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Preclinical activity of ilmofosine against human tumor colony forming units *in vitro*

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Ilmofosine (BM 41.440, 1-hexadecylthio-2-methoxymethyl-rac-glycero-3-phosphocholine) is a synthetic alkyl lysophospholipid analog with activity against a variety of tumor models *in vitro* and *in vivo*. The i.v. form is presently undergoing early clinical investigation in phase I trials. In order to help define types of tumors that might be clinically sensitive to this agent we have studied the anti-tumor effects of ilmofosine against a variety of freshly explanted human tumor specimens using an *in vitro* soft agar cloning system. Final concentrations of 1.0–30 μg/ml were used in continuous incubations experiments. Of 348 specimens tested, 134 (39%) were evaluable for determination of tumor growth modulating activity. The most common tumor types recruited included non-small cell lung, breast, colorectal, ovarian, renal cell cancer and melanoma. A concentration-dependent increase in the frequency of inhibited tumor specimens was observed with 6/134 (4%) sensitive specimens at 1 μg/ml as compared with 113/133 (85%) sensitive specimens at 30 μg/ml ($p < 0.0000005$). We conclude that ilmofosine is active against a variety of tumors *in vitro*. Clinical phase II trials with ilmofosine including the tumor types with *in vitro* sensitivity are warranted if adequate plasma concentrations of this agent can be reached in patients.

Key words: Anti-tumor effects, BM 41.440, 1-hexadecylthio - 2 - methoxymethyl - rac - glycero - 3 - phosphocholine, human tumor cloning assay, ilmofosine.

Introduction

Alkyl lysophosphates are analogs of lysophosphocholine, a cell membrane constituent which serves as an intermediate in cellular phospholipid turnover.[1] Various members of the alkyl lysophosphate family are active anti-tumor agents in a variety of *in vitro* and *in vivo* tumor models.[2,3] Ilmofosine, a thioetherlysophospholipid, is one of the most potent agents of this group and is active against leukemia and cancer cells.[2–4] It does not possess the platelet-aggregating activity of other alkyl lysophosphates.[4] Its primary mechanism of action is the disturbance of cell membrane composition and interference with phospholipid turnover.[5,6] This may lead to significant cell membrane damage with subsequent cytocidal consequences. However, a number of additional mechanisms of action including immunomodulating, anti-metastatic and anti-invasive effects has also been proposed.[7–9] Recently, our group has reported on a phase I clinical study with ilmofosine administered over 2 h every 28 days.[10] The dose-limiting toxicity and maximal tolerated dose have not been reached for other schedules of the drug. In the present study we have utilized a human tumor cloning system to determine the anti-tumor effects of ilmofosine against a variety of freshly explanted human tumor specimens *in vitro*.

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Materials and methods

Compounds

Ilmofosine (BM 41.440, 1-hexadecylthio-2-methoxymethyl-rac-glycero-3-phosphocholine) was kindly provided by the Boehringer Mannheim

Pharmaceuticals Corporation (Rockville, MD 20850). Stock solutions of 25 mg/ml were prepared in distilled water and stored at −20°C until used. Final concentrations ranged from 0.01 to 100 ng/ml and were prepared in distilled water.

Human tumor cloning system

After obtaining informed consent in accordance with federal and institutional guidelines, tumor specimens were collected by sterile standard procedures as part of routine clinical measures. Biopsies of solid tumors were stored in McCoy's 5A medium containing 10% newborn calf serum, 10 mM HEPES, 90 U/ml penicillin and 90 μg/ml streptomycin (all Gibco, Grand Island, NY) for transport to the laboratory. Preservative-free heparin (10 U/ml, O'Neill, Johns and Feldman, St Louis, MO) was added immediately after collection of fluids to prevent coagulation. Solid specimens were minced and repeatedly passed through metal meshes with mesh widths of 40 μm (EC Apparatus, St Petersburg, FL) to obtain a single cell suspension. Effusions were centrifuged at 150 × *g* for 5–7 min and passed through 25 gauge needles to obtain single cell suspensions when necessary. All specimens were suspended in McCoy's 5A medium (Gibco) containing 5% horse serum (HS), 10% fetal calf serum (FCS) (both Hyclone, Logan, UT), 2 mM sodium pyruvate, 2 mM glutamine, 90 U/ml penicillin, 90 μg/ml streptomycin and 35 μg/ml L-serine (all Gibco).

The human tumor cloning assay (HTCA) was performed using the two-layer system described by Hamburger and Salmon with several modifications.[11] Base layers contained 0.5% agar (Difco, Detroit, MI) in a mixture of McCoy's 5A medium as described above, 0.6% soy broth (Difco) and 100 μg/ml asparagine (Gibco). Cells were plated at a density of 5×10^5/dish in 35-mm Petri dishes (Corning) in a mixture of 0.3% agar in CMRL medium 1066 (Irvine Scientific) containing 15% HS, 2% FCS, 5 mg% vitamin C (Gibco), 90 U/ml penicillin, 90 μg/ml streptomycin, 0.1 mM non-essential amino acids, 2 mM glutamine (all Gibco), 2 U/ml insulin (Iletin I®, Eli Lilly), 2 μg/ml transferrin and 4 ng/ml hydrocortisone (both Sigma). Immediately prior to plating, HEPES (Gibco, 10 mM final concentration), asparagine (100 μg/ml final concentration) and sodium pyruvate (2 mM final concentration) were added. All determinations were done in triplicate. Each experiment included a control with orthosodium vanadate (10^{-3} M, Sigma) to assure the presence of a good single-cell suspension (positive control).[12] Plates were incubated at 37°C, 5% CO_2, 100% humidity. After 14 days, colonies were counted with an inverted microscope. An experiment was considered evaluable when the water control had ≥20 colonies/plate and the positive control showed ≤30% colony formation compared with the solvent control. A decrease in tumor colony formation was considered significant if survival of colonies was ≤0.5-fold compared with the control.

Statistical analysis

Data were expressed as means and standard deviations of triplicate determinations. Percent survival was calculated by expressing the average number of tumor colony forming units from ilmofosine treated cells as a percent of the average number of tumor colony forming units from untreated controls. Statistical analyses were performed using the McNemar's test.

Results

The effects of ilmofosine on tumor colony formation were studied in a total of 358 specimens from cancer patients using continuous exposure experiments. Ten specimens were confirmed benign and did not form colonies in controls. These specimens were excluded from further analyses. As summarized in Table 1, the largest tumor subgroups accrued were non-small cell lung, breast, colorectal, ovarian, renal cell cancer and melanoma. Final concentrations of ilmofosine ranged from 1 to 30 μg/ml. While only 6/134 (4%) evaluable specimens were inhibited at 1.0 μg/ml, 113/133 (85%) specimens showed a significant decrease in colony formation at 30 μg/ml ($p < 0.0000005$)

Table 1. Tumor types studied with ilmofosine

Tumor type	No. evaluable/no. attempted (%)
Lung, non-small cell	33/62 (53)
Breast	19/51 (37)
Colorectal	14/40 (35)
Ovary	23/33 (70)
Melanoma	9/26 (35)
Kidney	12/23 (52)
Gastric	2/14 (15)
Other tumor types	22/99 (22)
Total	134/348 (39)

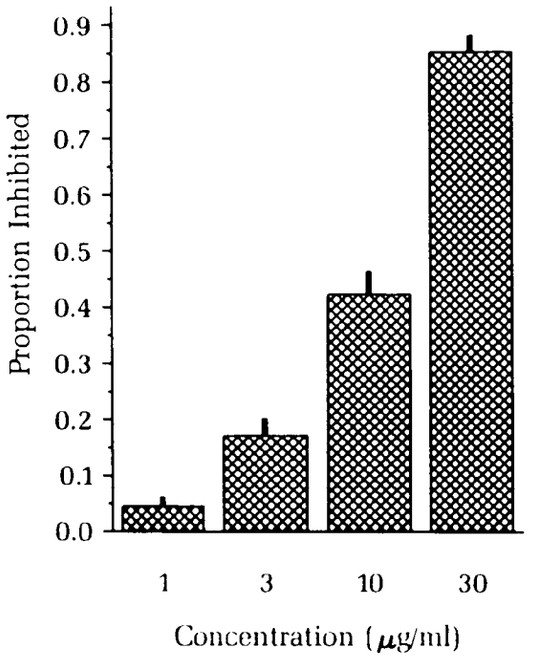

Figure 1. Concentration-dependent inhibition of human tumor colony forming units *in vitro* by ilmofosine. A statistically significant increase in the frequency of inhibited tumor specimens was observed ($p < 0.0000005$). Error bars represent 1 SD.

Table 2. Concentration-dependent inhibition of human tumor colony forming units *in vitro* by ilmofosine: subgroup analyses by tumor type

Tumor type	No. specimens with inhibition/no. specimens evaluable[a] at dose (μg/ml)				p^b
	1.0	3.0	10.0	30.0	
Lung, non-small cell	3/33	6/33	15/33	32/33	<0.0001
Ovary	0/23	2/24	7/24	21/23	<0.0001
Breast	1/19	2/19	6/19	14/19	<0.002
Colorectal	0/14	1/14	6/14	12/14	<0.002
Kidney	2/12	6/12	9/12	11/12	=0.027
Melanoma	0/9	3/9	7/9	8/9	=0.013
Other	0/24	0/24	7/24	15/23	=0.0003

[a] Colony survival ≤0.5 × lines control. [b] McNemar's test.

(Figure 1). A concentration-dependent increase in the frequency of significant growth inhibition was also notable for each tumor subgroup (Table 2).

Head-to-head comparisons of the anti-tumor activity of ilmofosine with standard anti-neoplastic agents were performed using a total of 19 different compounds. For 11 of these compounds sample sizes were large enough to permit a statistical analysis. As shown in Table 3, ilmofosine was active

Table 3. Comparison of the anti-tumor activity of ilmofosine (10.0 μg/ml; continuous exposure) and conventional anti-neoplastic agents

		Ilmofosine		
		Sensitive	Resistant	
Cisplatin	Sensitive[a]	2	2	$p^b = 0.0002$
(0.2 μg/ml)	Resistant	21	30	
VP-16	Sensitive[a]	1	0	$p = 0.001$
(3.0 μg/ml)	Resistant	12	12	
Vinblastine	Sensitive[a]	6	3	$p = 0.002$
(0.05 μg/ml)	Resistant	18	33	
5-Fluorouracil	Sensitive[a]	1	0	$p = 0.002$
(6.0 μg/ml)	Resistant	12	31	
Doxorubicin	Sensitive[a]	3	1	$p = 0.006$
(0.04 μg/ml)	Resistant	12	30	
Melhphalan	Sensitive[a]	0	1	$p = 009$
(0.1 μg/ml)	Resistant	11	15	
Mitomycin C	Sensitive[a]	3	3	$p = 0.01$
(0.1 μg/ml)	Resistant	15	23	
BCNU	Sensitive[a]	1	1	$p = 0.046$
(0.1 μg/ml)	Resistant	8	8	
Methotrexate	Sensitive[a]	2	3	$p = 0.2$
(0.3 μg/ml)	Resistant	8	10	
Bleomycin	Sensitive[a]	1	0	$p = 0.25$
(0.2 μg/ml)	Resistant	3	10	
Cyclophosphamide	Sensitive[a]	1	4	$p = 0.35$
(3.0 μg/ml)	Resistant	7	14	

[a] Colony formation ≤0.5 times control. [b] McNemar's test.

in a substantial number of tumor specimens with resistance to standard anti-neoplastic agents. Incomplete cross-resistance was observed with compounds subject to the multi-drug resistance gene mediated type of resistance but also included agents not involved in the mdr phenotype.

Discussion

Ilmofosine is a thioetherlysophospholid with anti-tumor activity in various preclinical tumor models.[3] As with other alkyl lysophospholipids, its biologic activity is believed to be the result of interference with the composition of the cell membrane as well as with cellular phospholipid turnover.[2,5]

In the present study we have investigated the effects of ilmofosine on *in vitro* tumor colony formation of freshly explanted human cancer specimens. Our results indicate that ilmofosine is active against a variety of different tumor types at concentrations of $\geq 10\ \mu g/ml$. Our data are confirming and expanding the findings of Neumann *et al.*[13] who have reported on anti-tumor activity of ilmofosine in a smaller series of tumors. These investigators have used a methylcellulose-based cloning system and have observed sensitivity of 15/30 (50%) specimens studied including non-small cell lung, small cell lung, colorectal, ovarian cancer, melanoma and soft tissue sarcoma specimens at $10\ \mu g/ml$. However, they have not performed a comparison of ilmofosine with other agents.

Among the individual tumor types we have analyzed, no specific entity could be identified with particular sensitivity or resistance towards this agent. A number of investigators, however, have reported on selective cytotoxicity of alkyl lysophospholipids on tumor cells and little effect on normal tissues.[4] The reasons for this observation are not completely understood but may include differences in membrane composition or enzyme affinities between benign and malignant cells.[14]

A comparison with the anti-tumor activity of standard anti-cancer agents demonstrated that ilmofosine remains active in a significant number of tumors resistant to standard chemotherapy. These data indicate that ilmofosine may possess additional clinical activity in patients with advanced cancer after pretreatment with conventional chemotherapy if sufficiently high plasma concentrations can be achieved.

In summary, we have demonstrated that ilmofosine is active against *in vitro* tumor colony forming units from a variety of human malignancies. Only partial cross-resistance has been observed with other chemotherapeutic agents. Based on our results and the results of Neumann *et al.*,[13] further clinical evaluation of ilmofosine is warranted if appropriate plasma concentrations can be achieved.

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