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ORIGINAL ARTICLE

Synergy of irofulven in combination with other DNA damaging agents: synergistic interaction with altretamine, alkylating, and platinum-derived agents in the MV522 lung tumor model

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Abstract
Purpose Irofulven (MGI 114, NSC 683863) is a semi-synthetic derivative of illudin S, a natural product present in the *Omphalotus illudins* (Jack O'Lantern) mushroom. This novel agent produces DNA damage, that in contrast to other agents, is predominately ignored by the global genome repair pathway of the nucleotide excision repair $(NER)^2$ system. The aim of this study was to determine the antitumor activity of irofulven when administered in combination with 44 different DNA damaging agents, whose damage is in general detected and repaired by the genome repair pathway.
Methods The human lung carcinoma MV522 cell line and its corresponding xenograft model were used to evaluate the activity of irofulven in combination with different DNA damaging agents.
Results Two main classes of DNA damaging agents, platinum-derived agents, and select bifunctional alkylating agents, demonstrated in vivo synergistic or super-additive interaction with irofulven. DNA helicase inhibiting agents also demonstrated synergy in vitro, but an enhanced interaction with irofulven could not be demonstrated in vivo. There was no detectable synergistic activity between irofulven and agents capable of inducing DNA cleavage or intercalating into DNA.
Conclusion These results indicate that the antitumor activity of irofulven is enhanced when combined with platinum-derived agents, altretamine, and select alkylating agents such as melphalan or chlorambucil. A common factor between these agents appears to be the production of intrastrand DNA crosslinks. The synergistic interaction between irofulven and other agents may stem from the nucleotide excision repair system being selectively overwhelmed at two distinct points in the pathway, resulting in prolonged stalling of transcription forks, and subsequent initiation of apoptosis.

Keywords Irofulven · Synergy · Carboplatin · Melphalan · Chlorambucil · Altretamine

Abbreviations

NER	Nucleotide excision repair
MTD	Maximum tolerated dose
ILS	Increase in life span
DMSO	Dimethylsulfoxide
TW	Tumor weight
RW	Relative weight

Introduction

Irofulven (NSC 683863), a semi-synthetic analogue (Fig. 1) of the natural fungal product illudin S [13], has demonstrated activity against a variety of tumor models including lung, breast, gastric, colon, prostate, myeloid leukemia, and intracranial glioblastoma multiforme xenografts [4, 18]. Irofulven induces DNA strand breakage, but not DNA

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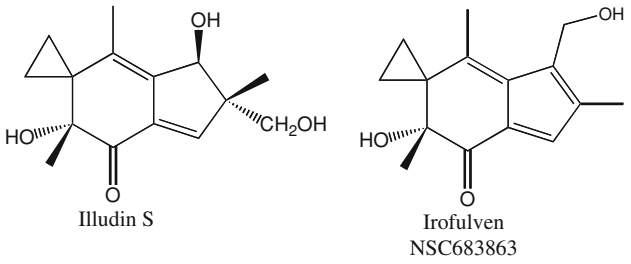

Fig. 1 Structure of irofulven and illudin S

intrastrand cross-links or DNA-protein cross-links [38]. Irofulven also differs from other DNA-damaging alkylating agents by displaying a preferential cytotoxicity towards cell lines deficient in the DNA repair helicases XPB and XPD [14, 20]. The exact nature of the DNA lesion produced by irofulven is unclear, but in contrast to other known DNA damaging agents, the lesion is not recognized by the global genome repair pathway and processed only by the transcription- and replication-coupled repair pathways [12]. This dependency on transcription-coupled repair was confirmed and a correlation was noted between drug cytotoxicity and cellular XPG expression [23]. Recent studies on the response of DNA damage pathways to irofulven emphasize the uniqueness of the DNA lesions induced by this drug. Irofulven induces ATM-dependent CHK2 activation leading to S phase arrest [34], but this pathway responds primarily to ionizing radiation induced DNA double strand breaks [10]. In contrast, the ATR and CHK1 pathway, which responds to most drug-induced DNA lesions, does not play a role in the irofulven-induced DNA damage response [34]. Recent studies have identified several of the DNA adducts induced by irofulven [5], but it is not clear which adducts are responsible for the unique DNA damaging properties of the drug, and which require repair by the transcription-coupled repair pathway.

A unique aspect of irofulven's antitumor activity is its ability to act as a selective inducer of apoptosis in tumor cells versus nontumor/immortalized cells [38]. This selective apoptosis occurs regardless of the p53 or p21 status [11], and is independent of Bcl-2 expression [8]. The ability of irofulven to induce apoptosis is related to activation of JNK and ERK ad caspase-mediated apoptosis [35]. In addition to these unusual properties, irofulven remains is effective against MDR1- and MRP1-positive xenografts that display resistance to conventional chemotherapeutic agents [15, 19].

On the basis of its unique properties, irofulven was chosen as the initial illudin-derived candidate for human trials. Irofulven was evaluated in a variety of phase I and II clinical trials with promising results [29, 30]. Partial or complete tumor regressions were noted in patients with pancreatic, liver, colon, ovarian, prostate or sarcoma tumors, which were nonresponsive to conventional chemotherapy. While irofulven has displayed substantial single-agent activity in clinical studies, a number of preclinical studies have found enhanced antitumor activity when the drug is combined with other agents. A synergistic activity has been noted between irofulven and topoisomerase I inhibitors, taxanes, and 5FU [1, 7, 16, 21, 22, 36]. Preliminary reports of clinical studies in which irofulven was administered in combination with other agents also demonstrate the potential utility of such combination approaches [9].

We began a systematic study to identify the potential of combining irofulven with other groups of chemotherapeutic agents. The MV522 lung carcinoma xenograft model was chosen to allow comparison to previous xenograft studies, and because this xenograft model is not responsive to a variety of conventional and experimental agents [15, 17–19, 21, 22]. Here we present evidence that irofulven interacts with platinum-derived and select bifunctional alkylating agents.

Materials and methods

Cell culture studies

The MV522 cell line was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) as previously described and routinely screened for mycoplasma [18]. 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 media 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. Results were compared to control cultures (no drug) and to cultures containing only an individual drug added at identical concentrations. Determination of whether a drug combination at a given concentration and ratio was synergistic, was performed by the median-effect principal of Chou (see "Statistical analysis and determination of synergistic activity") [3].

Athymic mice

Balb/c nu/nu 4 week old female mice weighing 18–22 g were obtained from Simonsen, Inc., (Gilroy, CA) and housed in groups of four in plastic cages vented with polyester fiber filter covers, and provided with sterilized food

and water ad libitum. 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. The University Institutional Animal Care and Use Committee approved all studies.

Compounds and drugs

Irofulven (NSC 683863) was obtained from MGI Pharma, (Bloomington, MN). Distamycin A, gliotoxin, and mithramycin A were obtained from Sigma-Aldrich (St. Louis, MO). Dactinomycin (Cosmegen), carboplatin (Paraplatin), melphalan (Alkeran), bleomycin (Blenoxane), and temozolomide (Temodar) were obtained from the UCSD Cancer Center Pharmacy. All other agents were obtained from the NCI DTP repository and included acodazole, altretamine (HMM), berenil, bisbenzamide, busulfan, BCNU, carboxypthalatoplatin, chlorambucil, chlorozotocin, chromomycin A3, cisplatin, cytemba, dacarbazine, daunorubicin, echinomycin, iproplatin, mAMSA, mechlorethamine, neocarzinostatin, netropsin, nogalamycin, penclomidine, pentamethylmelamine (PMM), pepleomycin, piperbroman, plicamycin, procarbazine, stallimycin prodrug (NSC 617595), streptozocin, tetraplatin, and treosulfan. All agents, unless noted, were prepared as stock solutions of 1–10 mg/ml in 40% DMSO/normal saline and diluted with 10% DMSO/normal saline as required. The maximum tolerated dose (MTD) for irofulven 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 (either 3 or 5 times per week) that produces a weight loss of $\leq$15%.

In vivo evaluation using the MV522 xenograft model

Mice were randomized into treatment groups prior to tumor load injection, earmarked, and followed individually throughout the experiment. The mice received s.c. injections of 8–10 million MV522 cells, obtain from cell culture, 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 (TW) estimated according to the formula: $w = [(\text{width})^2 \times \text{length}/2]$ [32]. Relative weights (RW) were calculated to standardized variability in tumor size amongst test groups at initiation of the treatment by using the formula RW = Wt/Wi, where Wi is the tumor weight for a given animal at the beginning of drug treatment and Wt is tumor weight at a subsequent time [31]. Tumor growth inhibition (TGI%) and mean % shrinkage were calculated as previously described [27].

Statistical analysis and determination of synergistic activity

The median-effect principle by Chou [3] was chosen to determine the interaction of irofulven with other agents based on a review by Greco of available approaches to quantify synergistic interaction between agents [6]. Median-effect computer software (CalcuSyn for Windows, Biosoft, Ferguson, MO) was used to generate the isoeffective dose (Dx) values which are used to generate the combination index (CI), where a CI value of <1, $= 1$, and >1 indicates synergism (i.e. the effect of drug combination is greater than anticipated from the additive effect of the individual agents), additive effect, and antagonism respectively [3].

To compare the relative tumor weights between the groups of animals, ANOVA followed by Dunnett's post-ANOVA analysis was performed. Comparison of survival curves between groups of animals was performed using the method of Kaplan and Meier. 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 (Graph Pad, Inc., La Jolla, CA USA).

Results

In vitro synergy studies

The activity of irofulven in combination with DNA-interacting agents was first examined in vitro using a continuous 48-h exposure with MV522 lung carcinoma cells. The MV522 cell line was chosen to allow comparison to previous in vitro and xenograft studies, and because the xenograft model is refractory to treatment with a variety of conventional and experimental agents [15, 17–19, 21, 22]. Previous studies using colony-forming assays demonstrated that irofulven produces its cytotoxic action on MV522 and other cell lines within 2 h [20]. Therefore, initial studies were performed adding irofulven prior to addition of the other agent. If a synergistic interaction was noted, the effect of schedule dependency was also determined by adding irofulven to the cell culture media either simultaneously, or 4 h after the addition of the other agent.

A summary of the in vitro screening results for agents demonstrating synergy (supra-additive) or additive interaction with irofulven is provided (Table 1). Other agents, such as DNA intercalators or agents inducing DNA strand cleavage, displayed an antagonistic interaction with irofulven, regardless of schedule of addition (data not shown).

Table 1 Summary of results of in vitro synergy study between irofulven and various DNA damaging agents based on Chou's CI values for cytotoxicity effects >0.25

Agent	NSC number	Irofulven added first	Combination index	Added together	Combination index	Irofulven second	Combination index
Bleomycin	125066	Additive	1.1 ± 0.2	Additive	1.0 ± 0.1	NT	
Bisbenzamide	322921	SYNERGY	0.7 ± 0.1	Additive	1.0 ± 0.2	Additive	1.0 ± 0.1
Carboplatin	201345	SYNERGY	0.8 ± 0.1	SYNERGY	0.8 ± 0.1	SYNERGY	0.8 ± 0.2
Chlorambucil	3088	Antagonism	1.4 ± 0.3	Antagonism	1.5 ± 0.1	Antagonism	2.2 ± 0.5
Cisplatin	119875	SYNERGY	0.8 ± 0.2	SYNERGY	0.8 ± 0.2	SYNERGY	0.8 ± 0.1
Dacarbazine	45388	Additive	1.2 ± 0.1	Additive	1.1 ± 0.1	Additive	1.0 ± 0.1
Dactinomycin	3053	SYNERGY	0.8 ± 0.2	Additive	1.2 ± 0.3	NT	
Daunorubicin	82151	SYNERGY	0.8 ± 0.2	Additive	1.1 ± 0.2	NT	
Distamycin A	82150	SYNERGY	0.6 ± 0.1	Additive	1.1 ± 0.2	Additive	1.0 ± 0.2
HMM	13875	SYNERGY[a]	1.1 ± 0.3	SYNERGY[a]	1.1 ± 0.3	SYNERGY[a]	1.0 ± 0.3
Mechlorethamine	762	SYNERGY	0.7 + 0.1	Additive	1.0 ± 0.1	Additive	1.1 ± 0.3
Melphalan	8806	Additive	1.2 ± 0.1	Additive	1.0 ± 0.1	Additive	1.1 ± 0.3
Mithramycin A	24559	SYNERGY	0.5 ± 0.2	Additive	1.00 ± 0.2	Additive	1.0 ± 0.2
Temozolomide	362856	Additive	1.0 ± 0.1	Additive	0.9 ± 0.1	Additive	0.9 ± 0.2

NT not treated

[a] Altretamine (HMM) displayed the unusual property of demonstrating antagonistic action at low doses and synergistic interaction at high doses. See text and Fig. 3 for specific details

Altretamine and platinum-derived agents displayed additive interaction with irofulven at low doses, and a synergistic interaction at high cytotoxic concentrations, that was independent of the schedule of addition (Figs. 2 and 3). The DNA helicase agents (distamycin A, mithramycin A) displayed a prominent schedule dependency. For example, if distamycin A was added after irofulven, then interaction of the two agents was synergistic (Fig. 4). In contrast, concomitant addition, or adding irofulven second, resulted in a predominantly additive profile for these agents. Dactinomycin, mechlorethamine, bisbenzamide, and daunorubicin displayed a synergistic profile when added after irofulven (Table 1), indicating that a strong schedule dependency was also present for these agents.

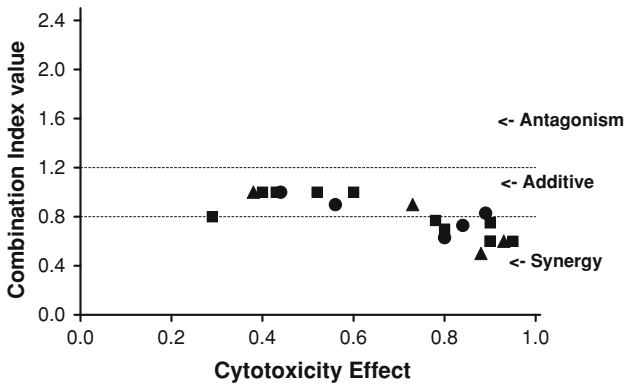

Fig. 2 Combination index (CI) plot displaying the in vitro interaction between irofulven and cisplatin (NSC 119875). The classifications of the extent of synergy are as defined previously by Chou et al. [2, 3]. Irofulven and cisplatin added simultaneously (*filled square*) irofulven added first (*open square*), and cisplatin added first (*filled circle*)

Xenograft synergy studies

Agents presented in Table 1 were chosen for in vivo studies using the MV522 lung carcinoma xenograft. In addition, agents that could not be studied in vitro as they required in vivo metabolic activation (cyclophosphamide, ifosfamide), were also studied in the xenograft model to determine their interaction with irofulven. Dosages and administration schedules for individual agents were chosen after a review of existing literature.

Cyclophosphamide, when combined with irofulven at subtoxic doses, demonstrated enhanced activity (ILS of 71%) as compared to monotherapy at the 2/3 MTD for either agent (Expt A, Table 2). The combination therapy, however, was less effective than that of irofulven administered alone at MTD. The combination of carboplatin and irofulven (at 2/3 MTD of each agent) was effective at inducing tumor shrinkage (Expt B, Table 2). The combination significantly extended life span as compared to that induced by either irofulven (ILS of 23% compared to control animals) or carboplatin (ILS of 4%) monotherapy ($p < 0.01$ for all irofulven-carboplatin treated groups versus irofulven monotherapy).

Melphalan produced a strong antitumor response when combined with irofulven (Expt. C, Table 2). The life span of animals treated with the melphalan-irofulven combination 2/3 MTD (ILS of 85%) was markedly extended as

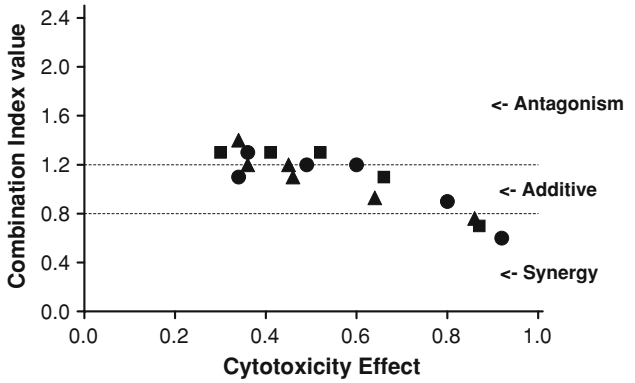

Fig. 3 Combination index (CI) plot displaying the in vitro interaction between irofulven and altretamine (NSC 13875). Irofulven and altretamine added simultaneously (*filled square*), irofulven added first (*open square*), and altretamine added first (*filled circle*)

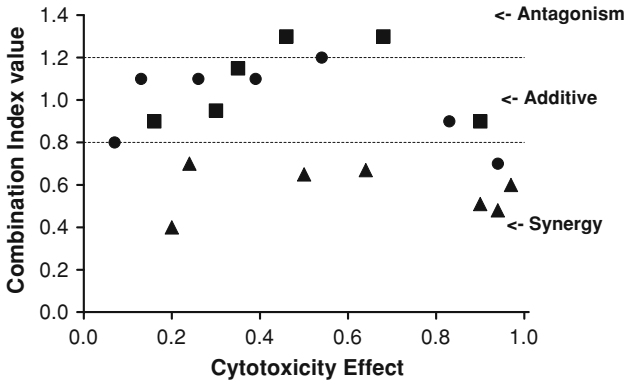

Fig. 4 Combination index (CI) plot displaying the in vitro interaction between irofulven and distamycin A (NSC 82150). Irofulven and distamycin A added simultaneously (*filled square*), irofulven added first (*open square*), and distamycin A added first (*filled circle*)

compared to animals treated with irofulven alone administered at MTD ($p < 0.05$). The enhanced activity of this combination was also evident when the drugs were combined at the 1/2 MTD doses (ILS of 64%). Chlorambucil, an agent structurally related to melphalan (Expt. D, Table 2), also produced a synergistic antitumor effect when combined with irofulven. The life span of chlorambucil-irofulven treated animals was also markedly extended as compared to control animals ($p < 0.001$), but was only equivalent to irofulven monotherapy at MTD treated animals ($p = 0.068$).

Based on the activity noted with cyclophosphamide, the related agent ifosfamide was studied as well as the melamine-derived agent altretamine (HMM). Both agents displayed enhanced activity when combined with irofulven at subtoxic doses as noted by the marked increase in tumor regression ($p < 0.05$ for both ifosfamide-irofulven and HMM-irofulven treated animals as compared to irofulven MTD treated animals)(Fig. 5), and in the number of animals demonstrating tumor shrinkage (Expt E, Table 2). The increase in life span of animals treated with both altretamine and irofulven at 2/3 MTD, or with ifosfamide and irofulven at 2/3 MTD, was extended as compared to control animals (ILS of 100%, $p < 0.01$; ILS of 57%, $p < 0.05$ respectively), and was equivalent to irofulven monotherapy at MTD (ILS of 57% versus control animals). The methane sulfonate alkylating agent busulfan also demonstrated evidence of synergy as the 2/3 MTD combination (test agent plus irofulven) treated animals had an increase in the number of animals displaying partial remissions when compared to the 2/3 MTD monotherapy (test agent or irofulven) treated animals (results not shown). Combining either daunorubicin or bleomycin with irofulven was noted to have some beneficial effect when compared to animals treated the agents at their respective 2/3 MTD, but survival did not exceed that of animals treated only with irofulven at MTD (data not shown). Other agents tested in the xenograft, but not demonstrating any beneficial interaction with irofulven, included dacarbazine, dactinomycin, mithramycin, BCNU, and temozolomide (data not shown).

Discussion

In this report, the effects of combining irofulven with other DNA damaging agents were examined. Among the agents most active when combined with irofulven were those of the nitrogen mustard class (chlorambucil and melphalan), the chloroethyl-phosphoamides (cyclophosphamide and ifosfamide), and platinum-based agents (carboplatin and cisplatin). In contrast, co-administration of BCNU (a nitrosourea agent) or the unique agent temozolomide, failed to enhance the antitumor activity of irofulven.

The most plausible explanation for the synergistic interaction between irofulven and select DNA damaging agents is the unique nature of the DNA damage induced by irofulven. Repair of DNA damage induced by most alkylating agents requires expression of ERCC1 [28], and inhibition of this enzyme results in sensitivity to alkylating agents [25]. Numerous studies have confirmed that the expression of the ERCC1-XPF complex is important for repair of DNA damage induced by platinum-based agents [24]. Irofulven, however, differs from other DNA-damaging alkylating agents by displaying a greater enhanced cytotoxicity in cell lines deficient in the DNA repair helicases XPD and XPB [20], and by failure of the global genome repair pathway to recognize irofulven-induced DNA lesions [12]. Expression of the latter helicase, XPB, has been deemed critical or rate limiting for NER repair capacity based on mRNA expression and transgenic over expression studies [33].

Thus, the synergistic interaction between irofulven and other DNA damaging agents may stem from the NER system being selectively overwhelmed at two distinct points in

Table 2 The number of MV522 tumor-bearing mice (#N) displaying partial tumor regression (PR) or complete tumor regression (CR) after receiving irofulven in combination with other agents

Group	Dose (mg/kg)	TGI[a] (%)	Mean percentage shrinkage	# N	Mice with partial shrinkage	Mice with complete shrinkage
Expt A: controls	40% DMSO			8	0	0
Irofulven MTD	10	[b]	91	8	7	1
Cyclophosphamide	39	16		8	0	0
2/3 MTD irofulven	6.7	27		8	0	0
2/3 MTD cyclophosphamide	26	17		8	0	0
2/3 MTD Cyclophosphamide + 2/3 MTD Irofulven	26 + 6.7	63	20	8	3	0
Expt B: Controls	40% DMSO			8	0	0
Irofulven MTD	10	65	38	8	2	0
Carboplatin MTD	60	47		8	0	0
2/3 MTD irofulven MTD	6.7	21		8	0	0
2/3 MTD carboplatin	40	3		8	0	0
2/3 MTD carboplatin + 2/3 MTD Irofulven	40 + 6.7	85	78	8	6	1
1/2 MTD Carboplatin + 1/2 MTD Irofulven	30 + 5.0	58		8	0	0
Expt C: controls	40% DMSO			16	0	0
Irofulven MTD	10	52	76	16	3	5
Melphalan MTD	10	75	75	16	11	0
2/3 MTD irofulven	6.7	30	30	16	1	0
2/3 MTD melphalan	6.7	68	71	16	2	0
2/3 MTD melphalan + 2/3 MTD irofulven	6.7 + 6.7	[b]	98	16	14	2
1/2 MTD melphalan + 1/2 MTD irofulven	5.0 + 5.0	72	90	16	10	2
Expt D: controls	40% DMSO			8	8	0
Irofulven MTD	10	[b]	90	8	8	0
Chlorambucil MTD	15	83	25	8	1	0
2/3 MTD irofulven	6.7	63	25	8	3	0
2/3 MTD chlorambucil	10	37		8	0	0
2/3 MTD chlorambucil + 2/3 MTD irofulven	10 + 6.7	[b]	55	8	8	0
1/2 MTD chlorambucil + 1/2 MTD irofulven	7.5 + 5.0	82	61	8	3	0
Expt E: controls	40% DMSO			8	8	0
Irofulven MTD	10	96	63	12	11	0
Altretamine MTD	200	40		6	0	0
2/3 MTD irofulven	6.7	81		6	0	0
2/3 MTD altretamine	133	14		6	0	0
2/3 MTD Altretamine + 2/3 MTD Irofulven	133 + 6.7	[b]	79	6	5	1
1/2 MTD altretamine + 1/2 MTD irofulven	100 + 5.0	88	45	6	4	0
Ifosfamide MTD	100	36		6	0	0
2/3 MTD ifosfamide	67	32		6	0	0
2/3 MTD ifosfamide + 2/3 MTD irofulven	67 + 6.7	[b]	85	6	6	0
1/2 MTD ifosfamide + 1/2 MTD irofulven	50 + 5.0	94	53	6	2	0

Drugs were administered i.p. three times per week for three weeks

[a] Values exclude mice with partial or complete shrinkage of tumor

[b] All treated mice exhibited complete or partial tumor, so the % TGI could not be calculated

the pathway. Helicases already committed to repairing irofulven-induced DNA damage would not be available for continued repair of DNA damage induced by other agents after the critical incision is made by the ERRC1-XPF complex. This delayed repair of DNA damage could result in prolonged stalling of transcription forks and induction of apoptosis [26, 39], the process by which irofulven kills tumor cells [35, 37]. Compatible with this concept is the

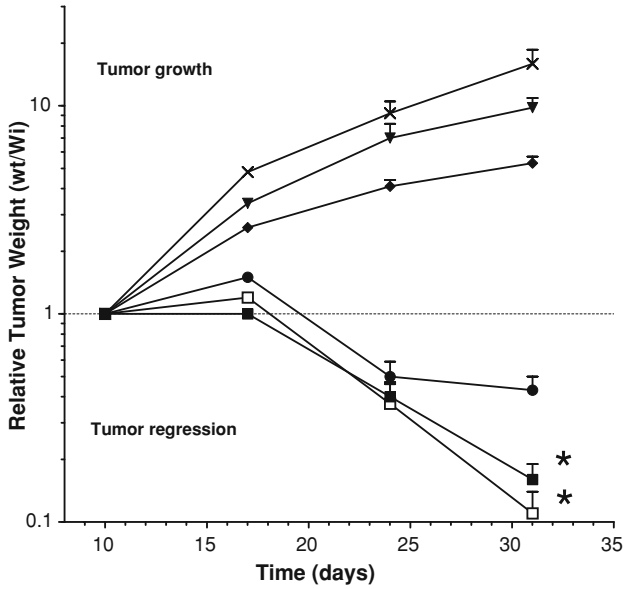

Fig. 5 Efficacy of irofulven in combination with hexamethylmelamine (HMM) or with ifosfamide versus single agent therapy in the MV522 xenograft model. Tumor bearing animals received 10% DMSO/saline as control (*multi symbol*), irofulven at the MTD of 10 mg/kg (*filled circle*), HMM at the MTD of 200 mg/kg (*filled inverted triangle*), ifosfamide at the MTD of 100 mg/kg (*filled diamond*), irofulven at 2/3 MTD + HMM at 2/3 MTD (*filled square*), irofulven at 2/3 MTD + ifosfamide at 2/3 MTD (*open square*). All drugs were administered i.p. three times a week for 3 weeks, starting on day 10 after tumor implantation, and study was terminated on day 31. *Data points* indicate means for each group and *bars* represent SE. The symbol "$_*$" indicates a p value less than 0.05 as compared to the irofulven MTD treated group

finding that topoisomerase inhibitors, expected to further compromise the processing of stalled replication forks, also sensitize cells to irofulven [16, 36]. The variation in ability of alkylating agents to enhance irofulven activity is probably related to the type of DNA damage induced by each agent. It appears that the alkylating agents demonstrating enhanced activity with irofulven are those agents that produce a high percentage of di-adducts.

In summary, irofulven produces DNA damage that is repaired predominately by transcription- and replication-coupled NER processes [12]. The significant synergistic activity of irofulven and combinations of platinum-derived or select DNA damaging agents (such as melphalan or hexamethylmelamine) indicates that further clinical evaluation of these combinations is warranted.

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