4-Demethylpenclomedine, an Antitumor-active, Potentially Nonneurotoxic Metabolite of Penclomedine


Abstract

Penclomedine [3,5-dichloro-4,6-dimethoxy-2-(trichloromethyl)pyridine], an antitumor agent, is currently in Phase I clinical trials and is believed to be a prodrug. In these studies, cerebellar effects have been dose limiting. Previous studies identified 4-demethylpenclomedine (4-DM-PEN) as the major plasma metabolite in rodents and humans. 4-DM-PEN was demonstrated to be an antitumor-active metabolite of penclomedine in vivo when evaluated against the penclomedine-sensitive MX-1 human breast tumor xenograft implanted either s.c. or intracerebrally and is believed to be on the metabolic activation pathway of penclomedine. Because earlier studies revealed an absence of neurotoxic cerebellar effects for 4-DM-PEN in contrast to penclomedine in a rat model, this metabolite may be a candidate for an alternative to penclomedine in the clinic for treatment of breast cancer or brain tumors, if the cerebellar effects of penclomedine preclude its further clinical development. Because neither penclomedine nor 4-DM-PEN were very active in vivo, the metabolism of penclomedine was also investigated using rat liver microsomes in an attempt to identify the ultimate active form of the drug. Metabolites and putative metabolites were prepared by chemical synthesis for antitumor evaluation in vitro and in vivo. A reductive metabolite, α,α-didechloro-PEN, was observed to be much more cytotoxic than penclomedine or 4-DM-PEN in vitro, but evaluation of this and the other metabolites and putative metabolites in vivo against the MX-1 tumor failed to identify any active metabolite among the structures evaluated other than 4-DM-PEN. The limited activity of 4-DM-PEN in vitro indicates that it, like penclomedine, is also a prodrug, demonstrating a need for additional studies on the metabolic activation of penclomedine to identify the ultimate active form of the drug.

Introduction

Penclomedine (Fig. 1) is currently undergoing Phase I clinical testing at Johns Hopkins University Oncology Center and the University of Wisconsin Comprehensive Cancer Center for possible use in the treatment of breast cancer, based on potent activity against human breast tumor xenografts and experimental mammary tumor models (1–6), and in the treatment of brain tumors, based on its activity against tumor xenografts in brain (6).

In these clinical evaluations, the principal side effects included dose-limiting neurotoxicity, which was related to peak plasma concentrations of penclomedine (4). Studies on the metabolism of penclomedine in rodents and humans led to the identification of 4-DM-PEN as the major plasma metabolite in both species (1, 2), and neuroanatomic studies of the cerebellar effects of penclomedine and 4-DM-PEN in rats revealed cerebellar damage only in the penclomedine-treated group (7). Because of the weak cytotoxicity of penclomedine in vitro, Reid et al. (8) suggested that metabolism is required for antitumor activity in vivo, and we report here additional studies on the microsomal metabolism of penclomedine in an attempt to identify the active form of the drug and on the antitumor activity of actual and putative metabolites in vitro against P388 murine leukemia cells and in vivo against human MX-1 breast tumor xenografts implanted s.c. or intracerebrally. These investigations support the possible use of 4-DM-PEN as an alternative to penclomedine for treatment of breast cancer or brain tumors in the event that the cerebellar effects of penclomedine preclude its further clinical development.

Materials and Methods

Penclomedine, formulated penclomedine in a lipid emulsion of 10 mg/ml, and [trichloromethyl-14C]penclomedine were provided by Dr. Joseph Covey (Pharmacology Branch, NCI, Bethesda, MD). 4-DM-PEN was prepared as reported (1), and the other metabolites were prepared from penclomedine by synthetic routes that will be reported elsewhere. All metabolites were characterized by nuclear magnetic resonance and mass spectrometry and by HPLC.

Antitumor Evaluation in Vivo. Antitumor evaluations were conducted as described previously (9). P388 murine leukemia cells were propagated in DMEM with 10% FBS and 2 mM L-glutamine. For evaluation of cytotoxic activity, eight replicate samples of P388 cells were treated with compound levels over a 5-log range. After 3 days of continuous exposure, the viable cells were analyzed using the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt dye conversion assay.

Antitumor Evaluation in Vivo. Antitumor evaluations were conducted as described previously (5, 6). Athymic NCR-nu/nu mice were obtained from various suppliers under contract with NCI and housed in sterile, filter-capped microisolator cages in a barrier facility. Human MX-1 breast tumor was obtained from the NCI Tumor Repository (Frederick, MD). For injection into mice, penclomedine and the actual and putative metabolites were prepared either as a suspension in aqueous hydroxypropyl cellulose (i.p. treatment) or as a lipid emulsion (i.v. treatment). For s.c. implants, MX-1 tumor fragments (30–40 mg) from in vivo passage were implanted into the axillary region of the mice. Treatment was initiated when the tumors reached approximately 300 mg in size and continued for 5 days. Each tumor was measured by caliper in two dimensions twice weekly and converted to tumor mass. Antitumor activity was assessed on the basis of tumor growth delay in comparison to a vehicle-treated control, tumor regressions (partial and complete), and tumor-free survivors.

For intracerebral implants, 0.03 ml of a MX-1 tumor brei (containing 106 cells) was implanted into the right hemisphere of the mice. Treatment was initiated 1 day after tumor implant and continued for 5 days. Mice were monitored daily for survival. Antitumor activity was assessed on the basis of the percentage increase in life span, in comparison to a vehicle-treated control and long-term survivors.

Microsomal Metabolism. S-9 fractions from livers of 200–250-g Sprague-Dawley male rats stimulated with arachol 1254 were purchased from Microbiological Associates (Rockville, MD), and microsomal fractions were prepared by differential centrifugation. Ten μl each of [14C]penclomedine (108 μCi/ml, 2 mg/ml), 10 μM NADPH, and microsomes (10 mg/ml protein) in
Table 2 Activity of penciomedine and actual and putative metabolites against s.c. implanted human MX-1 breast tumor xenograft (5,6)

<table>
<thead>
<tr>
<th>Drug or metabolite</th>
<th>i.p. dose (mg/kg/day)</th>
<th>No. of responders</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penclomedine</td>
<td></td>
<td></td>
<td>0.85</td>
</tr>
<tr>
<td>6-DM-PEN</td>
<td>1.35</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>DDM-PEN</td>
<td>1.35</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>PEN Acid</td>
<td>1.35</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Unsat. PEN Dimer</td>
<td>1.35</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Tris buffer (pH 7.4; 0.1 M; 70 µl) were incubated at 37°C for 30 min and either evaporated in vacuo or extracted with methylene chloride to produce an extract and an aqueous fraction for TLC or HPLC analysis. Preparative incubations were conducted on a 50-fold scale.

Chromatography. HPLC was conducted as described by Reid et al. (8) but with additional radioactivity detection using a FLO-ONE/Beta Series A-500 radiochromatogram detector (Radiomatic Instrument and Chemical Co., Meriden, CT). TLC utilized Analtech silica gel GF plates of 250 µm or 2 mm thickness (Analtech, Newark, DE).

Results and Discussion

Anticellular Evaluation in Vitro. Because the low cytotoxic potency of penclomedine in vitro in contrast to its potent antitumor activity in vivo suggested the likelihood of metabolic activation in vivo (8), penclomedine and the actual and putative metabolites were evaluated in vitro against P388 murine leukemia cells using procedures reported previously (9). The results are shown in Table 1 and indicate that only α,α-didechloro-PEN was significantly more cytotoxic than penclomedine against this cell line, which suggested that it might be an active metabolite and which led to its...
evaluation in vivo. Evaluation of 4-DM-PEN in NCI's in vitro tumor panel of 55 human tumor cell lines also failed to reveal any in vitro activity for 4-DM-PEN (data not shown).

Antitumor Evaluation in Vivo. Using procedures and protocols reported previously for evaluation of penclomedine against the MX-1 xenograft implanted s.c. (5, 6), penclomedine and the actual and putative metabolites were evaluated in vivo, giving the results shown in Table 2. The data indicate that only penclomedine and 4-DM-PEN were active against this xenograft. Evaluation of penclomedine and 4-DM-PEN against this same tumor xenograft implanted intracerebrally gave the data shown in Table 3, and the results indicate closely comparable activity of the two agents when administered either i.p. or p.o.

Microsomal Metabolites. Incubation of [14C]penclomedine with aroclor 1254-stimulated rat liver microsomes followed by TLC analysis of the total incubate in methylene chloride:methanol (3:1) on silica gel yielded three metabolites at Rf values of 0.5, 0.3, and 0.1 (Rf of penclomedine, 1.0). Co-TLC with synthetic standards identified the component at Rf 0.5 as 3,5-dichloro-4,6-dimethoxypicolinic acid and the component at Rf 0.1 as 3,5-dichloro-4,6-dihydroxypicolinic acid (Fig. 1). HPLC analysis of a methylene chloride extract of the incubation solution using conditions identical to those reported by Reid et al. (8) produced the chromatograms shown in Fig. 2, which are based on both UV and radioactivity detection methods. Penclomedine was identified by co-HPLC, and multiple separations were used to collect five fractions (I–V), as shown in Fig. 2. Mass spectral analysis identified V as PEN dimer, confirming the reported identification of this metabolite (8). Mass spectral analysis of components III and IV gave identical molecular ions and indicated unsaturated dimer structures corresponding to cis and trans isomers (Fig. 1). Co-HPLC with a synthetic standard confirmed the identity of III as one of the isomers. Although no metabolite was identified in II, mass spectral analysis of I identified α,α,α-tridechloro-PEN in this fraction, establishing side chain reductive dechlorination as an actual microsomal metabolic pathway.

Evaluation of actual metabolites and logically structured putative metabolites (Fig. 1) in vivo indicated that only 4-DM-PEN, the major plasma metabolite in rodents and patients (1, 2), is on the activation pathway for penclomedine and suggests the potential importance of this metabolite as a reasonable substitute for penclomedine as a clinical agent in the event that the neurotoxicity of penclomedine reduces its clinical utility, particularly because of the demonstration of the absence of cerebellar damage by 4-DM-PEN in a rat model in contrast to penclomedine (7). It is also of potential importance that 4-DM-PEN demonstrates antitumor activity against an intracerebrally implanted tumor xenograft, which suggests that it may be useful for treatment of brain tumors, whereas the utility of penclomedine for such treatment or for treatment of breast or other tumors may be compromised because of the possibility that therapeutically effective doses of penclomedine may cause lasting cerebellar damage (7).

α,α-Didechloro-PEN, which appeared to be a reasonable candidate for an activated metabolite based on its activity in vitro (Table 1), failed to demonstrate any activity in vivo against the penclomedine- and 4-DM-PEN-responsive MX-1 human breast tumor xenograft. Studies are continuing in an attempt to identify the ultimate active metabolite of penclomedine while Phase I clinical trials are in progress.

References
4-Demethylpenclomedine, an Antitumor-active, Potentially Nonneurotoxic Metabolite of Penclomedine
