THE IN VITRO METABOLISM OF PENCLOMEDINE IN MOUSE, RAT, AND HUMAN SYSTEMS

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ABSTRACT:

Penclomedine is a multi-chlorinated α-picoline derivative that has demonstrated activity in several murine breast cancer models and is currently in clinical testing for use against solid tumors. This study evaluates the metabolism of penclomedine in several in vitro hepatic models, including microsomes, fresh liver slices, and the isolated perfused rat liver (IPRL). Both human and mouse liver slices as well as human and mouse liver microsomes under aerobic conditions resulted in limited metabolism of penclomedine to several oxidized metabolites, including penclomic acid, 4-demethylpenclomedine, and 4-demethylpenclomedine. Microsomes under anaerobic conditions vigorously produced mainly reduced metabolites, primarily penclomedine dimers. This is in contrast to in vivo data, which showed rapid metabolism of penclomedine to primarily 4-demethylpenclomedine. The IPRL preparation, however, metabolized 50 μM penclomedine 90% within 90 min, producing primarily 4-demethylpenclomedine and penclomic acid. These were formed in roughly equimolar amounts and did not undergo significant further metabolism over 4 hr. Numerous highly polar biliary metabolites were also found. The IPRL preparation thus seems to most accurately reflect the in vivo situation.

Penclomedine (fig. 1), a multi-chlorinated derivative of α-picoline, is a potential new anti-tumor agent that is currently in phase I clinical trials ([1], [2], [3]). Preclinical activity was evaluated using several murine tumor models in vivo, and remarkable activity was found against several breast tumors including both murine tumors and human xenografts, although comparatively less activity was found against non-breast tumor models ([4], [5]). Intravenously administered penclomedine also had activity against intracerebrally implanted MX-1 xenografts, suggesting that it penetrates the blood-brain barrier ([6], [7]). The compound was approximately equally effective against murine tumors whether given orally or intravenously; however, pharmacodynamic data showed penclomedine to have an oral bioavailability of less than 2% ([8]). This, combined with the fact that penclomedine also demonstrates little activity in cell culture, suggests that penclomedine acts as a pro-drug. Previous work in this laboratory has shown that penclomedine is extensively metabolized by rats, mice, and man in vivo ([9], [10]). This is in contrast to earlier work in vitro ([8]), as well as preliminary work from this laboratory ([9], [10]), which showed significant penclomedine metabolism only in microsomes incubated under anaerobic conditions. To explain this difference, this laboratory has examined several in vitro hepatic models of penclomedine metabolism of varying approximations to the in vivo condition, including microsomes, fresh liver slices, and the isolated perfused rat liver (IPRL). 1

Materials and Methods

Human liver samples, medically unsuitable for transplantation, were acquired through the auspices of the Washington Regional Transplant Consortium (Washington, D.C.). Microsomes were prepared in this laboratory and stored at −70°C until use ([11]). Livers came from both male and female donors and were evaluated for cytochrome P450 activity in this laboratory (Ludden et al., 1995). Microsomes were prepared in this laboratory and stored at −70°C until use ([12]). CD2F1 male mice were obtained from Harlan Sprague-Dawley (Frederick, MD). They were acclimated for 7 days and were kept under standard rodent conditions and fed rodent chow and water ad libitum. Mouse livers were removed, and microsomes were prepared in this laboratory and stored at −70°C until use (Ludden et al., 1995). Freshly isolated human, mouse, and rat liver slices were procured from In Vitro Technologies (Baltimore, MD) (human liver tissue unsuitable for transplantation was obtained through the IIAM, Exton, PA). These were evaluated for viability and metabolic activity by In Vitro Technologies. Fresh human and mouse liver slices were used immediately upon receipt.

Penclomedine and 14C-labeled penclomedine (17.6 mCi/mmol, labeled as in fig. 1) were obtained from the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). 4-Demethylpen-

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1 Abbreviation used is: IPRL, isolated perfused rat liver.

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Fig. 1. Structure of penclomedine.
clomedine (IV), penicillic acid (II), and 4-demethylpenicillic acid (I) were synthesized as described below; dechloropenclomedine (V), dechloropenclomedine (VIII), 4-demethyldechloropenclomedine (III), 4,6-didemethylpenclomedine, and the unsaturated penclomedine dimer (VI, VII) were synthesized and generously provided by Dr. Robert Struck, Southern Research Institute, Birmingham, AL (Waud et al., 1997). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP⁺, and mollusk glucuronidase were obtained from Sigma. All other reagents and solvents were obtained from standard sources and were of the highest grade commercially available.

Metabolite Syntheses. Penclomedine was formed by heating a 10 mM solution of penclomedine in 50% aqueous acetonitrile at 80°C for 48 hr. The acetonitrile was evaporated, the solution was alkalinized with sodium bicarbonate, extracted with diethyl ether, and the aqueous phase was neutralized to crystallize the product. Product identification was confirmed by mass spectrometry. Product yield was not determined.

4-Demethylclomedine was formed by heating a 200 mM solution of penclomedine in anhydrous dimethyl sulfoxide at 150°C for 90 min. The principal products were 4-demethylpenclomedine and 4-demethylpenclomedine acid. The dimethyl sulfoxide was evaporated under a vacuum, the residue was dissolved in chloroform, and 4-demethylpenclomedine acid was extracted into water. The solvent was evaporated, and 4-demethylclomedine was purified by precipitation from methanol/water. Product identification was confirmed by mass spectrometry and NMR. Product yield was not determined.

Microsome Assays. Microsomes were diluted to 1 mg of protein/ml with 0.1 M Tris buffer, pH 7.8. An NADPH generating system consisting of 10 mM glucose-6-phosphate, 1 mM NADP⁺, and 1.2 units/ml glucose-6-phosphate dehydrogenase (final concentrations) was provided. [14C]Penclomedine was added as a 10% ethanol or polyethylene glycol 400 solution (final concentration of cosolvents, 0.2%). The final concentration of penclomedine was 50 μM, and the final incubation volume was 5 ml. Incubations were maintained at 37°C in a shaking water bath. Anaerobic incubations were performed under a blanket of humidified nitrogen added directly to the gauze-stoppered flask with interruptions for sample removal. Controls included boiled microsomes and incubations without the NADPH generating system. At 0, 10, 20, and 40 min, 1-ml aliquots of incubation mixture were withdrawn and added to 3 ml of ethyl acetate with 200 μl of 0.7 M ammonium phosphate buffer, pH 2.7. This mixture was vortexed and centrifuged, and the organic layer was drawn off. 50 μl of dimethyl sulfoxide was added to the organic layer to prevent complete evaporation, and the ethyl acetate was evaporated under nitrogen to approximately 100 μl. 50 μl of acetonitrile was added to this residue, and the resulting solution was analyzed by HPLC. The aqueous phase was washed with 5 ml of ethanol, centrifuged, and the ethanol-insoluble precipitate was washed again with 5 ml of ethanol. After centrifugation, the pellet was disrupted with 0.5 ml of 6 M guanidinium chloride, added to 10 ml of 3a70B scintillation cocktail (Research Products International Corp., Mount Prospect, IL), and counted for radioactivity using a Packard 2500 TR liquid scintillation analyzer (Packard Instrument Co., Downers Grove, IL).

GC/EI/MS Metabolite Identification. Metabolites were identified by comparing the mass spectra of the incubation extracts with those of synthetic standards. Mass spectra were obtained on a Hewlett-Packard gas chromatograph equipped with a model 5971 mass selective detector (Hewlett Packard Co., Palo Alto, CA). The column used was an Alltech Adsorbosphere HS C18 5 μ 3.0 m × 3.18 mm column (Alltech Associates, Deerfield, IL). The system used gradient elution consisting of 100% 10 mM ammonium phosphate buffer, pH 2.7, progressing to 100% acetonitrile over 25 min at 1 ml/min. Detection was by means of ultraviolet absorbance at 240 nm as well as by detection of radioactivity using a Radiomatic Flo-One β A140 equipped with a 500-μl liquid cell and using Flo-Scint VI scintillation cocktail at a 2:1 ratio (Packard Instrument Co., Downers Grove, IL).

Results

When human microsomes (fig. 2A) or liver slices (fig. 3A) were incubated with [14C]penclomedine under aerobic or anaerobic conditions, at least seven radioactive components could be separated by HPLC. Preliminary evidence as to the identity of this penclomedine-derived radioactivity was obtained by comparison of the retention times and ultraviolet spectra of this material with those of synthetic penclomedine analogues thought to be potential metabolites. Identification was confirmed by comparing GC/EI/MS analysis of the corresponding metabolites and analogues. Many compounds could be analyzed by GC/EI/MS as isolated; others required silylation with N,O-bis(trimethylsilyl)trifluoroacetamide (Supelco Inc., Bellefonte, PA) in acetonitrile at 60°C for 20 min and injected without further processing.

Liver Slice Assays. Human liver slices (7 × 0.3 mm) were incubated in 1 ml of Krebs-Henseleit buffer, pH 7.4, with 2.25% bovine serum albumin in 24-well cluster plates, two slices per well. [14C]Penclomedine was added as above to a final concentration of 50 μM, and the slices were incubated on a rocking platform at 37°C under an atmosphere of humidified air with 5% CO₂. Anaerobic incubations were blanketeted with humidified nitrogen introduced through a hole bored in the cover of the plate with interruptions for sample removal. At the end of varying intervals up to 8 hr, the slices and medium were collected. The medium was processed in the same manner as the microsome suspensions; the slices were homogenized in 0.5 ml of Krebs-Henseleit buffer with 2.25% bovine serum albumin and were then processed in the same manner as the microsome suspensions. Mouse liver slices (6 × 0.3 mm) were incubated and processed identically to human liver slices, except three slices were used per well as opposed to two.

IPRL Incubations. Male Sprague-Dawley rats (250–300 g, Charles River, Wilmington, MA) housed at Walter Reed Army Institute of Medicine and were kept under standard rodent conditions and fed rodent chow and water ad

lubium. They were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally), and livers were isolated using standard techniques with modifications (Leo et al., 1996). Each liver was placed on a glass platform inside a thermostatically controlled (37°C) cabinet and was perfused with 100 ml of a modified Krebs-Henseleit buffer solution containing 20% washed sheep red blood cells, 1% bovine serum albumin, and 0.1% glucose via a constant flow recirculating system at a rate of 1.0 ml/g liver weight/min. Sodium taurocholate (40 μmol/hr) was continuously infused into the perfusate reservoir to simulate enterohepatic bile acid cycling and to normalize the composition of bile (Wolkoff et al., 1987). The perfusate was oxygenated with a mixture of O₂/CO₂ (95%/5%) by means of diffusion of the gas through a silastic membrane. [14C]Penclomedine was added as a bolus to the perfusate from a 5 mM stock solution containing 2% bovine serum albumin, 25% polyethylene glycol 400, and 1% ethanol (final penclomedine concentration 50 μM). One-ml perfuse samples were taken from the reservoir at times 0, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 210, and 240 min postdose. Fresh perfuse at 37°C was added to the reservoir to replace the volume of samples removed. Bile was collected at hourly intervals; volume was estimated by weight. Liver viability was assessed by normal visual appearance, steady oxygen consumption, sustained bile production, constant perfusion portal pressure, and steady perfusate pH. Perfuse “plasma” was separated from red cells by centrifugation; plasma, red cells, bile, and liver samples were frozen at −80°C until assay. Plasma and red cells were processed similarly to microsomes; livers were processed similarly to liver slices; bile samples were diluted with an equal volume of HPLC mobile phase and chromatographed without further processing.

HPLC Assays. The HPLC system consisted of a Hewlett-Packard Series II 1090 liquid chromatograph with diode array detector (Hewlett Packard Co., Palo Alto, CA). The column used was an Alltech Adsorbosphere HS C18 5 μ 250 × 4.6-mm column (Alltech Associates, Deerfield, IL). The system used gradient elution consisting of 100% 10 mM ammonium phosphate buffer, pH 2.7, progressing to 100% acetonitrile over 25 min at 1 ml/min. Detection was by means of ultraviolet absorbance at 240 nm as well as by detection of radioactivity using a Radiomatic Flo-One β A140 equipped with a 500-μl liquid cell and using Flo-Scint VI scintillation cocktail at a 2:1 ratio (Packard Instrument Co., Downers Grove, IL).

GC/EI/MS Metabolite Identification. Metabolites were identified by comparing the mass spectra of the incubation extracts with those of synthetic standards. Mass spectra were obtained on a Hewlett Packard 5890 series II gas chromatograph equipped with a model 5971 mass selective detector (Hewlett Packard Co., Palo Alto, CA). The compounds were separated in a 20 m × 0.25-mm i.d. DB-5 fused silica capillary column with a 0.4-micron film thickness (Alltech Associates, Deerfield, IL). Helium was used as the carrier gas at a flow rate of 0.6 ml/min, and the injector and transfer lines were 200 and 270°C, respectively. The column temperature was held at 150°C for 4 min after sample injection and then linearly programmed to 290°C at a rate of 10°C/min.

Sample Preparation for GC/EI/MS. Media from the liver tissue incubations were extracted with ethylacetate, and the organic extract was evaporated with a stream of dry nitrogen. Samples were either reconstituted in ethyl acetate and injected directly (2 μl) into the GC or reacted with 50% N,O-bis(trimethylsilyl)trifluoroacetamide (Supelco Inc., Bellefonte, PA) in acetonitrile at 60°C for 20 min and injected without further processing.

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dant metabolite in the previous in vivo studies (Hartman et al. 1996; O’Reilly et al., 1996). Mass spectral confirmation of the identity of this compound is shown in fig. 4E. The silylated derivative produced a molecular ion at m/z 381 containing five chlorine atoms. Major fragmentation of this compound occurred through loss of a methyl or chlorine radical producing fragment ions of m/z 366 and m/z 346, respectively. Peak V could be identified without silylation and corresponded to mono-dechlorinated penclomedine (fig. 4F). The molecular ion cluster observed contained four chlorine atoms producing a parent ion at m/z 289 if one assumes a fragmentation ion (loss of H radical) at m/z 288. Deconvolution of the isotopic content in this region of the mass spectrum using a procedure previously described (Anderson et al., 1987) produced an isotopic cluster that was consistent with a 1:1 mole ratio of the ion species M⁺:(M−1)⁺ for a compound containing four chlorine atoms. Compounds VI and VII were observed when liver microsomes were incubated anaerobically with penclomedine (fig. 2, A2 and B2). These two compounds were well separated on the GC, and electron impact mass spectral identification determined their structure as dimers of didechloropenclomedine (fig. 4G), most likely cis and trans isomers. A molecular ion was observed for both compounds at m/z 506 consistent with the presence of six chlorine atoms. The most prominent ion in the spectra at m/z 471 arose through loss of a chlorine radical from the parent ion. No monodechloropenclomedine dimer as reported by Reid (Reid et al., 1992) was observed in the GC/MS analysis of liver incubation extracts; however, it is possible that this compound is converted to didechloropenclomedine dimers in the GC injector port. One additional compound (VIII) not observed in the radiochromatograms of the media extracts from the penclomedine liver incubations was detected during the GC/MS analysis and identified by electron mass spectra as didechloropenclomedine (fig. 4H). The molecular ion cluster contained three chlorine atoms and a molecular ion at m/z 255. Loss of a chlorine radical produced the ion fragment at m/z 220.

When human microsomes were incubated with 50 μM [14C]penclomedine under aerobic conditions, approximately 12% of the total radioactivity could be accounted for by peaks I through V in 20 min (fig. 2A1). Under anaerobic conditions, however, the rate of reaction was extremely rapid, with a 50 μM starting concentration of penclomedine being nearly totally eliminated in 20 min (fig. 2A2). Under these conditions, the major metabolites were the dimer metabolites VI and VII, as well as the dechlorinated metabolite V. Microsomes obtained from several individuals metabolized penclomedine to different extents (fig. 5A). Most human microsome data were obtained with microsomes from liver #2. Similar experiments performed using mouse microsomes (fig. 2, B1 and B2) produced results nearly identical both qualitatively and quantitatively to those from human liver #2 (fig. 2, A1 and A2).

Metabolism in human liver slices was measured primarily in terms of the release of metabolites into the culture medium. Under aerobic conditions, metabolism by human liver slices (fig. 3A1) was qualitatively very similar to that seen in human liver microsomes (fig. 2A1). The results obtained from slices incubated under anaerobic conditions (fig. 3A2) more closely resembled that seen in aerobic slices (fig. 3A1) than that seen in anaerobic microsomes (fig. 2A2). There was a slight shift toward less polar metabolites in the anaerobic incubations; however, there were none of the major dimer peaks that dominated the anaerobic microsomal incubations. When the slices themselves were extracted rather than the medium, small amounts of these dimers were detected in both aerobic and anaerobic incubations (data not shown). Although there were some differences in the amounts of individual metabolites among different liver donors, the amount of total metabolism was relatively constant at approximately 12% both aerobically and anerobically.
and anaerobically at 8 hr. The results from mouse liver slices were very similar in both rate and amount of metabolite to those seen from human liver slices (fig. 3B), as were those from rat liver slices (data not shown).

When 14C-labeled penclomedine was incubated with either microsomes or liver slices, a small but reproducible amount of radioactivity was bound to tissue macromolecules and was not removable with ethanol washing (fig. 5B). This binding was not observed when the NADPH generating system was omitted or when heat-denatured microsomes were used and corresponded with the amount of total metabolites produced when microsomes from different donors were compared (fig. 5A). The bound fraction did not account for more than 2% of the total radioactivity added. When washed protein samples demonstrating this binding were hydrolyzed in 6 N HCl, all radioactivity was recovered as 2,4-didemethylpenclomic acid.

Penclomedine was rapidly metabolized to primarily penclomic acid (II) and 4-demethylpenclomedine (IV) in the IPRL system. These compounds were cleared very slowly from the perfusate, with negligible disappearance noted for either metabolite observed over the 4-hr perfusion, though small amounts of 4-demethylpenclomic acid (I), a potential metabolic product of both penclomic acid and 4-demethylpenclomedine, could be detected at later time points (figs. 5 and 6). The homogenized liver extract from these preparations generally resembled the perfusate extracts; however, traces of other metabolites could also be discerned, including possibly one of the dimers (fig. 7B). Analysis of the bile from this preparation revealed many poorly resolved radioactive peaks, essentially all of which had a polarity equal to or greater than that of penclomic acid (fig. 7C). Biliary metabolites accounted for approximately 12 percent of the total radioactivity, most of which was present in the 1- and 2-hr samples. The identity of these metabolites is not known at this time; however, several of the peaks noted in fig. 7C seemed to shift to later retention times upon incubation with mollusk glucuronidase (H-2, Sigma #G0876, which also contains sulfatase activity), suggesting the presence of glucuronide or sulfate conjugates.

**Discussion**

From previous research, it seems that although penclomedine therapy is effective in some in vivo breast cancer models, it is probably a metabolite of penclomedine which is responsible for the activity.

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**(A)** Penclomedine, GC Rₜ 16.1 min; (B) 4-demethylpenclomedine, silyl derivative, GC Rₜ 17.4 min; (C) penclomic acid, silyl derivative, GC Rₜ 15.6 min; (D) 4-demethyl-dechloropenclomedine, silyl derivative, GC Rₜ 15.9 min; (E) 4-demethylpenclomedine, silyl derivative, GC Rₜ 17.9 min; (F) dechloropenclomedine, GC Rₜ 14.4 min; (G) didechloropenclomedine dimer, GC Rₜ 27.7 and 28.5 min; (H) didechloropenclomedine, GC Rₜ 12.5 min.

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**Fig. 4.** Mass spectral analysis of penclomedine and penclomedine metabolites.
4-Demethylpenclomedine has been shown to have antitumor activity in both *in vivo* and *in vitro* systems (Waud *et al.*, 1997) and can be shown to reach high levels in both human and animal subjects (Hartman *et al.*, 1996). It is therefore encouraging that penclomedine is metabolized to similar metabolites and to a similar extent by both human and murine *in vitro* preparations, albeit that the extent of metabolism seen in liver slice and microsome preparations is much smaller than that predicted from *in vivo* measurements.

Penclomedine is remarkable in that it shows considerable microsomal metabolism under anaerobic conditions. *In vitro* metabolism under anaerobic conditions can be demonstrated for a number of drugs and other substances (Ahr *et al.*, 1980, 1982; Workman and Walton, 1990); however, it is generally slow and does not account for much metabolism *in vivo*. The *in vitro* anaerobic metabolism of penclomedine is very energetic compared with its aerobic metabolism, however, and given that the oxygen tension of the liver is only about 35% that of other tissues (Jungermann and Katz, 1982), it is reasonable to propose that some anaerobic metabolism may be present under physiological conditions.

There are many mechanisms for anaerobic metabolism, not all of which involve cytochrome P-450 (fig. 8). The most likely mechanism is probably the one proposed by Reid *et al.* (1992), which involves the binding of penclomedine to the oxygen site of cytochrome P-450 and thus receiving the electrons that would have gone to reduce oxygen. After receiving the first electron, a chloride ion would be released with formation of free radical, which could then either receive a second electron to form a stable species or else diffuse away to abstract a second electron elsewhere. This is probably the mechanism by which metabolites V and VIII are formed, as well as the dimers VI and VII. In dimer formation, presumably one penclomedine free radical either attacks a penclomedine molecule with loss of a chloride free radical or else coalesces with another penclomedine free radical, resulting in the unsaturated penclomedine dimer. This compound would then lose two chlorines to form metabolites VI and VII. Dimers would only be predicted to form when excess penclomedine is present, such as in a microsome study. Although the formation of reduced metabolites under anaerobic conditions is rapid and extensive, it is unlikely that these are responsible for the activity of penclomedine because none of the anaerobic metabolites (V, VI, VII, and VIII in this article) showed any activity in *in vivo* systems (Waud *et al.*, 1997). The only metabolite that showed activity *in vivo* was 4-demethylpenclomedine.
Under aerobic conditions, both human and murine liver slice studies correlate with the microsome data. One does not see the great increase in penclomedine metabolism in liver slices incubated anaerobically that one sees in microsomes, though. The reason for this is unclear; however, presumably because the liver slice assay may more accurately model the whole animal, these data may be the more physiologically applicable. It is interesting to note that small but reproducible amounts of the dimer metabolites were formed in both aerobic and anaerobic liver slice studies, as well as in the IPRL liver extracts, suggesting that these metabolites may indeed be formed in the intact animal.

It is possible that cytochrome P-450 may not be required for some of the metabolic steps implied by our data. The production of penclonic acid from penclomedine is not actually an oxidative process, as addition of oxygen is driven by loss of electronegative chlorine, and therefore no net change in oxidation state occurs. This reaction can occur spontaneously over several days in solutions containing water; however, it occurs much more quickly in the presence of microsomes and an NADPH generating system. At physiological pH, 4-demethylenclomedine is converted to 4-demethylpenclomic acid within minutes in the absence of enzyme, although the presence of plasma protein greatly retards this reaction.

Previous in vivo metabolism studies report a much higher degree of penclomedine metabolism than the results obtained in microsome or liver slice systems in this investigation (Hartman et al., 1996; O’Reilly et al., 1996), suggesting that a major portion of penclomedine metabolism is extrahepatic. The IPRL studies were performed to further investigate the role of hepatic metabolism. These studies show rapid, primarily oxidative metabolism that substantially resembles that seen in the whole animal, suggesting that penclomedine is probably metabolized in vivo primarily by the liver. It is possible that the reduced rate of metabolism noted for microsome and liver slice systems may be because of an activated metabolite of penclomedine binding to and inactivating cytochrome P450. This effect may not be noticeable in the IPRL and living systems owing to a number of effects, such as the greater availability of metabolic enzymes or glutathione or the more effective oxygenation of the IPRL system. More advanced enzyme studies need to be performed to evaluate this. Extrahepatic metabolism may occur; however, the extent of metabolism observed in the IPRL preparations seems sufficient to account for the degree of metabolism seen in vivo.

The mechanism of action of penclomedine is unclear; however, it has been found that those cell lines resistant to alkylating agents are cross-resistant to penclomedine (Harrison et al., 1991), suggesting that penclomedine chemotherapy includes alkylation. In this investigation, some metabolite of penclomedine has been shown to bind irreversibly with tissue macromolecules. This concurs with results seen in other laboratories (Benvenuto et al., 1995) and may be related to the persistent plasma radioactivity seen in in vivo studies (Hartman et al., 1996; O’Reilly et al.,

![Diagram of proposed in vitro metabolic scheme for metabolites elucidated in this study.](image)
The nature of this metabolite has not been established; however, upon acid hydrolysis of these macromolecules, 2,4-dimethylenepicolonic acid can be detected in the hydrolysate. Penclomedine, penclomonic acid, 4-demethylpenclomedine, and 4-demethylpenclomonic acid are all converted to 2,4-dimethylenepicolonic acid by acid hydrolysis; therefore, the identity of the actual binding species is ambiguous. However, the fact that the bound radioactivity is released by acid suggests that an acylation is involved. Mitotane (Cai et al., 1995) and chloramphenicol (Pohl et al., 1978) covalent binding to macromolecules has been shown to be the result of acylation, apparently owing to an acyl chloride intermediate. As can be seen from fig. 8, acyl chlorides may be involved in the formation of both penclomonic acid and 4-demethylpenclomonic acid. Trapping agents might be useful in clarifying the mechanism of this binding. It is not known what significance if any this has to the mechanism of action of penclomedine; however, considering the high plasma levels of 4-demethylpenclomedine (Hartman et al., 1996; O’Reilly et al., 1996) and the rapid formation of 4-demethylpenclomonic acid from 4-demethylpenclomedine, it is reasonable to propose that 4-demethylpenclomedine may be at least the immediate precursor of the active species.

Although in vitro systems are useful experimental adjuncts in terms of simplicity and in terms of limiting the use of experimental animals, in this case the simpler in vitro systems gave a very poor prediction of the in vivo metabolic fate of penclomedine. The IPRL system provided a much better model of the in vivo situation; however, this is a technically demanding system that is difficult to use in cases where a large number of compounds need to be evaluated and is especially difficult to implement in human systems. For this investigation it seems that an ideal in vitro system remains to be discovered and underscores the necessity of evaluating in vitro predictions in living systems before these results can be relied upon.

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