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ORIGINAL ARTICLE

Imexon-based combination chemotherapy in A375 human melanoma and RPMI 8226 human myeloma cell lines

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Abstract
Purpose This study evaluated the cytotoxic effects of imexon (NSC-714597) in tumor cells when combined with a broad panel of chemotherapeutic drugs.
Methods The sulforhodamine B (SRB) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assays were used to analyze the degree of growth inhibition for the combination studies in the A375 human malignant melanoma and RPMI 8226 human multiple myeloma cell lines, respectively. Cells were continuously exposed to both drugs at a constant molar ratio for 4–5 days. Combination effects were analyzed using the Median Effect method. Statistical significance was inferred if the 95% confidence interval for the combination interaction (C.I.) values for a particular two-drug combination did not include 1.0 (additivity). Synergy was inferred for C.I. values < 1.0 and antagonism for CI values > 1.0.
Results Imexon was synergistic when combined with DNA-binding agents (cisplatin, dacarbazine, melphalan) and pyrimidine-based antimetabolites (cytarabine, fluorouracil, gemcitabine) in both cell lines. Antagonistic combinations with imexon included methotrexate and the topoisomerase I (TOPO I) and II (TOPO II) inhibitors irinotecan, doxorubicin, mitoxantrone and etoposide. Docetaxel was synergistic with imexon in both cell lines whereas paclitaxel and fludarabine showed a mixed result. Dexamethasone and the proteasome inhibitor bortezomib showed synergy in myeloma cells and additivity in the melanoma cells. The vinca alkaloid, vinorelbine, and the multi-targeted antifol, pemetrexed, were additive with imexon in both cell lines.
Discussion The consistent synergy seen for imexon and alkylating agents may relate to the sulfhydryl-lowering effect of imexon, which would render cells more sensitive to electrophilic species from the alkylators. The marked synergy noted with pyrimidine-based antimetabolites was unexpected and may relate to the induction of cell cycle arrest in S-phase. The strong antagonism noted for imexon with topoisomerase I and II inhibitors may be due to the effect of imexon at increasing oxidant levels which are known to antagonize the cytotoxic effects of topoisomerase poisons. In contrast, the synergy seen with bortezomib in myeloma cells may be related to an increase in reactive oxygen species (ROS) from both drugs. These results suggest that combinations of imexon with alkylating agents and pyrimidine-based antimetabolites are rational to pursue in therapeutic studies in vivo.

Keywords Combination chemotherapy · Imexon · Glutathione · Drug interactions · Synergy

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Introduction

Imexon (4-imino-1,3-diazabicyclo-[3.1.0] hexan-one) is an aziridine-based small molecule that exhibits

antitumor activity in multiple myeloma and melanoma cell lines, animal tumor models, as well as in humans. It is also nonmyelosuppressive in vivo [13, 15]. Imexon has been shown to induce mitochondrial swelling and loss of mitochondrial membrane potential [16, 17]. This is associated with the accumulation of reactive oxygen species (ROS) and activation of the intrinsic pathway of apoptosis, involving caspases 3 and 9 [18]. In the recently completed phase I trial of imexon in patients with advanced cancers, the maximally tolerated dose was 875 mg/m^2/day × 5 days, every other week. This dose produced no myelosuppression ≥ grade 3 [15], suggesting that imexon could be combined with other myelosuppressive anticancer agents at full doses of both drugs.

The advantages of combination chemotherapy have been proven numerous times both in vitro and in vivo [21, 23, 26]. They include the ability to maximize cell death while minimizing toxicity through the use of drugs with nonoverlapping mechanisms of action and toxicities [8, 12, 26]. Therefore, the purpose of this study was to test imexon in combination with other classes of chemotherapy drugs to identify effects that are greater than additive in vitro. Seven alkylating agents were evaluated in this study along with six antimetabolites, four topoisomerase inhibitors, three tubulin-binding agents, the proteasome inhibitor, bortezomib, and the glucocorticosteroid, dexamethasone. The results show consistent patterns of synergy, additivity and antagonism among the different mechanistic classes of anticancer agents when combined with imexon.

Materials and methods

Chemicals

Imexon (NSC 714597) was obtained through a RAID grant to RTD from the National Cancer Institute. It was manufactured to GMP specifications by Series Laboratory Incorporated (Santa Rosa, CA). Cisplatin was obtained from Bayer Corp (Spokane, WA). Cytarabine was purchased from Bedford Laboratories (Bedford, OH); dexamethasone, chlorambucil, and mechlorethamine were purchased from Sigma (St. Louis, MO); doxorubicin was obtained from Fujisawa USA (Deerfield, IL); and dacarbazine (DTIC) was purchased from Bayer Corp (West Haven, CT). Fluorouracil was purchased from Allergan Inc. (Irvine, CA); gemcitabine (Gemzar®) and pemetrexed (Alimta®) were purchased from Eli Lilly and Co. (Indianapolis, IN); melphalan and vinorelbine were obtained from GlaxoWellcome, Inc. (Research Triangle Park, NC); and methotrexate was obtained from Bristol (Syracuse, NY). Paclitaxel, etoposide, carboplatin, and mitomycin C were purchased from Bristol Myers Squibb (Princeton, NJ) and docetaxel (Taxotere®) was obtained from Sanofi-Aventis (Bridgewater, NJ). Mitoxantrone (Novantrone™) was obtained from Wyeth Laboratories (Collegeville, PA). Bortezomib (Velcade®) was purchased from Millenium Pharmaceuticals (Cambridge, MA). Irinotecan was purchased from Pfizer Oncology (New York, NY). Fludarabine was obtained from Berlex Laboratories (Richmond, CA).

Cells and culture conditions

Human malignant melanoma A375 cells (CRL-1619) and human myeloma RPMI 8226 cells (CCL-155) were obtained from the American Type Culture Collection (Manassas, VA). The steroid-sensitive MM.1S multiple myeloma cell line was kindly supplied by Dr. Steven Rosen (Northern University, Chicago, IL) [24]. All cell lines were cultured in RPMI 1640 media (Gibco-BRL Products, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated bovine calf serum (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator containing 5% CO_2 at 37°C.

Cytotoxicity studies

Cytotoxic drug activity was determined using standard 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) or sulforhodamine B (SRB) analysis. The drug concentrations and ratios used in the combination studies were determined from the IC_{50} values of single-drug experiments in each cell line. The drug concentration ranges used for each combination study were developed by making incremental concentration changes above and below the IC_{50} value for each antitumor drug as a single agent. In order to apply the Median Effect synergy calculation, the IC_{50} of each antitumor agent was compared to the IC_{50} value for imexon and a fixed constant ratio was established for use in the subsequent combination drug exposure experiments. Five days after drug addition, 96-well plates containing RPMI 8226 cells were analyzed using the MTT (mitochondrial reductase) assay while plates containing A375 cells were analyzed using the SRB (protein-based) assay [41]. A fixed constant ratio was used to allow a manageable matrix of experiments that could be applied to all classes of drugs.

Simultaneous exposure to both drugs (imexon plus one of the anticancer agents) was chosen as the test method. This was based on the fact that most

chemotherapy combination regimens do not specify particular sequences of drug administration, and thus, most drugs are simultaneously present in the patient's bloodstream.

Median Effect analysis for drug combination

The combination interactions were analyzed using the median effect principle using commercial software (CalcuSyn®, Median Effect Program, Biosoft Corp., Ferguson, MO) [9]. This method is based on Michaelis–Menton kinetics and reduces combination effects to a single numeric indicator, the combination index (C.I.). Synergy is inferred at C.I. values less than 1.0, additivity at C.I. values approximating 1.0 and antagonism at C.I. values greater than 1.0.

Statistical analysis

It is possible to see mixed effects over a range of fraction-effected values. For that reason, the different C.I. values for a particular two-drug combination were analyzed statistically using the 95% confidence interval test. Statistical significance for the different drug combinations was assessed by calculating the 95% confidence interval, for the combination indexes (typically 8–10), across a range of two-drug concentrations effecting cell viability from 100% viability (0 fraction effected) to 1–5% cell viability (99–95% fraction effected). To reduce the bias from large positive combination index values, the C.I. values were first transformed into their natural logarithm for calculation of the 95% confidence intervals. Final results were then reconverted into the antilog of these values for summary reporting in Tables 1 and 2. If the 95% confidence interval range included 1.0, the interaction was deemed additive (the null hypothesis). Those combinations wherein the 95% C.I.s were > 1.0 were deemed antagonistic, and those < 1.0 were deemed synergistic.

Results

RPMI 8226 myeloma cell line

Several classes of anticancer agents demonstrated synergistic activity with imexon in the RPMI 8226 multiple myeloma cell line (Table 1). These include five of the seven alkylating agents tested. Alkylators which showed statistically significant synergy included chlorambucil, cisplatin, dacarbazine, mechlorethamine and melphalan. Figure 1 shows the combination indices plotted as a

Table 1 Median Effect analysis of imexon combinations in RPMI 8226 human multiple myeloma cells

Drug class agent	Molar drug concentration ratio (drug:imexon)	Combination index mean (SD)	95% C.I.	Interaction
DNA-binding agents				
Carboplatin	1:3.2	0.99 (0.04)	0.96–1.06	Additive
Chlorambucil	1:2.3	0.66 (0.16)	0.53–0.82	Synergism[a]
Cisplatin	1:4.85	0.78 (0.06)	0.66–0.92	Synergism[a]
Dacarbazine	1:4.25	0.71 (0.25)	0.65–0.99	Synergism[a]
Mechlorethamine	1:21.7	0.66 (0.12)	0.61–0.73	Synergism[a]
Melphalan	1:1.8	0.65 (0.07)	0.58–0.73	Synergism[a]
Mitomycin C	1:1,347	0.77 (0.60)	0.09–2.46	Additive
Antimetabolites				
Cytarabine	1:110	0.64 (0.24)	0.50–0.81	Synergism[a]
Fludarabine	0.85:1	1.10 (0.12)	1.03–1.13	Antagonism[a]
Fluorouracil	1:7.8	0.52 (0.11)	0.40–0.67	Synergism[a]
Gemcitabine	1:5,000	0.42 (0.21)	0.22–0.85	Synergism[a]
Methotrexate	1:213	1.13 (0.11)	1.02–1.24	Antagonism[a]
Topoisomerase inhibitors				
Doxorubicin	1:150	1.72 (0.76)	1.2–2.94	Antagonism[a]
Etoposide	1:25,877	1.16 (0.06)	1.08–1.44	Antagonism[a]
Irinotecan	1:112.3	1.66 (1.32)	1.59–4.39	Antagonism[a]
Mitoxantrone	1:1,000	1.69 (1.06)	1.30 – 2.21	Antagonism[a]
Tubulin-binding agents				
Docetaxel	1:2,667	0.60 (0.14)	0.52–0.69	Synergism[a]
Paclitaxel	1:533	2.19 (0.89)	1.21–3.07	Antagonism[a]
Vinorelbine	1:500	0.99 (0.28)	0.68–1.43	Additive
Proteasome inhibitor				
Bortezomib	1:4,000	0.73 (0.19)	0.60–0.86	Synergism[a]
Corticosteroid				
Dexamethasone (MM.1S myeloma)	1:2	0.67	0.42–0.93	Synergism[a]

[a] Statistically significant difference from additivity per 95% confidence analysis

Table 2 Median Effect analysis of imexon combinations in A375 human malignant melanoma cells

Drug class agent	Molar drug concentration ratio (drug:imexon)	Combination index mean (SD)	No. of combination doses evaluated	95% C.I.	Interaction
DNA-binding agents					
Carboplatin	1:3.75	0.86 (0.318)	8	0.42–1.63	Additive
Chlorambucil	1:1.8	2.97 (1.56)	8	1.67–3.24	Antagonism[a]
Cisplatin	1:5.6	0.56 (0.20)	10	0.48–0.96	Synergism[a]
Dacarbazine	1:3.36	0.85 (0.16)	12	0.64–1.14	Synergism
Mechlorethamine	1:27	4.18 (2.16)	8	3.41–5.12	Antagonism[a]
Melphalan	1:2.5	0.74 (0.04)	8	0.69–0.79	Synergism[a]
Mitomycin C	1:468	0.75 (0.13)	6	0.61–0.89	Synergism[a]
Antimetabolites					
Cytarabine	1:76	0.54 (0.16)	12	0.39–0.74	Synergy[a]
Fludarabine	1:7.8	0.49 (0.35)	8	0.20–0.71	Synergy[a]
Fluorouracil	1:5	0.33 (0.16)	8	0.16–0.68	Synergy[a]
Gemcitabine	1:4,667	0.66 (0.18)	16	0.47–0.94	Synergism[a]
Methotrexate	1:280	1.29 (0.22)	8	1.16–1.43	Antagonism[a]
Pemetrexed	1:1,000	1.02 (0.77)	7	0.70–1.32	Additive
Topoisomerase inhibitors					
Doxorubicin	1:108	1.56 (0.28)	10	1.39–1.74	Antagonism[a]
Etoposide	1:1,403	1.50 (0.09)	8	1.42–1.58	Antagonism[a]
Irinotecan	1:106	1.44 (0.23)	8	1.28–2.82	Antagonism[a]
Mitoxantrone	1:1,000	1.49 (0.61)	15	1.19–1.81	Antagonism[a]
Tubulin-binding agents					
Docetaxel	1:8,235	0.84 (0.06)	9	0.79–0.88	Synergism[a]
Paclitaxel	1:200	0.92 (0.06)	12	0.85–0.99	Synergism[a]
Vinorelbine	1:483	0.96 (0.14)	8	0.88–1.14	Additive
Proteasome inhibitor					
Bortezomib	1:1,000	0.86 (0.06)	7	0.59–1.27	Additive
Corticosteroid					
Dexamethasone	45:1	1.03 (0.03)	8	0.99–1.07	Additive

[a] Statistically significant difference from additivity per 95% confidence analysis

function of the fractional cell survival for prototypical DNA-binding agent including the platinum-containing agent cisplatin, the bischloroethyl/based alkylators, melphalan and mechlorethanine, and the DNA methylating agent, dacarbazine. Two alkylating agents did not show synergy when combined with imexon in the myeloma cells: carboplatin demonstrated additivity, and mitomycin C demonstrated a mean combination index of 0.77 suggesting moderate synergy, however, the large variability precluded statistical significance (95% C.I. of 0.09–2.46). Synergy was also observed for the combination of imexon with several pyrimidine-based antimetabolites, including cytarabine (Fig. 2a), fluorouracil (Fig. 2d) and gemcitabine (Fig. 2c). In contrast, fludarabine (Fig. 2b) and methotrexate (Fig. 3a) were antagonistic when combined with imexon in the myeloma cells.

In contrast to the alkylating agents and the pyrimidine-based antimetabolites, consistent antagonism was noted for combinations of imexon with all four topoisomerase inhibitors tested in the myeloma cells. These include inhibitors of TOPO I such as irinotecan (Fig. 3b) and inhibitors of TOPO II such as doxorubicin (Fig. 3c), mitoxantrone and etoposide (Fig. 3d). Mixed results were identified when imexon was combined with the tubulin-binding agents: synergy was noted with docetaxel, but the paclitaxel combination was antagonistic. Additivity was observed with vinorelbine in the myeloma cell line (Table 1). The proteasome inhibitor, bortezomib, showed moderate synergy with imexon in the myeloma cells.

Two different myeloma cell lines were evaluated with the combination of imexon and dexamethasone. The RPMI 8226 cell line exhibited additive effects when dexamethasone was combined with imexon (Table 1). Because this cell line is known to be relatively insensitive to glucocorticoid-induced cell death [11], we also evaluated the combination of dexamethasone and imexon in a steroid-sensitive myeloma cell line, MM.1S. In this case, synergy was noted for the combination.

Imexon combinations in the A375 malignant melanoma cell line

Overall, the findings were very similar to the myeloma results in the A375 malignant melanoma cell line (Table 2). Synergy was again noted for imexon and most

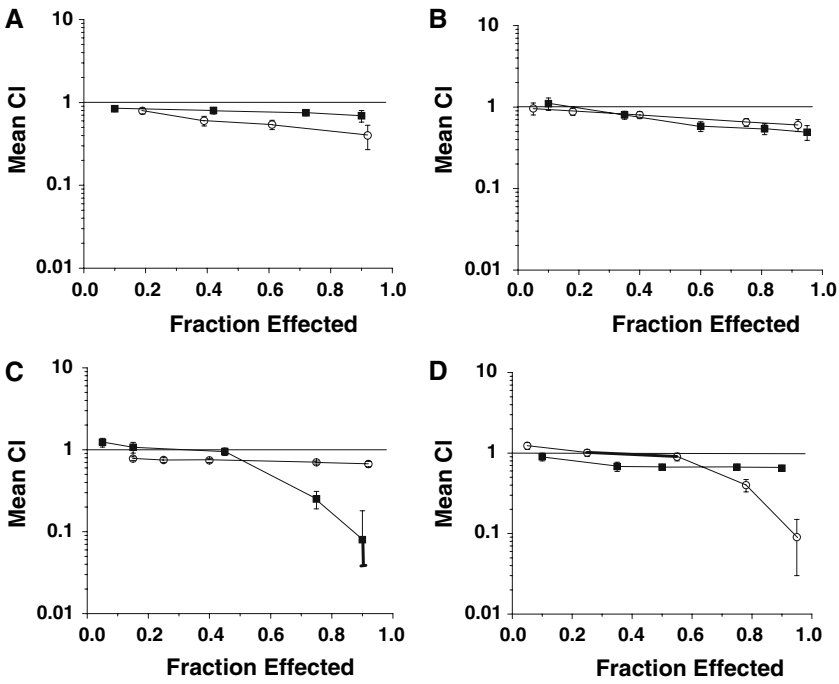

Fig. 1 Combination indices of representative DNA-binding agents with imexon. The mean combination indexes (logarithmic *y-axis*) are plotted versus the fraction of cells effected (% of control growth, *x-axis*), for DNA-binding agents combined with imexon in RPMI 8226 multiple myeloma cells (*closed symbols*) and in A375 malignant melanoma cells (*open symbols*). The *vertical bars* indicate one standard deviation for the combinations of imexon with: **a** cisplatin, **b** dacarbazine, **c** melphalan and **d** mitomycin. The *horizontal bar at 1.0* indicates the line of simple additivity

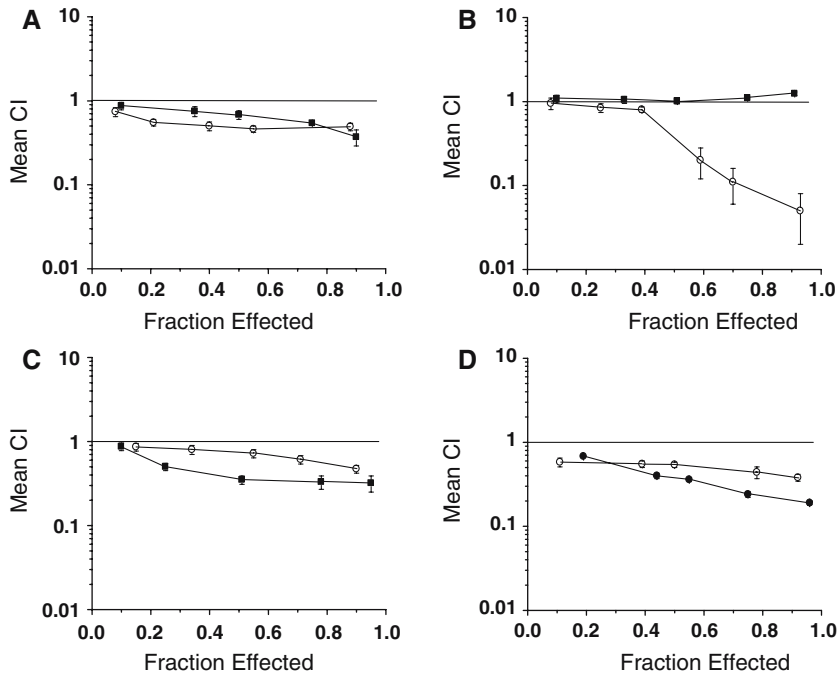

Fig. 2 Combination indices of antimetabolites with imexon. The mean combination indexes (logarithmic *y-axis*) are plotted versus the fraction of cells effected (% of control growth, *x-axis*), for pyrimidine-based antimetabolites combined with imexon in RPMI 8226 multiple myeloma cells (*closed symbols*) and in A375 malignant melanoma cells (*open symbols*). The *vertical bars* indicate one standard deviation for the combinations of imexon with: **a** cytarabine, **b** fludarabine, **c** gemcitabine and **d** fluorouracil

DNA-binding agents (Fig. 1 and Table 2). Carboplatin was again only additive when combined with imexon. One striking exception was the combination of imexon and mechlorethamine (nitrogen mustard), which demonstrated marked antagonism (mean C.I. = 4.18, Table 2). Synergy again was noted with imexon combined with all four pyrimidine-based antimetabolites: cytarabine (Fig. 2a), fludarabine (Fig. 2b), fluorouracil (Fig. 2d) and gemcitabine (Fig. 2c). As in the myeloma cells, pemetrexed showed only additivity, and methotrexate was antagonistic when combined with imexon (Fig. 3a). Similarly, all of the topoisomerase inhibitors were antagonistic when combined with imexon in the melanoma cell line (Fig. 3b–d and Table 2).

One difference with the melanoma cell line as compared to the myeloma cells was the finding that both taxanes were synergistic with imexon (Table 2). The vinca alkaloid vinorelbine was again additive in the

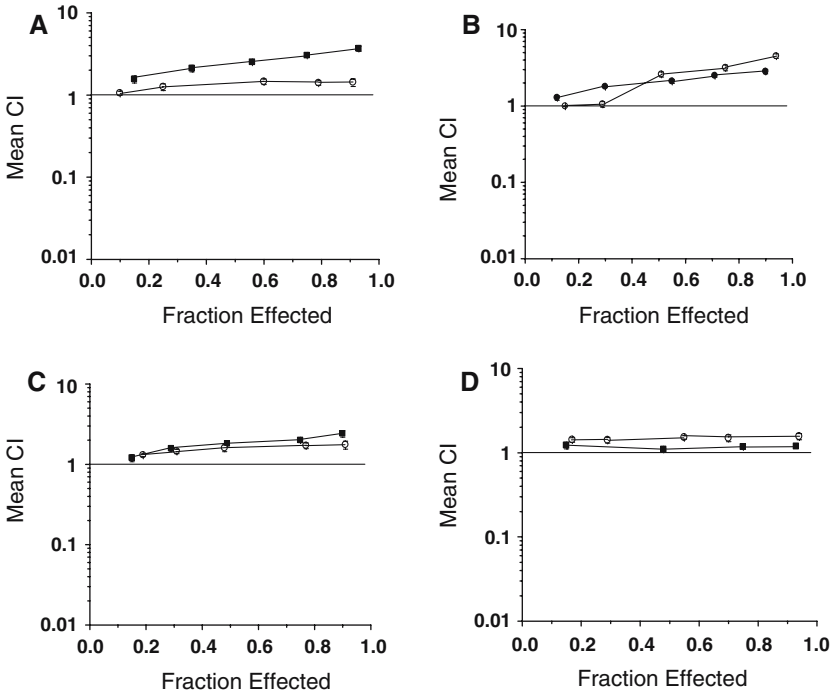

Fig. 3 Combination indices of topoisomerase inhibitors with imexon. The mean combination indexes (logarithmic *y-axis*) are plotted versus the fraction of cells effected (% of control growth, *x-axis*), for agents that were antagonist when combined with imexon in RPMI 8226 multiple myeloma cells (*closed symbols*) and in A375 malignant melanoma cells (*open symbols*). The *vertical bars* indicate one standard deviation for the combinations of imexon with: **a** methotrexate, **b** irinotecan, **c** doxorubicin and **d** etoposide

melanoma cell lines. Other drugs which were additive when combined with imexon in the melanoma cell line include the proteasome inhibitor, bortezomib, and the corticosteroid, dexamethasone (Table 2). The additivity of bortezomib in the melanoma cell line contrasts with the synergy seen with bortezomib seen in the 8226 myeloma cell line.

Discussion

Results of these imexon combination studies in two human tumor cell lines present a common theme. Imexon is synergistic with several anticancer drug classes including alkylating agents and pyrimidine-based antimetabolites. The results with two taxanes were mixed. Bortezomib and dexamethasone were only synergistic in the RPMI 8226 and MM.1S myeloma cell lines, respectively. In contrast, all of the topoisomerase inhibitors, including agents targeting TOPO I and TOPO II, were antagonistic when combined with imexon in both cell lines. The vinca alkaloid, vinorelbine, and the multi-targeted antifol, pemetrexed, were consistently additive when combined with imexon.

The synergy noted for imexon with most of the alkylating agents tested may be due to the effects of imexon on sulfhydryls [27]. Imexon binds to sulfur atoms in reduced thiols resulting in drug–thiol conjugates. In some cases, such as with cysteine, there is formation of a unique thiazoline intermediate [27]. Conversely, when imexon binds to glutathione (GSH) the reaction appears to proceed by the more common nucleophilic attack at the sulfur by the carbonium ion liberated from the aziridine moiety [27]. Imexon has been shown to decrease stores of reduced sulfhydryls in RPMI 8226 myeloma cells exposed to high drug concentrations in vitro [16]. Such a reduction in reduced sulfhydryls should render the cells much less able to neutralize reactive electrophilic species produced by the alkylating agents [3]. Indeed, reduced thiols are documented to be antagonists of several alkylating agents [2–6]. For example, dacarbazine cytotoxicity is synergistically increased when combined with the glutathione synthesis inhibitor, L-buthionine sulfoximine [43], and exogenous thiols directly counteract the cytotoxic effects of decarbazine in cell culture [42]. Glutathione and other sulfhydryls are also known to modulate cellular sensitivity and resistance to the platinum-containing agent, cisplatin [2, 4, 5, 36, 39]. In that regard, the consistent lack of synergy for imexon and carboplatin was striking, but may relate to the slower activation and lower thiol reactivity of carboplatin compared to cisplatin [31, 34]. The alkylating agent, melphalan, also showed synergy in both cell lines when combined with imexon. The relationship between melphalan resistance and an increase in cellular GSH has been shown in many different types of cancer [3], including RPMI 8226 myeloma cells [6].

A glutathione-depletion mechanism likely cannot account for the consistent synergy seen with imexon

and pyrimidine-based antimetabolites. Cytarabine and gemcitabine are primarily metabolized by cytidine deaminase, a ubiquitous enzyme with a known dependence on reduced sulfhydryls for proper activity [7]. However, pilot studies with gemcitabine have suggested that cytidine deaminase activity is not impaired by imexon [40]. Rather, the combined activity of imexon with antimetabolites may relate to the induction of cell cycle arrest in S-phase [16] or G_2-phase [40]. This would allow for greater DNA incorporation of activated metabolites, leading to an enhanced cell kill. A similar cell cycle mechanism has been reported for the synergistic combination of gemcitabine with the ribonucleotide reductase inhibitor, hydroxyurea [44, 45]. Other drugs which have been reported to be synergistic with gemcitabine include pemetrexed [22], bortezomib [30], fluorouracil [10] and oxaliplatin [19]. The molecular basis for these other drug interactions is not known.

Several classes of drugs demonstrated antagonism when combined with imexon: the antifols, represented by methotrexate, and the topoisomerase I and II inhibitors. Methotrexate, which was antagonistic with imexon in both cell lines, is known to require reduced sulfhydryls for optimal cell uptake [32]. The markedly reduced activity of this combination in the current studies may be related to reduced intracellular drug accumulation. In contrast, the marked antagonism imexon seen in combinations of the topoisomerase inhibitors suggests that these agents may have reduced activity in the presence of increased ROS. Indeed, irreversible thiol oxidants such as maleimide and *N*-ethylmaleimide (NEM [29]), can not only inhibit TOPO IIa catalytic activity but also antagonize the cytotoxic effects of TOPO II poisons in several different assays [29]. Maleimide and NEM have been shown to (1) reduce etoposide-induced DNA strand breaks, (2) prevent trapping of α and β isoforms and (3) reduce cytotoxicity in a colony-forming assay [29]. Indeed, maleimide, at noncytotoxic concentrations, was able to efficiently antagonize the cytotoxicity of daunorubicin, idarubicin, mitoxantrone and teniposide in four different murine and human cancer cell lines in vitro [29]. These results suggest that TOPO II requires reduced redox conditions for full inhibition by well-characterized TOPO II poisons. Imexon may therefore be acting similarly to maleimide, by oxidizing SH groups and thereby blocking the stabilization of the TOPO-II DNA-cleavable complexes.

The antineoplastic drug, taxotere, was also synergistic with imexon in the melanoma and myeloma cell lines. It has been demonstrated that increased glutathione-*S*-transferase (GST) activity is related to a cellular resistance to taxotere and that a decrease in GST activity leads to increased taxotere activity [37]. The same changes, favoring increased expression of redox genes including GST, have been observed in the pre-treatment biopsy tissues of breast cancer patients who do not respond to docetaxel [28]. By depleting cellular GSH levels with imexon, GST activity slows, and taxotere activity may thereby increase. In contrast, paclitaxel was antagonistic in the myeloma cell line (mean C.I. = 2.19) and only moderately synergistic in the melanoma cell line (mean C.I. = 0.92). The antagonism with paclitaxel in the RPMI 8226 myeloma cells is similar to a previous study involving MCF-7 human breast adenocarcinoma cells and A549 human lung adenocarcinoma, which demonstrated that GSH depletion by BSO leads to antagonism of paclitaxel cytotoxicity [33]. No clear mechanism is known to account for the modest synergy noted for paclitaxel and imexon in the melanoma cell line.

Dexamethasone and imexon produced additive effects in the A375 melanoma cells, additive effects in the RPMI 8226 myeloma cells and synergy in the steroid-responsive MM.1S myeloma cells. These data suggest that inherent sensitivity to corticosteroid-induced apoptosis is required for synergy between imexon and glucocorticosteroids.

The synergy noted for the combination of imexon and bortezomib in the myeloma cell line may involve additive effects on the generation of ROS, as bortezomib has been shown to increase ROS in tumor cells [35, 38]. Furthermore, the superoxide inhibitor, Tiron® (disodium 4,5-dihydroxybenzene-1,3-disulfonate), has been shown to block bortezomib-induced cytotoxicity in human tumor cell lines, including melanoma cell lines [21]. Another agent which produces ROS and was shown to increase cytotoxicity from bortezomib is the nonsteroidal anti-inflammatory drug sulindac [35]. Thus, a likely mechanism for the synergy between bortezomib and imexon is increased production of ROS by both drugs.

Because simultaneous drug exposures were used in these studies, different results might be obtained if different sequences of drug exposure were evaluated [1]. However, true sequence specificity for clinical drug combinations is rare, and therefore simultaneous drug exposure probably best approximates the clinical setting of combination chemotherapy. Recent studies in SCID mice bearing human MiaPaCa2 pancreatic cancer cells have shown that the synergistic interaction between imexon and gemcitabine can be maintained when both drugs are administered concurrently on a weekly schedule, thereby obviating the need for repeated daily imexon injections. This schedule is

currently being used in the phase I trial of imexon and gemcitabine, with both agents administered IV on days 1, 8 and 15 of a 4-week cycle.

The current findings suggest that further therapeutic studies with imexon should be aimed at combinations with at least four anticancer drug classes: (1) the alkylating agents, (2) pyrimidine-based antimetabolites, (3) taxotere and (4) bortezomib. Imexon combinations with topoisomerase I and II inhibitors and combinations with methotrexate were consistently antagonistic and should be avoided. Based on these findings, two phase I trials are currently underway with imexon in patients with advanced malignancies: a combination of imexon with gemcitabine in metastatic pancreatic cancer and imexon with dacarbazine in patients with metastatic malignant melanoma.

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References

1. Adel AL, Dorr RT, Liddil JD (1993) The effect of anticancer drug sequence in experimental combination chemotherapy. Cancer Investig 11:15
2. Andrews PA, Murphy MP, Howell SB (1989) Characterization of cisplatin resistance in COLO 316 human ovarian carcinoma cells. Eur J Cancer Clin Oncol 25:619
3. Arrick BA, Nathan CF (1984) Glutathione metabolism as a determinant of therapeutic efficacy: a review. Cancer Res 44:4224
4. Bedford P, Walker MC, Sharma HL, Perera A, McAuliffe CA, Masters J, Hill BT (1987) Factors influencing the sensitivity of two human bladder carcinoma cell lines to *cis*-diamminedichloroplatinum(II). Chem Biol Interact 61:1
5. Behrens BC, Hamilton TC, Masuda H, Grotzinger KR, Whang-Peng J et al (1987) Characterization of a *cis*-diamminedichloroplatinum(II) resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. Cancer Res 47:414
6. Bellamy WT, Dalton WS, Gleason MC, Grogan TM, Trent JM (1991) Development and characterization of a melphalan-resistant human multiple myeloma cell line. Cancer Res 51:995
7. Camiener GW (1967) Studies of the enzymatic deamination of cytosine arabinoside-II. Biochem Pharmacol 16:1681
8. Capizzi RL, Keiser LW, Sartorelli AC (1977) Combination chemotherapy—theory and practice. Semin Oncol 4:227
9. Chou TC, Talalay P (1984) Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27
10. Correal P, Cerretani D, Marsili S, Pozzessere D, Petrioli R, Messinese S, Sabatino M, Roviello F, Pinto E, Francini G, Giorgi G (2003) Gemcitabine increases systemic 5-fluorouracil exposure in advanced cancer patients. Eur J Cancer 39:1547
11. Dalton WS, Durie BG, Alberts DS, Gerlach JH, Cress AE (1986) Characterization of a new drug-resistant human myeloma cell line that expresses P-glycoprotein. Cancer Res 46:5125
12. DeVita VT, Young RC, Canellos GP (1975) Combinations versus single agent chemotherapy: a review of the basis for selection of drug treatment of cancer. Cancer 35:98
13. Dorr RT, Liddil JD, Klien MK, Hersh EM (1995) Preclinical pharmacokinetics and antitumor activity of imexon. Investig New Drugs 13:113
14. Dorr RT, Raymond MA, Landowski TH, Roman NO, Fukushima S (2005) Induction of apoptosis and cell cycle arrest by imexon in human pancreatic cancer cell lines. Int J Gastrointest Cancer 36:15
15. Dragovich T, Gordon M, Mendeslon D, Wong L, Modiano M, Chow S, Samulitis B, O'Day S, Greiner K, Hersh E, Dorr R (2007) Phase I trial of imexon in patients with advanced malignancy. J Clin Oncol (in press)
16. Dvorakova K, Payne CM, Tome ME, Briehl MM, McClure T, Dorr RT (2000) Induction of oxidative stress and apoptosis in myeloma cells by the aziridine-containing agent imexon. Biochem Pharmacol 60:749
17. Dvorakova K, Waltmire CN, Payne CM, Tome ME, Briehl MM, Dorr RT (2001) Induction of mitochondrial changes in myeloma cells by imexon. Blood 97:3544
18. Dvorakova K, Payne CM, Landowski TH, Tome ME, Halperin DS, Dorr RT (2002) Imexon activates an intrinsic apoptosis pathway in RPMI 8226 myeloma cells. Anticancer Drugs 13:1
19. Faivre S, Raymond E, Woynarowski JM, Cvitkovic E (1999) Supraadditive effect of 2′.2′-difluorodeoxycytidine (gemcitabine) in combination with oxaliplatin in human cancer cell lines. Cancer Chemother Pharmacol 44:117
20. Fernandez Y, Miller TP, Denoyelle C, Esteban JA, Tang W-H, Benstson AL, Soengas ME (2006) Chemical blockage of the proteasome inhibitory function of bortezomib: impact on tumor cell death. J Biol Chem 281:1107–1118
21. Frei E (1972) Combination cancer therapy: presidential address. Cancer Res 32:2593
22. Giovannetti E, Mey V, Danesi R, Mosca I, Del Tacca M (2004) Synergistic cytotoxicity and pharmacogenetics of gemcitabine and pemetrexed combination in pancreatic cancer cell lines. Clin Cancer Res 10:2936
23. Goldin A (1980) Combined chemotherapy. Oncology 37:3
24. Greenstein S, Krett NL, Kurosawa Y, Ma C, Chauhan D, Hideshima T, Anderson KC, Rosen ST (2003) Characterization of the MM.1 human multiple myeloma (MM) cell lines: a model system to elucidate the characteristics, behavior, and signaling of steroid-sensitive and -resistant MM cells. Exp Hematol 31:271
25. Heinemann V, Hertel LW, Grindey GB, Plunkett W (1988) Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. Cancer Res 48(14):4024
26. Henderson EH, Samaha RJ (1969) Evidence that drugs in multiple combinations have materially advanced the treatment of human malignancies. Cancer Res 29:2272
27. Iyengar BS, Dorr RT, Remers WA (2004) Chemical basis for the biological activity of imexon and related cyanoaziridines. J Med Chem 47:218
28. Iwao-Koizumi K, Matoba R, Ueno N, Kim SJ, Ando A, Miyoshi Y, Maeda E, Noguchi S, Kato K (2005) Prediction of docetaxel response in human breast cancer by gene expression profiling. J Clin 23:422
29. Jenson LH, Renodon-Corniere A, Wessel I, Langer SW, Sokilde B, Carstensen EV, Sehested M, Jensen PB (2002) Maleimide is a potent inhibitor of topoisomerase II in vitro and in vivo: a new mode of catalytic inhibition. Mol Pharmacol 61:1235

30. Kamat AM, Karashima T, Davis DW, Lashinger L, Bar-Eli M, Millikan R, Shen Y, Dinnery CPN, McConkey DJ (2004) The proteasome inhibitor bortezomib synergizes with gemcitabine to block the growth of human 253JB-V bladder tumors in vivo. Mol Cancer Ther 3:279
31. Knox RJ, Friedlos F, Lydall DA et al (1986) Mechanism of cytotoxicity of anticancer platinum drugs: evidence that *cis*-diamminedichloro-platinum(II), and *cis*-diammine-(1,1-cyclobutanedicarboxylato)-platinum(II) differ only in the kinetics of their interactions with DNA. Cancer Res 46:1972
32. Leszczynska A, Pfaff E (1982) Activation by reduced glutathione of methotrexate transport into isolated rat liver cells. Biochem Pharmacol 31:1911
33. Liebmann JE, Hahn SM, Cook JA, Lipschulz C, Mitchell JB, Kaufman DC (1993) Glutathione depletion by L-buthionine sulfoximine antagonizes taxol cytotoxicity. Cancer Res 53:2066
34. Micetich KC, Barnes D, Erickson LC (1985) A comparative study of the cytotoxicity and DNA-damaging effects of *cis*-(diammino)(1,1-cyclobutanedicarboxylato)-platinum(II) and *cis*-diamminedichloroplatinum(II) on L1210 cells. Cancer Res 45:4043
35. Minami T, Adachi M, Kawamura R, Zhang Y, Shinomura Y, Imai K (2005) Sulindac enhances the proteasome inhibitor bortezomib-mediated oxidative stress and anticancer activity. Cancer Ther Preclin 11:5248
36. Mistry P, Kelland LR, Abel G, Sidhar S, Harrap KR (1991) The relationships between glutathione, glutathione-*S*-transferase and cytotoxicity of platinum drugs and melphalan in eight human ovarian carcinoma cell lines. Br J Cancer 64:215
37. Park JS, Yamamoto W, Sekikawa T, Matsukawa M, Okamoto R, Sasaki M, Ukon K, Tanimoto K, Kumazaki T, Nishiyama M (2002) Cellular sensitivity determinants to docetaxel in human gastrointestinal cancers. Int J Oncol 20:333
38. Pei XY, Dai Y, Grant S (2004) Synergistic induction of oxidative injury and apoptosis in human multiple myeloma cells by the proteasome inhibitor bortezomib and histone deacetylase inhibitors. Clin Cancer Res 10:3839
39. Richon VM, Schulte N, Eastman A (1987) Multiple mechanisms of cellular resistance to platinum coordination complexes. Cancer Res 47(8):2056
40. Roman NO, Fukushima S, Raymond MA, Landowski T, Dorr RT (2005) Imexon and gemcitabine are synergistic against human pancreatic cancer cells in vitro and in vivo. In: AACr 96th annual meeting, abst # 5884, vol 46, p 1385
41. Rubinstein LV, Shoemaker RH, Paull KD, Simon RM, Tosini S, Skehan P, Scudiero DA, Monks A, Boyd MR (1990) Comparison of in vitro anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. J Natl Cancer Inst 82:1113
42. Saunders PP, Schultz GA (1970) Studies of the mechanism of action of the antitumor agent 5(4)-(3,3-dimethyl-1-triazeno) imidazole-4(5)-carboxamide in *Bacillus subtilis*. Biochem Pharmacol 19:911
43. Thrall BD, Raha GA, Springer DL, Meadows GG (1991) Differential sensitivities of murine melanocytes and melanoma cells to buthionine sulfoximine and anticancer drugs. Pigment Cell Res 4:234
44. Wong SJ, Myette MS, Wereley JP, Chitambar CR (1999) Increased sensitivity of hydroxyurea-resistant leukemic cells to gemcitabine. Clin Cancer Res 5:439
45. Zhou B, Mi S, Mo X, Shih J, Tsai J, Hu E, Hsu M, Kay K, Yen Y (2002) Time and sequence dependence of hydroxyurea in combination with gemcitabine in human KB cells. Anitcancer Res 22:1369