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ORIGINAL ARTICLE

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Acyl derivatives of demethylpenclomedine, an antitumor-active, non-neurotoxic metabolite of penclomedine

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Abstract *Purpose*: The purpose of this investigation was to compare the antitumor activities of a series of acyl derivatives of 4-demethylpenclomedine (DM-PEN), the major plasma metabolite of penclomedine (PEN) observed to be an active antitumor agent in vivo and non-neurotoxic in a rat model with that of DM-PEN. *Methods*: Acyl derivatives were prepared from DM-PEN and evaluated in vivo against human MX-1 breast tumor xenografts implanted subcutaneously (s.c.) or intracerebrally (i.c.). Several derivatives were also evaluated against other human tumor xenografts and murine P388 leukemia cell lines. *Results*: Several of the acyl derivatives were found to be superior to DM-PEN against MX-1, human ZR-75-1 breast tumor, human U251 CNS tumor and the P388 leukemia parent cell line and lines resistant to cyclophosphamide and carmustine. 4-Demethyl-4-methoxyacetylpenclomedine showed inferior activity to current clinical brain tumor drugs against a glioma cell line, superior activity to temozolomide and procarbazine against the derived mismatch repair-deficient cell line, and superior activity to cyclophosphamide and carmustine but inferior activity to temozolomide against two ependymoma cell lines, all of which were implanted s.c. *Conclusion*: Proposed mechanisms of activation and action of DM-PEN and the acyl derivatives support the potential clinical superiority of the acyl derivatives.

Keywords Penclomedine · 4-Demethylpenclomedine · Antitumor evaluation in vivo · Human tumor xenografts

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Introduction

Penclomedine (PEN) was evaluated in phase I clinical trials at Johns Hopkins University Oncology Center, the University of Wisconsin Comprehensive Cancer Center and the Western General Hospital in Edinburgh [1, 2, 3, 4, 5] for possible use in the treatment of breast cancer, based on activity against human breast tumor xenografts and experimental mammary tumor models [6, 7], and in the treatment of brain tumors, based on its activity against tumor xenografts in the brain [7]. In all of these trials, dose-limiting neurotoxicity was observed after both intravenous and oral administration and was related to peak plasma levels of PEN [4]. 4-Demethylpenclomedine (DM-PEN) has been identified as the major plasma metabolite in patients and rodents [1, 2], and neuroanatomic studies of PEN and DM-PEN in rats have revealed cerebellar damage only in PEN-treated animals [8].

These observations led to the synthesis and antitumor evaluation of DM-PEN, and the notable activity of DM-PEN against human MX-1 breast tumor xenografts implanted both subcutaneously (s.c.) and intracerebrally (i.c.) led to discussions with the National Cancer Institute (NCI) and commercial clients about possible clinical development [9]. During the course of these discussions, a series of acyl derivatives of DM-PEN were prepared and evaluated against MX-1 tumor xenografts, several other human tumor xenografts and murine P388 leukemia. The results of the synthesis and antitumor evaluation of these novel agents are described in this report and support the preferential clinical development of a representative acyl

derivative of DM-PEN instead of DM-PEN, and NCI funding has been awarded for preclinical toxicology studies to support an Investigational New Drug (IND) application and for subsequent clinical evaluation against brain tumors in a phase I/phase II study.

Materials and methods

DM-PEN was prepared by a modification of the previously reported method [1] and was obtained as a white crystalline solid which was characterized by mass spectral, NMR and elemental analysis.

Synthesis of acyl derivatives of DM-PEN

In a typical preparation, DM-PEN (500 mg) in methylene chloride was cooled in an ice bath and treated with one equivalent of triethylamine followed by dropwise addition of one equivalent of an acyl halide in methylene chloride. The reaction solution was stirred at room temperature 4 h after the addition and evaporated in vacuo. The residue was triturated with acetone and filtered to remove triethylamine hydrochloride. The filtrate was concentrated in vacuo and separated by preparative thin-layer chromatography (TLC) on silica gel in hexane/methylene chloride (1:1). The major UV-visible band was collected and eluted with acetone. Filtration and evaporation of the filtrate yielded a TLC-homogeneous product as a crystalline solid or oil, which was characterized by fast atom bombardment mass spectrometry.

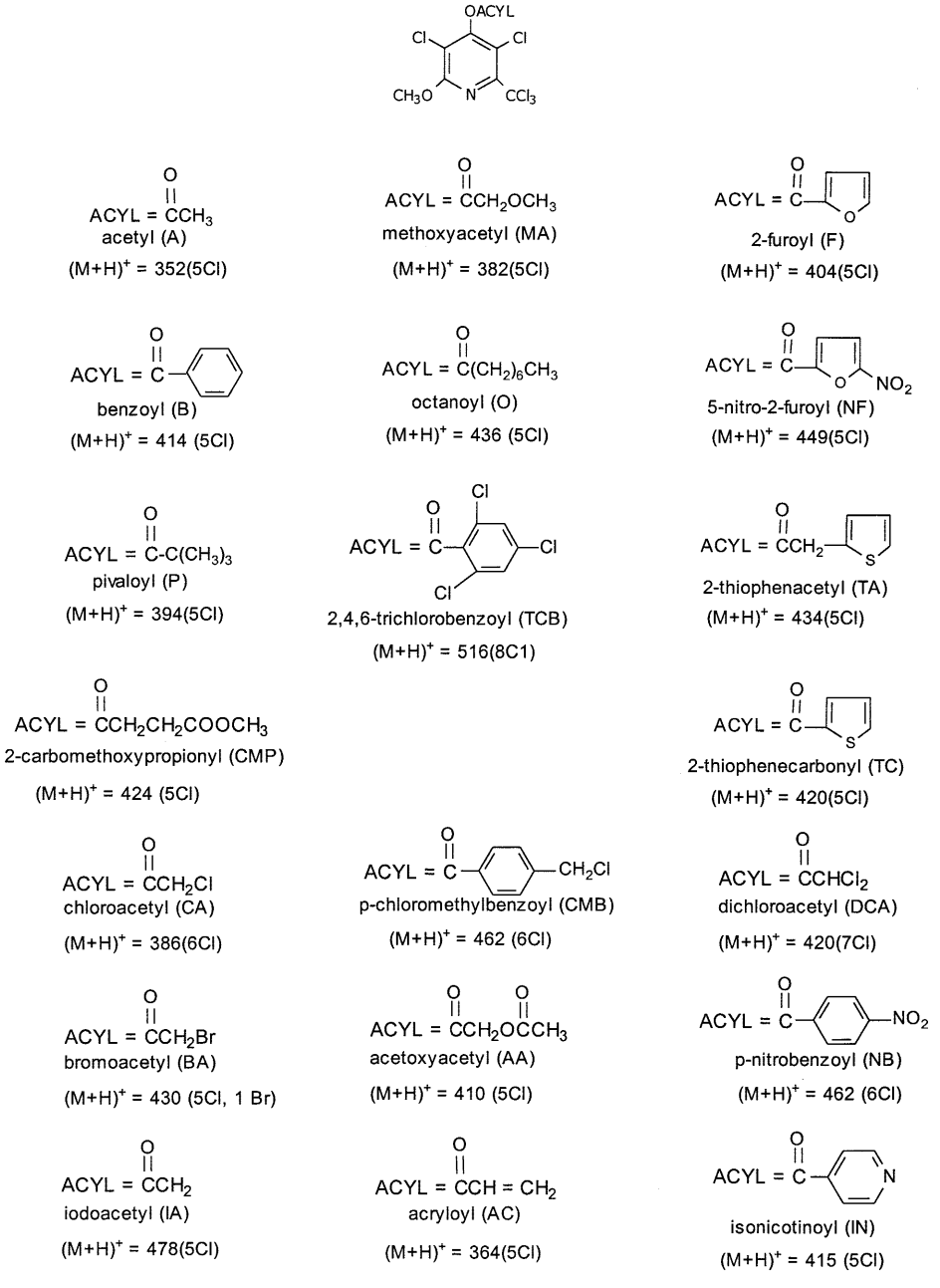


Fig. 1 Structures of 20 acyl derivatives of DM-PEN

Antitumor evaluation in vivo

Antitumor evaluations were conducted as described previously [6, 7]. Athymic NCr-*nu*/*nu* and $CD2F_1$ 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 and ZR-75-1 breast tumors, human U251 CNS tumor, and murine P388 leukemia cells (parent line) were obtained from the NCI Tumor Repository (Frederick, Md.). P388 lines resistant to cyclophosphamide and carmustine were derived at the Southern Research Institute [10]. Human D-245 MG glioma, D-245 MG PR glioma and D-528 EP and D-612 EP ependymomas were established and characterized by procedures described previously [11]. For intraperitoneal (i.p.) injection into mice, DM-PEN and the acyl derivatives were prepared as a suspension in aqueous hydroxypropyl cellulose. The same vehicle was used for oral treatment. For s.c. implants, tumor fragments (30–40 mg) from in vivo passage were implanted into the axillary region of the mice.

Treatment of groups of five mice each was initiated when the tumors reached approximately 300 mg in mass and was continued for 5 days for all treatment groups. Each tumor was measured by caliper in two dimensions twice weekly and converted to tumor

Table 1 Summary of the in vivo antitumor activity of acyl derivatives DM-PEN against human MX-1 mammary tumor (*A* acetyl, *AA* acetoxyacetyl, *AC* acryloyl, *B* benzoyl, *BA* bromoacetyl, *CA* chloroacetyl, *CMB* *p*-chloromethylbenzoyl, *CMP* 2-carbomethoxypropionyl, *DCA* dichloroacetyl, *F* 2-furoyl, *IA* iodoacetyl, *IN* isonicotinoyl, *MA* methoxyacetyl, *NB* *p*-nitrobenzoyl, *NF* 5-nitro-2-furoyl, *O* octanoyl, *P* pivaloyl, *TA* thiophenacetyl, *TC* thiophenecarbonyl, *TCB* 2,4,6-trichlorobenzoyl)

Derivative	Tumor site[a]	Optimal i.p. dosage ($< LD_{10}$) (mg/kg/dose)	Schedule (days)	Median percent ILS (dying mice only)[b]	T–C (days)[c]	Tumor free (survivors/total)
DM-A-PEN	s.c.	90	12–16	–	> 41.0	5/5
	s.c.	60	14–18	–	> 38.3	4/5
	s.c.	135[d,e]	13–17	–	> 38.5	5/5
	s.c.	60 (LD_{20})	15–19	–	> 37.2	3/5
	i.c.	60	1–5	+ 60	–	0/5
	i.c.	135[d,e]	1–5	+ 88	–	0/5
DM-B-PEN	s.c.	135[d,e]	13–17	–	–1.4	0/5
	s.c.	135[e]	11–15	–	> 42.7	5/5
	i.c.	135[e]	1–5	+ 84	–	0/5
	i.c.	135[d,e]	1–5	+ 12	–	0/5
DM-P-PEN	s.c.	135[d,e]	13–17	–	0.9	0/5
	s.c.	135[e]	11–15	–	> 42.7	5/5
	i.c.	135[e]	1–5	+ 64	–	0/5
	i.c.	135[d,e]	1–5	+ 20		
DM-CMP-PEN	s.c.	135[d,e]	13–17	–	> 38.5	2/5
	s.c.	135[e]	11–15	–	> 42.7	3/5
	i.c.	90[d]	1–5	+ 76	–	1/5
	i.c.	90	1–5	+ 87	–	1/5
DM-CA-PEN	s.c.	60	12–16	–	> 41.0	3/5
	s.c.	135[d,e]	12–16	–	> 41.0	5/5
	i.c.	135[d,e]	1–5	+ 84	–	2/5
	i.c.	135[e]	1–5	+ 140	–	2/5
DM-BA-PEN	s.c.	135[e]	12–16	–	> 41.0	2/5
DM-IA-PEN	s.c.	60 (LD_{40})	12–16	–	20.3	1/5
DM-MA-PEN	s.c.	135[d,e]	12–16	–	> 41.0	5/5
	s.c.	90[d]	1–5	+ 110	–	1/5
	i.c.	90	1–5	+ 126	–	1/5
DM-O-PEN	i.c.	90	1–5	+ 121	–	0/5
DM-TCB-PEN	i.c.	135	1–5	+ 92	–	1/5
DM-CMB-PEN	i.c.	[90]	1–5	+ 73	–	0/5
DM-AA-PEN	i.c.	135[d,e]	1–5	+ 115	–	2/5
	i.c.	135[e]	1–5	+ 67	–	0/5
DM-F-PEN	s.c.	[90]	12–16	–	> 41.2	5/5
	i.c.	135[e]	1–5	+ 108	–	2/5
DM-NF-PEN	s.c.	[90]	12–16	–	> 41.2	4/5
	i.c.	135[e]	1–5	+ 59	–	0/5
DM-AC-PEN	s.c.	[60]	12–16	–	30.0	1/5
	i.c.	[60]	1–5	+ 55	–	0/5
DM-TA-PEN	s.c.	[90]	12–16	–	> 41.2	3/5
	i.c.	[90]	1–5	+ 75	–	0/5
DM-TC-PEN	s.c.	[90]	12–16	–	> 41.2	4/5
	i.c.	135[e]	1–5	+ 100	–	0/5
DM-DCA-PEN	s.c.	135[e]	12–16	–	> 38.6	2/5
DM-IN-PEN	s.c.	90 (LD_{20})	12–16	–	> 38.6	3/5
DM-NB-PEN	s.c.	135[e]	12–16	–	> 38.6	5/5

[a] Athymic mice (NCr-nu) were implanted with human MX-1 mammary tumor (either i.c. with 10^6 cells or s.c. with 30–40-mg fragments)
[b] Where no value is indicated, the mice were killed when their tumors reached 4 g, so an ILS value would be meaningless
[c] The difference in the median time poststaging for tumors of the treated (T) group to double twice in mass compared to the median of the control (C) group
[d] Oral treatment (by gavage)
[e] Highest dosage tested

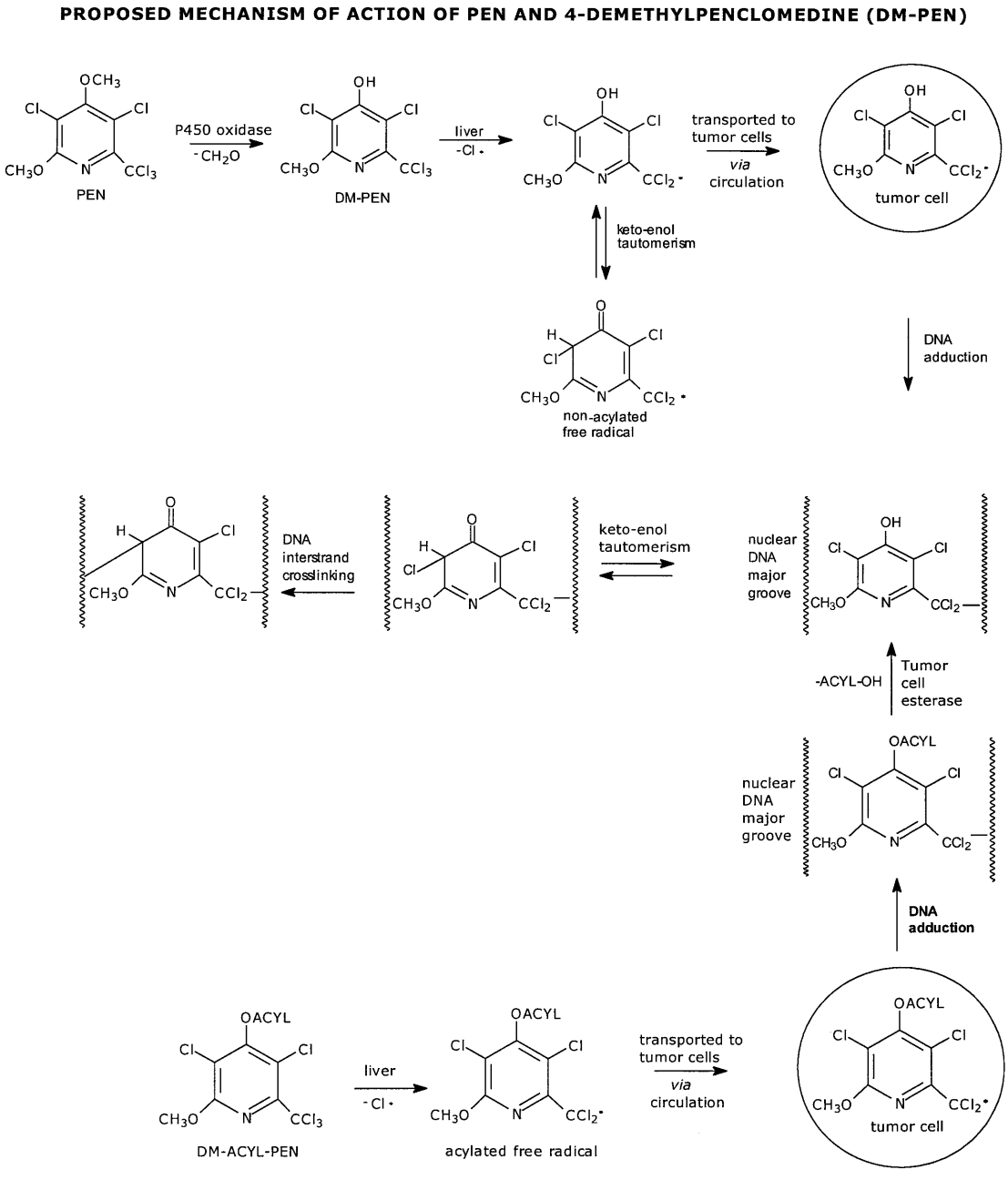
Table 2 Comparative activity of PEN, DM-PEN and acyl derivatives of DM-PEN against i.c.-implanted human MX-1 mammary tumor at optimal dosages (LD_{10} or less) and schedule (daily days 1–5) administered i.p. (*CA* chloroacetyl, *F* 2-furoyl, *MA* methoxyacetyl, *O* octanoyl, *TC* thiophenecarbonyl)

Agent	Median percent ILS[a]	Survivors/total
PEN	88	0/5
DM-PEN	60	0/5
DM-CA-PEN	140	2/5
DM-MA-PEN	126	1/5
DM-O-PEN	121	0/5
DM-F-PEN	108	2/5
DM-TC-PEN	100	0/5

[a]Based on dying mice only

mass. Antitumor activity was assessed on the basis of tumor growth delay in comparison to a vehicle-treated control (T–C, i.e. the difference in the median time poststaging for tumors of the treated (T) group to double twice in mass compared to the median of the control (C) group), tumor regressions (partial and complete), and tumor-free survivors, and experiments were terminated when the control tumors attained a mass of 1 g, which was typically 57–61 days. For i.c. implants, 0.03 ml of a MX-1 tumor brei (containing 10^6 cells) was implanted into the right hemisphere of the mice. For cross-resistance studies, 0.1 ml of either the P388 parent line or a drug-resistant line (containing 10^6 cells) was implanted i.p. into $CD2F_1$ mice. Treatment of both i.c. and i.p. implants was initiated 1 day after tumor implantation and continued for 5 days. Mice were monitored daily for survival. Antitumor activity was assessed on the basis of the percentage increase in lifespan (ILS) in comparison to a vehicle-treated control, and long-term survivors.

Scheme 1 Proposed mechanism of action of DM-PEN and on acyl derivative



Results

Figure 1 shows the structures of 20 acyl derivatives of DM-PEN prepared as described in Materials and methods accompanied by corresponding mass spectral data of the appropriate molecular ion peak.

Each acyl derivative was evaluated simultaneously with a DM-PEN control initially against MX-1 tumor implanted s.c. or i.c. with i.p. or oral treatment, and the results are shown in Table 1. A range of dosages of 135, 90 and 60 mg/kg per dose, were used typically including the maximum tolerated dose. Several of the derivatives yielded superior activity to DM-PEN, which produced two or three of five tumor-free survivors against s.c.-implanted tumor, i.e. DM-A-PEN, DM-B-PEN, DM-P-PEN, DM-CA-PEN, DM-MA-PEN, DM-F-PEN, DM-NF-PEN, DM-TC-PEN and DM-NB-PEN. DM-A-PEN was evaluated twice against s.c.-implanted tumors with i.p. treatment, confirming the results of the first experiment.

Table 2 shows the results of the most active analogs administered i.p. against i.c.-implanted MX-1 tumor. The results indicate analogs with activity superior to that of both PEN and DM-PEN and identify major candidates for possible activity against brain tumors or tumors metastatic to the brain. Notable activity (>100% ILS) was also observed for DM-AA-PEN (115% ILS) in this tumor model, which yielded two of five survivors following oral administration.

The acetyl derivative, DM-A-PEN, was compared with DM-PEN for activity against other human tumor xenografts implanted s.c. and showed superior activity against ZR-75-1 breast tumor (T–C of 14.1 days vs 0.4 days) and U251 CNS tumor (T–C of 15.7 days vs 6.9 days). DM-A-PEN was also evaluated against P388 leukemia line and lines resistant to cyclophosphamide and carmustine. Using the same dosages and schedule used for the xenograft experiments, DM-A-PEN produced 5.0 $\log_{10}$ units of cell kill vs 3.1 for DM-PEN in the parent line, 5.7 $\log_{10}$ units vs 1.1 for DM-PEN in the cyclophosphamide-resistant line, and 2.3 $\log_{10}$ units vs 0.8 for DM-PEN in the carmustine-resistant line, indicating generally superior activity for the acyl derivative.

One of the acyl derivatives, DM-MA-PEN (4-demethyl-4-methoxyacetylpenclomedine), was evaluated against several glioblastoma multiforme and ependymoma cell lines implanted s.c. in comparison with current agents used in the treatment of brain tumors. Against D-245 MG glioma, DM-MA-PEN was less active than temozolomide, cyclophosphamide, procarbazine and carmustine, but against D-245 MG PR, a mismatch repair-deficient line, it was more active than temozolomide and procarbazine but less active than cyclophosphamide and carmustine. Against D-528 ependymoma it was more active than cyclophosphamide and carmustine but less active than temozolomide (procarbazine not evaluated), and against D-612 EP, it was more active than cyclophosphamide and carmustine, equally active to procarbazine and less active than temozolomide.

As an indication of the possible absence of neurotoxicity for the acyl derivatives, DM-A-PEN was evaluated in a behavioral model of neurotoxicity in mice, and, while PEN caused tremors, DM-A-PEN and DM-PEN did not.

Discussion

A possible reason for the superior activity of the acyl derivatives in comparison to DM-PEN may be provided by consideration of proposed mechanisms of activation and action of the two agents. As shown in Scheme 1, DM-PEN can be fully activated by free radical formation in the liver for subsequent transport via the circulation to a tumor cell in which adduction to nuclear DNA can occur through the free radical moiety followed by crosslinking through the 5-chloro moiety, which can be activated via keto–enol tautomerism to produce an α-haloketo function, a recognized alkylating group. In contrast, as also shown in Scheme 1, an acyl derivative is reasonably converted to a free radical in the liver, in an analogous manner to DM-PEN, for transport to a tumor cell but can remain a partial prodrug until after DNA adduction, following which nuclear esterases could subsequently deacylate the monoadduct, allowing it to tautomerize to its α-haloketo form for completion of a DNA crosslink via alkylation. Based on previous studies comparing PEN with numerous other anticancer agents, an alkylating agent mechanism of action is similarly reasonable [6]. In addition, PEN, DM-PEN and the acyl derivatives react positively with the standard alkylating agent reagent, *p*-nitrobenzylpyridine.

It is also possible that the acyl derivatives are simply prodrug forms of DM-PEN and owe their superior antitumor activity to this possibility. Pharmacology studies to investigate this possibility are in progress.

The results of the behavioral model of neurotoxicity that revealed no neurotoxicity for DM-PEN or DM-A-PEN, consistent with a previous study in rats [8], indicate that neurotoxicity may not be dose-limiting for an appropriate acyl derivative as it is for PEN.

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