 4'-demethylpodophyllotoxin, and /β-peltatin versus α-peltatin) results in slight loss in activity; (d) comparisons of the activities of podophyllotoxin and 4'-demethylpodophyllotoxin with epipodophyllotoxin and 4'-demethyllepidopodophyllotoxin, respectively, indicate that the C-4 enantiomers (in which C-4 is in S configuration) have about 7- to 8-fold reduced activity in comparison to the epimers which have R configuration at the C-4 position; (e) picropodophyllotoxin, which is the C-2 epimer of podophyllotoxin, is about 17-fold less toxic in comparison to podophyllotoxin; (f) the lactone ring D in podophyllotoxin is not essential for its activity, inasmuch as its opening as in podophyllinic acid causes no reduction in the activity of the compound. The activity of podophyllinic acid, i.e., NSC 35475, in our system has been surprising, because Kelleher (14) has earlier reported that this compound was inactive in competing for the binding of [3H]colchicine to tubulin and hence had inferred that this compound was actually in the picro form (i.e., picropodophyllinic acid). However, the nonessential nature of the lactone group for the activity of podophyllotoxin is also shown by the studies of Loike et al. (18), in which replacement of the carbonyl group by a methylene group resulted in only a 2-fold loss in its activity; (g) substitution of bulky groups, e.g., glucopyranoside, at ether the C-4 or C-5 positions results in a drastic loss (300- to 500-fold) in the activity of the compounds (e.g., cf. podophyllotoxin versus podophyllotoxin β-D-glucopyranoside and /β-peltatin versus /β-peltatin-β-D-glucopyranoside) indicating that these substituents probably interfere with the binding of podophyllotoxin to its target site (18). In the present study, the loss in relative activity that results upon the above substitution is comparable for both podophyllotoxin and /β-peltatin; however, a much greater effect of such substitution upon /β-peltatin (=3000-fold) as compared to podophyllotoxin (=350-fold) has been reported by Kelleher (14); and (h) the lack of cross-resistance of the Pod mutants to VM-26 and VP16-213 confirms that these compounds do not act in the same manner as does podophyllotoxin. Recently, mutants of CHO cells resistant to VM-26 and VP16-213 have also been isolated in our laboratory, and these mutants do not exhibit any cross-resistance to podophyllotoxin or its other analogues which possess microtubule-inhibitory activity. These results together provide strong evidence that the mechanism and the site of action of these 2 analogues are different from that of podophyllotoxin-like compounds.

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Podophyllotoxin-resistant Mutants of Chinese Hamster Ovary Cells: Cross-Resistance Studies with Various Microtubule Inhibitors and Podophyllotoxin Analogues

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