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ORIGINAL ARTICLE

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Enhanced antitumor activity of irofulven in combination with thiotepa or mitomycin C

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Abstract *Purpose:* Irofulven (6-hydroxymethylacylfulvene, MGI 114, NSC 683863) is a semisynthetic derivative of illudin S, a toxin present in the *Omphalotus* mushroom. Irofulven has demonstrated activity against a broad range of solid tumors in both xenograft models and human trials. The potential application of administering irofulven in combination with aziridine-containing chemotherapeutic agents was evaluated in this study. *Methods:* Human lung carcinoma MV522 cells and BALB/c athymic mice bearing the human lung carcinoma MV522 xenograft were used to evaluate the activity of irofulven in combination with aziridine-containing drugs. *Results:* Irofulven in combination with either thiotepa or mitomycin C demonstrated a strong synergistic (supraadditive) activity both in vitro and in vivo, that exceeded results obtained with monotherapy at the same or higher doses of these agents. The majority of xenograft-bearing animals that received subtoxic doses of irofulven, and either thiotepa or mitomycin C, demonstrated a complete cure. In contrast, there was no detectable synergistic activity between irofulven and other aziridine-containing drugs, including AZQ and thiotepa metabolites such as TEPA or AZD. *Conclusions:* These results indicate that the therapeutic activity of irofulven is enhanced when combined with mitomycin C or thiotepa, and further evaluation of these combinations is therefore warranted.

Keywords Irofulven · Thiotepa · TEPA · Illudin · Mitomycin C

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Introduction

The illudins are natural sesquiterpene products isolated from the mushroom *Omphalotus illudens* and related species of basidiomycetes [27, 28]. The interest in illudin-derived agents as antineoplastic agents stems from the discovery that they are active against solid tumor cells at nano- to picomolar concentrations with short exposure times [14, 15], and produce a unique type of DNA damage [16, 17]. The preferential cytotoxicity towards tumor cells stems from a combination of intracellular drug accumulation [13, 19, 21, 22] and sensitivity of tumor cells to illudin-induced apoptosis [42]. Normal cells display marginal levels of apoptosis even after prolonged exposure to high concentrations of illudin-derived agents [41].

Irofulven (MGI 114, HMAF, NSC 683863) is a semisynthetic analogue of the fungal toxin illudin S [18, 29]. Irofulven has demonstrated significant antitumor activity against a variety of tumor models such as MV522 lung carcinoma, MX1 breast, and HT29 carcinoma xenografts [18, 26]. In addition, MDR1- and MRP1-positive xenografts retain sensitivity to irofulven, but display resistance to conventional chemotherapeutic agents [20, 23]. On this basis, irofulven was chosen as the initial illudin-derived candidate for human trials. Irofulven has been evaluated in a variety of phase I and II clinical trials with promising results [8, 38], and a large phase III trial in gemcitabine-refractory pancreatic cancer was initiated this year (Dr. John MacDonald, MGI PHARMA, Bloomington, Minn., personal correspondence).

While irofulven has displayed impressive single-agent activity in several clinical trials, a number of studies examining irofulven in combination with other antineoplastic agents have demonstrated an enhanced antitumor activity in various cell lines and xenografts. A synergistic or supraadditive activity has been noted between irofulven and topoisomerase I inhibitors in several studies, paclitaxel, and 5FU [2, 12, 24, 39]. We began a

systematic study to further define the potential of coadministering irofulven with other chemotherapeutic agents. Here we present evidence of a synergistic interaction between the aziridine-containing drugs mitomycin C and thiotepa, but not with aziridine-containing thiotepa metabolites or the aziridine prototype drug AZQ.

Materials and methods

Athymic mice

Balb/c nu/nu 4-week-old female mice weighing 18–22 g were obtained from Simonsen (Gilroy, Calif.) and maintained in the Athymic Mouse Colony of the University of California, San Diego, under pathogen-free conditions using HEPA filter hoods. The animals were housed in groups of four in plastic cages vented with polyester fiber filter covers, and provided with sterilized food and water ad libitum. Clean, sterilized gowns, gloves, masks, shoe and hood covers were worn by all personnel handling the animals. Studies were conducted in accordance with the guidelines of the National Research Council "Guide for Care and Use of Laboratory Animals", and the University of California, San Diego, guidelines for assessing illness and morbidity in rodents used in studies involving experimental neoplasia. All studies were approved by the University Institutional Animal Care and Use Committee.

Cell lines

The following cell lines were maintained in either RPMI-1640 or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) as previously described [14]: human lung adenocarcinoma cell line MV522 [37], colon adenocarcinoma line HT29 [25], and human breast carcinoma cell lines MCF7 [7]. All cell lines were routinely screened for mycoplasma. For determining the cytotoxic activity of irofulven in combination with other agents, cells were plated in 96-well plates, allowed to recover overnight, and various concentrations of the desired drug(s) were added. After 48 h incubation, the medium was removed, cells were washed twice with sterile saline, and cell viability determined using MTT. Briefly, the synergy studies were performed by adding the selected drugs together at various concentrations, but always maintaining a fixed ratio of drug A to drug B within an individual experiment [3, 4, 5]. Results were compared with those from control cultures (no drug) and cultures containing only an individual drug added at identical concentrations. The median-effect principal of Chou was used to determine whether a drug combination at a given concentration and ratio was synergistic (see Statistical analysis).

Drugs

Pharmaceutical grade irofulven (NSC 683863) was obtained from MGI PHARMA (Minneapolis, Minn.). Pharmaceutical grade thiotepa (NSC 6396) and mitomycin C (NSC 26980) were obtained from the UCSD Pharmacy. TEPA (NSC 54054), the aziridine analog (NSC 524926), and AZQ (NSC 182986) were obtained from the NCI DTP repository. Thiotepa and mitomycin C were reconstituted with sterile saline. TEPA, aziridine, and AZQ were dissolved in 1 ml 100% DMSO and diluted with sterile normal saline until a 10% DMSO/normal saline solution was achieved. Irofulven was dissolved in 100% ethanol and diluted with normal saline until a 10% ethanol/normal saline solution was achieved. The maximum tolerated dose (MTD) for irofulven and mitomycin C in this strain of mice had previously been determined [18] and is defined as the maximum dose administered for 3 weeks on a given schedule that produces a weight loss of $\leq 15\%$. Initial starting doses for thiotepa and AZQ were chosen on the basis of previous reports [10, 31].

In vivo evaluation using the MV522 xenograft model

Mice were randomized into treatment groups of eight animals each. Each animal was earmarked and followed individually throughout the experiment. The mice received s.c. injections of $8–10\times10^6$ MV522 cells over the shoulder. All drugs were administered i.p. three times a week for 3 weeks, starting on day 10 after tumor implantation. Tumor size was measured in two perpendicular diameters and tumor weight estimated according to the equation $w = (width^2 \times length/2)$ [33]. Relative weights (RW) were calculated to standardized variability in tumor size amongst test groups at initiation of the treatment using the equation $RW = Wt/Wi$, where Wi is the tumor weight for a given animal at the beginning of drug treatment and Wt is the tumor weight at a subsequent time [33].

Statistical analysis and determination of synergistic activity

To compare the relative tumor weights between the groups of animals, ANOVA followed by Tukey-Kramer multiple comparison post-ANOVA analysis was performed. Survival curves between groups of animals was compared using the method of Kaplan and Meier [1]. Probability values less than 0.05 were considered statistically significant. The relative tumor weight data and life-span data were analyzed using Instat (version 2.02) and Prism (version 3.0) software packages (GraphPad, La Jolla, Calif.).

To determine whether synergy existed between irofulven and other agents, we used the median-effect principle of Chou [3, 4, 5] to determine the dose-effect parameters for two drugs individually and for their different combinations. Median-effect computer software (CalcuSyn for Windows, Biosoft, Ferguson, Mo.) was used to generate the isoeffective dose (Dx) values which were used to generate the combination index (CI), where a CI values of < 1, $= 1$, and > 1 indicate synergism (i.e. the effect of the drug combination is greater than anticipated from the additive effect of the individual agents), an additive effect, and antagonism, respectively [3, 4, 5].

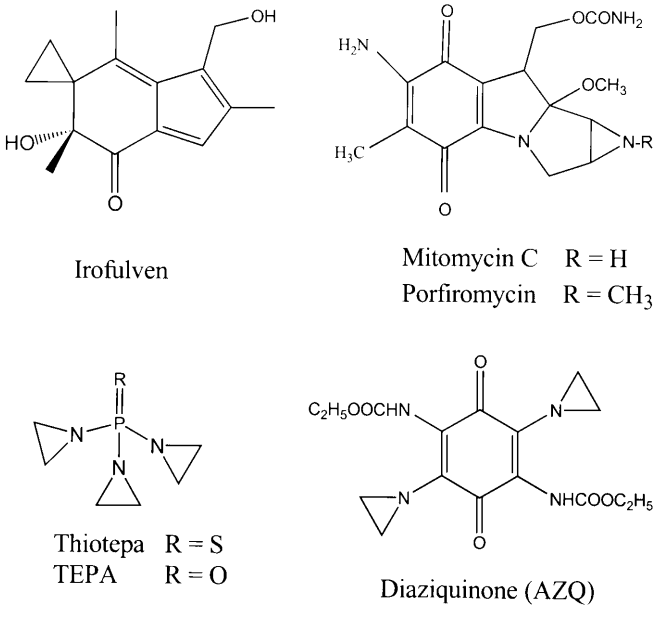

Fig. 1. Structures of irofulven and other agents used in this study

Results

Mitomycin C and thiotepa studies

The activity of irofulven (Fig. 1) in combination with either mitomycin C or thiotepa was examined in vitro using a continuous 48-h exposure in MV522 cells. Analysis by the median- effect principal revealed an increasing CI with increasing cytotoxicity (Fig. 2), which can be classified as strong synergy [3, 4, 5]. An initial in vivo study indicated that both irofulven and mitomycin C at the MTD were capable of inhibiting tumor growth (Fig. 3) and producing partial remissions, in accordance with the findings of previous studies [18]. Thiotepa at its MTD, however, had only a minor effect and produced tumor growth inhibition but not overt tumor regression. With the exception of one irofulven-treated animal, all tumors recurred within days in animals treated with single agents. When irofulven was coadministered with either mitomycin C or thiotepa, at the nontoxic dose of two-thirds MTD for each drug, there was marked tumor regression in all eight treated animals (Fig 3, Table 1). All eight animals in the combination irofulven and mitomycin treatment group were alive on day 120, and five had no evidence of tumor by palpation. All eight animals in the combination irofulven and thiotepa treatment groups were alive on day 120 and four had no evidence of tumor by palpation. In contrast, only one irofulven-treated animal survived until day 120, and there were no surviving animals in any of the mitomycin C- or thiotepa-treated groups (Table 1).

The median survival times were 27 days in control animals, 35 days in animals receiving thiotepa at its MTD, 60 days in animals receiving mitomycin C at its MTD, 75 days in animals receiving irofulven at its

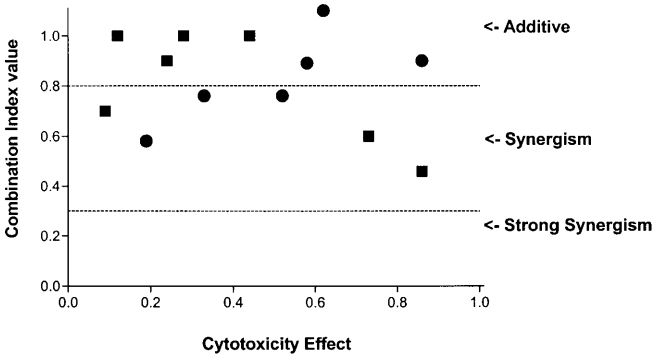

Fig. 2. Combination index (CI) plot displaying the in vitro interaction between irofulven and either mitomycin C or thiotepa. The classifications of the extent of synergy are as defined previously by Chou et al. [4, 5] (*circles* irofulven and mitomycin C together, *squares* irofulven and thiotepa together)

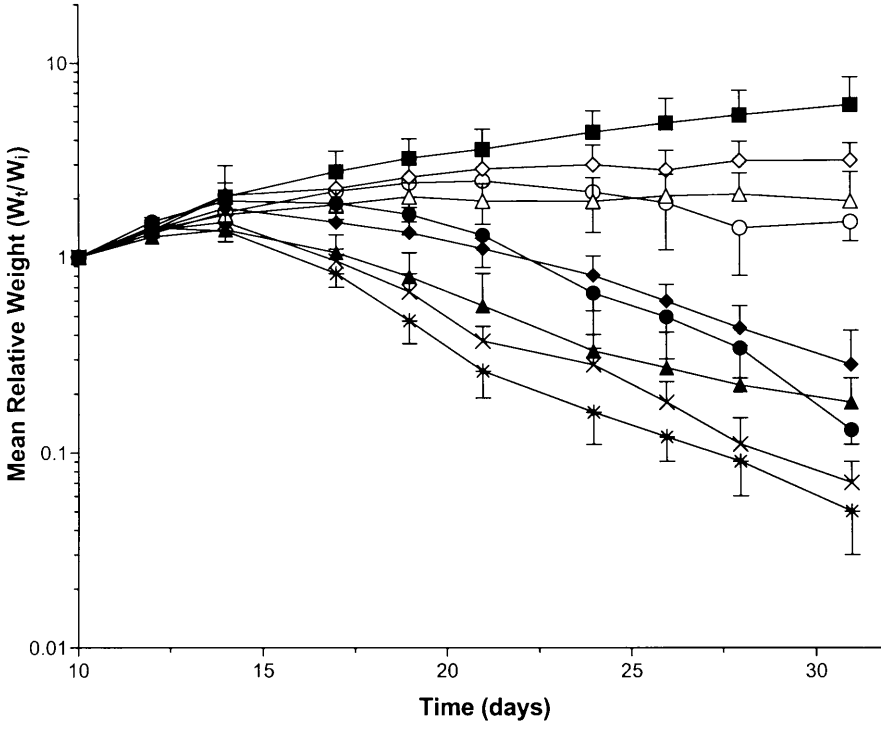

Fig. 3. Efficacy of irofulven in combination with thiotepa or mitomycin C versus single-agent therapy in the MV522 xenograft model. MV522-bearing animals received 10% DMSO/saline as control (*solid squares*), thiotepa at the MTD of 10 mg/kg (*solid diamonds*), thiotepa at two-thirds the MTD or 6.67 mg/kg (*open diamonds*), irofulven at the MTD of 10 mg/kg (*solid triangles*), irofulven at two-thirds the MTD of 6.67 mg/kg (*open triangles*), mitomycin C at the new MTD of 1.8 mg/kg (*solid circles*), mitomycin C at two-thirds the MTD of 1.2 mg/kg (*open circles*), irofulven at two-thirds the MTD plus mitomycin at two-thirds the MTD (*crosses*), irofulven at two-thirds the MTD plus thiotepa at two-thirds the MTD (*asterisks*). All drugs were administered i.p. three times a week for 3 weeks, starting on day 10 after tumor implantation. There were eight animals per group and the data points indicate means for each group and the bars represent SE

Table 1. The number of MV522 human lung tumor xenograft-bearing mice displaying partial tumor remission (PR) or complete tumor remission (CR) after receiving irofulven in combination with other aziridine drugs

Experiment A				Experiment B				Experiment C			
Drug	Dose	PR	CR	Drug	Dose	PR	CR	Drug	Dose	PR	CR
Normal saline/ DMSO (control)		0	0	Normal saline/ DMSO (control)		0	0	Normal saline/ DMSO (control)		0	0
Irofulven	10 mg/kg (MTD)	7	1	Irofulven	10 mg/kg (MTD)	7	1	Irofulven	10 mg/kg (MTD)	6	2
Mitomycin C	1.8 mg/kg (MTD)	8	0	Mitomycin C	1.8 mg/kg (MTD)	8	0	AZQ	2.5 mg/kg (MTD)	0	0
Thiotepa	10 mg/kg (MTD)	7	0	Thiotepa	10 mg/kg (MTD)	3	0	TEPA	10 mg/kg (MTD)	0	0
Irofulven	6.7 mg/kg (two-thirds MTD)	0	0	Irofulven	6.7 mg/kg (two-thirds MTD)	2	0	Irofulven	6.7 mg/kg (two-thirds MTD)	0	0
Mitomycin C	1.2 mg/kg (two-thirds MTD)	0	0	Mitomycin C	1.2 mg/kg (two-thirds MTD)	3	0	AZQ	1.25 mg/kg (two-thirds MTD)	0	0
Thiotepa	6.7 mg/kg (two-thirds MTD)	0	0	Thiotepa	6.7 mg/kg (two-thirds MTD)	0	0	TEPA	5 mg/kg (two-thirds MTD)	0	0
Irofulven/ mitomycin C	Both two-thirds MTD	3	5	Irofulven/ mitomycin C	Both two-thirds MTD	4	4	Irofulven/ AZQ	Both two-thirds MTD	0	0
Irofulven/ thiotepa	Both two-thirds MTD	4	4	Irofulven/ thiotepa	Both two-thirds MTD	4	4	Irofulven/ TEPA	Both two-thirds MTD	0	0

MTD, and >120 days in animals receiving both the irofulven plus mitomycin C and the irofulven plus thiotepa combinations. The animals receiving either the combination irofulven and mitomycin C treatment, or the irofulven plus thiotepa treatment, had significant increases in life-span when compared with control animals ($P < 0.001$ for both groups) and when compared with animals receiving irofulven at its MTD ($P = 0.033$ and $P = 0.026$, respectively), indicating that the combination therapy was markedly effective at prolonging life-span as compared with single-agent treatment. A second study (experiment B, Table 1) was performed to confirm the synergistic activity of irofulven with either mitomycin C or thiotepa. Again, when irofulven was coadministered with either mitomycin C or thiotepa, there was marked tumor regression in all treated animals (Table 1), and 50% of each group had no evidence of tumor by palpation on day 120.

TEPA and AZQ studies

The activity of thiotepa has been attributed, in part, to its metabolism to TEPA and subsequent metabolic conversion to aziridine moieties [1, 30]. Therefore, we determined whether the synergistic activity noted with thiotepa could be attributed to one of these metabolites. The antitumor activities of TEPA and the prototype aziridine drug, AZQ, were determined independently and in combination with irofulven. TEPA and AZQ at their MTD were minimally active in the MV522 xenograft model and produced only limited tumor growth inhibition (Fig. 4, Table 1). The combinations of irofulven, and either TEPA or AZQ, demonstrated minimal activity in vitro (Fig. 4, Table 1), which is in contrast to the in vitro activity noted with the combination of irofulven and either thiotepa or mitomycin C (Fig. 2).

Because of the unexpected lack of synergy with TEPA and AZQ, the in vitro screening studies were performed using these agents as well as porfiromycin (mitomycin-related analog) and AZD, an aziridine prototype thiotepa metabolite. There was no detectable synergy between irofulven and either TEPA or AZQ, in accordance with the xenograft results (Fig. 5). Schedule dependency was examined by adding one agent prior to the addition of the other agent. Regardless of whether irofulven was added first or second, there was still no detectable synergy between the drug and either TEPA or AZQ (Fig. 5). There was in vitro synergy between porfiromycin and irofulven that was independent of schedule (Fig. 6). However, there was no detectable synergy between irofulven and AZD (Fig. 6). To determine whether the synergy between thiotepa and irofulven was unique to MV522 lung carcinoma cells, we also examined their activity in colon HT29 and breast MCF7 carcinoma cells. There was also synergy between irofulven and thiotepa in both of these cell lines, but not between irofulven and TEPA (data not shown), indi-

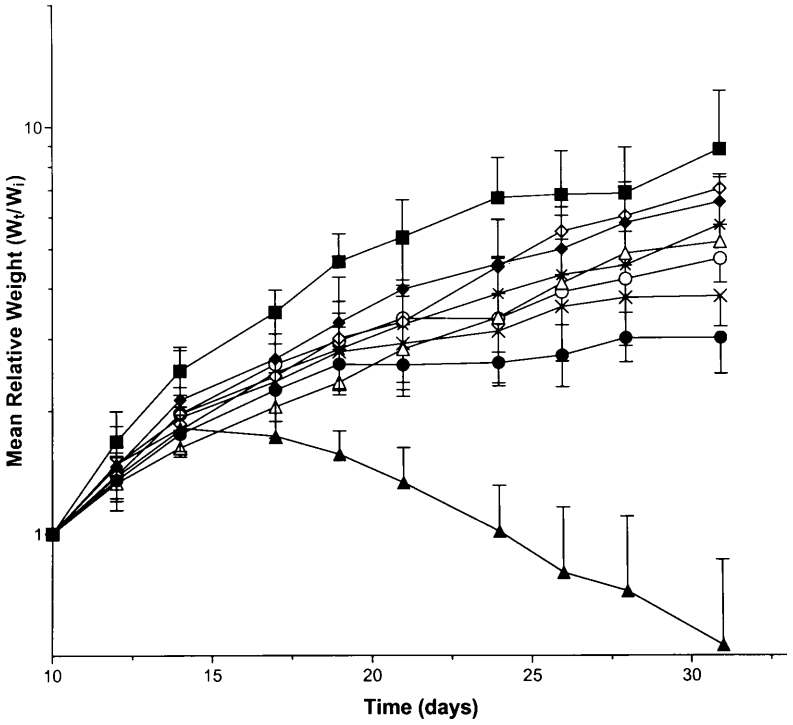

Fig. 4. Efficacy of irofulven in combination with TEPA or AZQ versus single-agent therapy in the MV522 xenograft model. MV522-bearing animals received 10% DMSO/saline as control (*solid squares*), TEPA at the MTD of 10 mg/kg (*solid diamonds*), TEPA at two-thirds the MTD or 5.0 mg/kg (*open diamonds*), irofulven at the MTD of 10 mg/kg (*closed triangles*), irofulven at two-thirds the MTD of 5.0 mg/kg (*open triangles*), AZQ at the MTD of 2.5 mg/kg (*solid circles*), AZQ at two-thirds the MTD of 1.25 mg/kg (*open circles*), irofulven at two-thirds the MTD plus TEPA at two-thirds the MTD (*crosses*), irofulven at two-thirds the MTD plus AZQ at two-thirds the MTD (*asterisks*). All drugs were administered i.p. three times a week for 3 weeks, starting on day 10 after tumor implantation. There were eight animals per group and the data points indicate means for each group and the bars represent SE

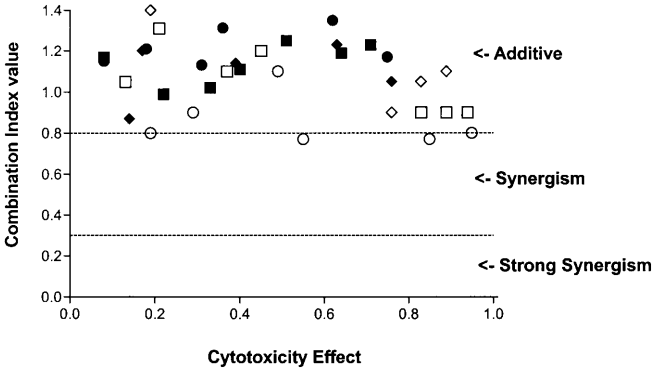

Fig. 5. Combination index (CI) plot displaying the in vitro interaction between irofulven and either TEPA or AZQ. The classifications of the extent of synergy are as defined previously by Chou et al. [4, 5] (*solid squares* irofulven and TEPA added together, *solid diamonds* irofulven added first and TEPA added 4 h later, *solid circles* TEPA added first and irofulven added 4 h later, *open squares* irofulven and AZQ added together, *open diamonds* irofulven added first and AZQ added 4 h later, *open circles* AZQ added first and irofulven added 4 h later)

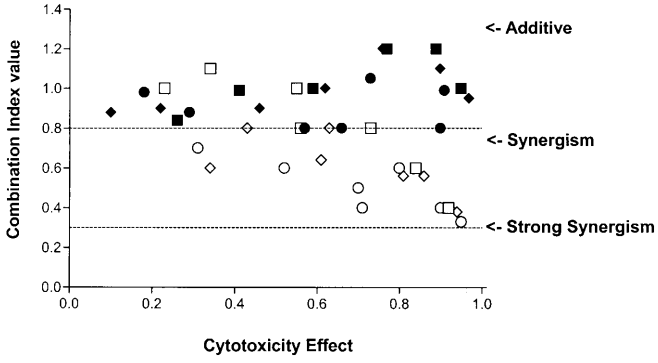

Fig. 6. Combination index (CI) plot displaying the in vitro interaction between irofulven and either AZD or porfiromycin. The classifications of the extent of synergy are as defined previously by Chou et al. [4, 5] (*solid squares* irofulven and AZD added together, *solid diamonds* irofulven added first and AZD added 4 h later, *solid circles* AZD added first and irofulven added 4 h later, *open squares* irofulven and porfiromycin added together, *open diamonds* irofulven added first and porfiromycin added 4 h later, *open circles* porfiromycin added first and irofulven added 4 h later)

cating that the synergy was not unique to the MV522 cell line.

Discussion

It is tempting to assign the in vitro and in vivo synergistic activity noted between irofulven and mitomycin C or thiotepa to the presence of the aziridine group in the latter drugs. However, other aziridine-containing compounds (TEPA, AZD, AZQ) did not demonstrate any enhanced antitumor activity with irofulven in either the in vitro or the in vivo studies.

The enhanced cytotoxic activity noted in this study may have been due in part to additive effects of combining a cell cycle-specific agent with agents that are not cell cycle-specific. Irofulven blocks the G_1/S phase interface suggesting either a selective death of cells synthesizing DNA or complete inhibition of DNA synthesis. In contrast, thiotepa is not cell cycle-specific as the active alkylating radicals, the ethylenimine moieties, are metabolically produced [9]. Similarly, the cytotoxic action of mitomycin C is also reportedly not cell cycle-specific, although the cytotoxic effect is maximized if cells are treated in late G_1 or early S phase.

The metabolic activation of thiotepa to a cytotoxic species is mediated by a microsomal process that is both NADPH- and oxygen-dependent [36]. It has been suggested that the cytotoxicity of mitomycin C is in part due to oxygen free radicals which contribute to DNA strand breaks. It has recently been reported that the combination of irofulven and radiation, the latter mediating toxicity in part via oxygen-derived radicals, also enhances cytotoxicity [40]. Thus, the common factor for enhanced cytotoxicity between irofulven and these agents may be the production of oxygen-derived radicals by the latter. In support of this hypothesis is the finding that AZQ, which displays no synergistic activity with irofulven, demonstrates radical production and cytotoxicity independent of oxygen tension [11].

The enhanced activity of the combination of irofulven and either mitomycin C or thiotepa may be via a decreased ability to repair DNA. In contrast to other agents, functional helicase activity is critical to the repair of illudin-induced damage and a deficiency in these helicase enzymes results in sensitivity to illudins [16, 19]. However, functional helicase activity is also required for the nucleotide excision repair system to handle lesions induced by other agents including mitomycin C [35]. Thus, combining irofulven with other DNA-damaging agents may result in DNA repair being selectively overwhelmed. Helicases already committed to repairing damage induced by mitomycin C or thiotepa would not be available for repair of illudin-induced damage, resulting in a functional depletion of activity, and a further sensitivity to irofulven.

It is plausible that the enhanced activity noted between irofulven and mitomycin C or thiotepa is not due to decreased DNA repair, but production of a specific type of DNA lesion associated with the latter agents and not with other aziridine-containing agents. In support of this hypothesis is the intriguing finding noted by others that thiotepa produces interstrand DNA crosslinks, whereas TEPA produces alkali-labile DNA lesions [6]. Mitomycin C also produces predominantly interstrand DNA crosslinks [34]. Although AZQ is capable of producing interstrand crosslinks, the predominant DNA damage appears to be alkali-labile sites [32], similar to that noted with TEPA [6]. This difference, in the type of DNA damage produced, may explain why some aziridine-containing drugs are synergistic in combination with irofulven while others are not.

In summary, the exact nature of the synergistic action between irofulven and mitomycin C or thiotepa is not clear, but likely involves production of reactive oxygen species, a specific type of DNA damage, or a combination of these two processes. The unique synergistic activity of combinations of irofulven and mitomycin C or thiotepa suggests that further evaluation of these combinations is warranted.

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