Pediatr Blood Cancer 2008;50:757–760

Synergy of Karenitecin and Mafosfamide in Pediatric Leukemia, Medulloblastoma, and Neuroblastoma Cell Lines

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Background. A major barrier to treatment of leptomeningeal disease is the lack of proven combination chemotherapy regimens for intrathecal administration. The purpose of this study was to determine the cytotoxic effects of karenitecin and mafosfamide in vitro against leukemia, medulloblastoma, and neuroblastoma cell lines. ***Procedure.*** A modified methyl tetrazolium (MTT) assay was used to determine the sensitivity of the cells to karenitecin and mafosfamide. Cells were exposed to drug for 72 hr, after which the number of surviving cells was quantitated. For drug combination experiments, cells were exposed to medium alone (controls), single drugs alone (mafosfamide only, karenitecin only) or to different concentrations of the combination of the two drugs (karenitecin + mafosfamide), for a total of 36 concentration pairs per plate. The universal response surface approach (URSA) was used to analyze the cytotoxic effects of the combination of karenitecin and mafosfamide. ***Results.*** The IC_{50}s of karenitecin and mafosfamide for the various cell lines were similar. For both drugs nearly complete inhibition of cell growth was demonstrated at higher concentrations in all cell lines. In the neuroblastoma cell lines (SK-N-DZ; SK-N-SH) and the DAOY medulloblastoma cell line, the combination of karenitecin and mafosfamide were synergistic. In the D283 medulloblastoma and both the leukemia cell lines (JM1 and Molt-4), the drug interaction was additive. Antagonism was not seen in any cell line. ***Conclusions.*** Karenitecin and mafosfamide are additive or synergistic in vitro against tumor types that disseminate to the leptomeninges. These results provide guidance for the choice of potential combination intrathecal regimens. Pediatr Blood Cancer 2008;50:757–760.

Key words: intrathecal therapy; karenitecin; mafosfamide; synergy

INTRODUCTION

Despite major advances in prevention and treatment, leptomeningeal cancer remains a difficult clinical problem. Combination chemotherapy forms the basis for most modern cancer treatment regimens. Drug combinations for systemic administration are usually selected based on considerations of known single agent activity of the drugs to be tested, non-overlapping toxicity profiles of the individual agents, and potential favorable interactions based on the drugs' mechanisms of action. One of the major barriers to treatment of leptomeningeal disease is the lack of proven combination chemotherapy regimens for intrathecal administration.

Most systemically administered anticancer agents penetrate poorly into the central nervous system (CNS) because of the blood brain barrier (BBB) [1]. The goal of intrathecal chemotherapy, which circumvents the BBB, is to maximize drug exposure in the cerebrospinal fluid (CSF) while minimizing drug toxicity. Because the volume of CSF is much smaller than that of plasma, a higher drug concentration can be achieved in CSF using a much smaller dose [2]. Intrathecal administration has been shown to be effective in decreasing the incidence of CNS relapse in patients with leukemia [1]. However, only a limited number of agents can be administered by intrathecal route. In addition, the process of conducting clinical trials of new intrathecal agents is slow, and it is critical to prioritize the agents that are to be studied.

Karenitecin belongs to the camptothecin class of topoisomerase I inhibitors, which also includes topotecan and irinotecan. It is highly lipophilic and maintains a high percentage of total drug in the lactone form, which may be clinically advantageous compared with other topoisomerase I inhibitors [3]. Karenitecin has shown in vitro activity against adult tumor cell lines including lung, prostate, breast, colon, ovarian, melanoma, and head and neck tumors [4,5], and against pediatric malignancies including medulloblastoma, neuroblastoma, and rhabdomyosarcoma [6].

Mafosfamide (ASTA Z7557) is a preactivated cyclophosphamide analog with in vitro activity equivalent or superior to that of activated cyclophosphamide [7]. Mafosfamide has demonstrated activity in vitro against leukemia and solid tumor cell lines [1]. In addition, mafosfamide has undergone phase 1 studies of intrathecal administration that demonstrate that concentrations that are cytotoxic in vitro are clinically achievable [25]. The purpose of the present study was to determine the cytotoxic and potential synergistic effects of karenitecin and mafosfamide in vitro against cell lines representative of pediatric tumors with a propensity for leptomeningeal dissemination.

MATERIALS AND METHODS

Drugs

Karenitecin was obtained from BioNumerik Pharmaceuticals (San Antonio, TX) and mafosfamide was obtained from Baxter Oncology (Halle/Westphalia, Germany, former ASTA Medica). Karenitecin was diluted with phosphate buffered saline (PBS) to an initial concentration of 0.1 mg/ml (220 μM). Mafosfamide was diluted with PBS to an initial concentration of 3.0 mg/ml (5,500 μM). Each drug was then diluted in cell culture media to the final concentrations used in each experiment.

This article contains Supplementary Material available at http://www.interscience.wiley.com/jpages/1545-5009/suppmat.

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Grant sponsor: National Cancer Institute; Grant number: #5K12CA90433; Grant sponsor: National Institute of Nursing Research; Grant number: 1K23NR9192-01; Grant sponsor: NIH; Grant number: P41-EB001978.

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Received 12 April 2007; Accepted 5 July 2007

DOI 10.1002/pbc.21330

Cell Line and Culture

Six human cell lines were used in the experiments: JM1, a B-cell leukemia line; Molt-4, a T-cell leukemia line; DAOY and D283, medulloblastoma cell lines; and SK-N-DZ and SK-N-SH, neuroblastoma lines. All cell lines except JM1 were cultured in RPMI-1640 medium with L-glutamine (Invitrogen Corp., Grand Island, NY) with heat inactivated 10% fetal bovine serum (Invitrogen Corp.) in 5% CO_2 at 37°C. JM1 was cultured using Iscove's Modified Dulbecco's Medium (IMDM) with L-glutamine, 25 mM HEPES buffer and 2 Beta-mercaptoethanol (Invitrogen Corp.).

Cytotoxicity Assays

A modified methyl tetrazolium assay (MTT; 3-(4,5 dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) [8,9] was used to determine the sensitivity of the cell lines to karenitecin and mafosfamide. For single drug experiments, 135 μl of 1×10^5 cells/ml of each cell line was plated into 96-well microtiter plates using an automated drug delivery system (Biomek Automated Laboratory Workstation, Beckman Coulter, Inc., Fuller, CA). All cells were growing exponentially within the time frame of the experiments. The effect of evaporation in the wells was minimized by using only the inner wells for analyses.

Twenty-four hours later, drug at specified concentrations was added to each well. Replicates of six wells were used at each concentration. After 72 hr of drug exposure, 15 μl of MTT (5 mg/ml) was added to each well, the plates were incubated for 4 hr at 37°C, the medium was replaced with 150 μl of DMSO to solubilize the formazan precipitate and the optical density (OD) was measured at 550 nm using a microplate spectrophotometer (Anthos Labetc Instruments, Salzbers, Austria). Cell survival was calculated by subtracting the background OD of media alone and then dividing the OD of test wells by the OD of the control (untreated) wells. Two replicate plates were used for each experiment.

For drug combinations, similar experiments were performed except that in each 96-well plate, cells were exposed to medium alone (controls), single drugs alone (mafosfamide only, karenitecin only) or to different concentrations of the combination of two drugs (karenitecin + mafosfamide), for a total of 36 concentration pairs per plate. All combination drug concentrations were tested in four replicate plates.

Analysis of Drug Effects

The average percent cell survivals for each drug alone were calculated and plotted against the drug concentrations. The estimated concentrations at which cell growth was inhibited by 10%, 25%, 75%, and 90% (i.e., the IC_{10}, IC_{25}, etc.) from the single drug experiments were used to choose the range of concentrations of each drug for the combination experiments. The universal response surface approach (URSA) [10] as implemented in ADAPT II [11] was used to analyze the cytotoxic effects of the combination of karenitecin and mafosfamide. The following equation was fitted to experimental data using nonlinear regression:

$$1 = \frac{Da}{IC_{50a}\left(\frac{E}{100-E}\right)^{1/ma}} + \frac{Db}{IC_{50b}\left(\frac{E}{100-E}\right)^{1/mb}} + \frac{\alpha \cdot Da \cdot Db}{IC_{50a}IC_{50b}\left(\frac{E}{100-E}\right)^{1/2ma}\left(\frac{E}{100-E}\right)^{1/2mb}}$$

where Da is the concentration of drug a, Db is the concentration of drug b, IC_{50} is the median effective drug concentration, E is the measured effect (fraction of cells surviving), and ma or mb is the slope parameter of the individual drug's concentration-effect curve [12]. When α is positive, Loewe synergy is indicated, whereas a negative α reflects Loewe antagonism. The interaction is considered additive if the 95% confidence interval (CI) for α encompasses zero [10,12].

RESULTS

Single Drug Cytotoxicity

The single toxicity curves for karenitecin and mafosfamide for the leukemia, medulloblastoma, and neuroblastoma cell lines are illustrated in Supplemental Figure 1. The IC_{50}s of karenitecin for the various cell lines ranged from 0.6 nM for D283 and Molt-4 to 9.0 nM for SK-N-DZ. Mafosfamide also demonstrated a narrow range of IC_{50}s, from 0.4 μM for Molt-4 to 4.3 μM for SK-N-SH. For both drugs nearly complete inhibition of cell growth was demonstrated at higher concentrations in all cell lines.

Cytotoxicity of Drug Combinations

The concentration-effect curves for leukemia (Supplemental Fig. 2), medulloblastoma (Supplemental Fig. 3), and neuroblastoma (Supplemental Fig. 4) cell lines are illustrated with the different concentrations of karenitecin and mafosfamide. Table I shows the results of the synergy analysis. Karenitecin and mafosfamide are synergistic ($\alpha > 0$) in the neuroblastoma cell lines SK-N-DZ and SK-N-SH and the medulloblastoma cell line DAOY. In the medulloblastoma line, D283 and both the leukemia cell lines (JM1 and Molt4), the interaction between karenitecin and mafosfamide is additive ($\alpha = 0$). Antagonism was not observed in any cell line.

DISCUSSION

An important challenge for the development of combination intrathecal chemotherapy is to define and prioritize the interactions between agents in candidate combinations. It remains very difficult, however, to determine even preclinically whether the activity of a drug combination is the same as, greater than, or less than expected from the known activities of the single drugs, or in other words, whether the combination is additive, synergistic, or antagonistic. The results of these experiments show that the cytotoxic effects of the combination of karenitecin and mafosfamide are additive for JM1, Molt4, and D283, and synergistic for SK-N-DZ, SK-N-SH, and DAOY.

A number of different methods (combination index, median-effect analysis, or isobologram analysis) have been proposed for determining the types of interactions of drug combinations. Discussion of the controversies surrounding the ideal methodology for analysis of drug interactions of drug combinations is beyond the scope of this article but has been reviewed in detail elsewhere [13–15]. In our study, we used the URSA to evaluate drug interactions. This rigorous approach has certain advantages, particularly that the slopes (m) of the individual drugs' concentration-survival curves do not have to be the same; that the mechanism of the drugs' interaction does not have to be specified; and that the ratio of the drugs in the in vitro combinations does not have to be held constant [14]. Thus the finding of synergy or

TABLE I. Cytotoxic Effects of the Combination of Karenitecin + Mafosfamide

Cell lines	Drug	IC_{50}	m	alpha	95% CI	Effects
JM1	Karenitecin (nM)	<0.01	1.75	<0.01	(−0.20, 0.20)	+
	Mafosfamide (μM)	1.55	16.70			
Molt-4	Karenitecin (nM)	<0.01	2.71	<0.01	(−0.18, 0.18)	+
	Mafosfamide (μM)	1.54	14.15			
SKNDZ	Karenitecin (nM)	0.09	1.56	1.63	(0.78, 2.48)	++
	Mafosfamide (μM)	7.24	1.81			
SKNSH	Karenitecin (nM)	0.04	0.84	2.90	(1.14, 4.65)	++
	Mafosfamide (μM)	7.64	1.84			
DAOY	Karenitecin (nM)	<0.01	0.58	1.35	(0.34, 2.40)	++
	Mafosfamide (μM)	3.87	0.56			
D283	Karenitecin (nM)	<0.01	1.83	<0.01	(−0.11, 0.12)	+
	Mafosfamide (μM)	1.49	1.11			

Note: IC_{50} is the concentration at which cell survival is inhibited by 50%; m is the slope parameter that indicates sigmodicity of dose-response curve; and 95% CI is the 95% confidence interval around estimate of alpha. The + indicates that the drug interaction is additive where alpha is 0 and 0 is within 95% CI. The ++ indicates that the drug interaction is synergistic where alpha is greater than 0 and 0 is not within 95% CI.

additivity in our experiments is unlikely to be an artifact of experimental design but rather reflects actual drug interaction.

We exposed the cells simultaneously to both drugs. The sequential administration of other camptothecins, such as topotecan with cisplatin has been examined both in preclinical and clinical studies [16–20]. A significant schedule-dependent synergistic cytotoxicity was found in ovarian and breast cancer cell lines, with cisplatin prior to topotecan showing the most pronounced activity [17–20]. In contrast, the topotecan followed by cisplatin schedule yielded most pronounced activity in the lung cancer cell line [17]. Future in vitro experiments to examine whether the combined cytotoxic effects of karenitecin and mafosfamide would be different when administered sequentially may be warranted. Because it is most feasible to administer agents for combination intrathecal therapy simultaneously rather than sequentially, however, simultaneous exposure in vitro experiments is appropriate for analyzing potential intrathecal combinations.

Mafosfamide has been given safely into the CSF in a small number of children who had leptomeningeal dissemination of brain tumors [1,21,22]. While karenitecin is still in development, another topoisomerase inhibitor, topotecan, has been studied in children with CNS tumors [23]. In addition, intrathecal topotecan can be safely administered to children and adults with neoplastic meningitis [24]. Thus combinations of mafosfamide with a topoisomerase I inhibitor for intrathecal administration should be feasible. In patients receiving 5 mg of mafosfamide by the intraventricular route, mafosfamide concentrations remain above 1 μM for approximately 6 hr [25]. Topotecan concentrations above 1 μM were maintained for approximately 8 hr after a 0.4 mg intraventricular dose [24]. Thus the concentrations used for these in vitro synergy studies are in the range of those achieved in CSF after intraventricular dosing in humans.

In summary, we have shown that karenitecin and mafosfamide are additive or synergistic in vitro against tumor types that disseminate to the leptomeninges. These results provide important support for the development of this combination for intrathecal therapy. Further preclinical experiments in a nonhuman primate model are planned to evaluate the safety and tolerability of combined karenitecin and mafosfamide for intrathecal administration.

ACKNOWLEDGMENT

This study was supported in part by the National Cancer Institute #5K12CA90433, National Institute of Nursing Research 1K23NR9192-01, and NIH grant P41-EB001978.

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