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## Preclinical report

# Enhancement of fotemustine (Muphoran®) cytotoxicity by amifostine in malignant melanoma cell lines

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**Fotemustine (Muphoran®, S10036), a nitrosourea derivative active in the treatment of malignant melanoma and primary brain tumors, was evaluated in combination with the free radicals cytoprotective agent amifostine (Ethyol®, WR-2721) and its alkaline phosphatase (AP)-generated active metabolite WR-1065 in four human melanoma (RPMI-7950, SK-MEL2, SK-MEL5 and WM-115) and lung fibroblast (MRC-5) cell lines. No difference in AP activity was found among the melanoma cell lines, but AP was found to be significantly higher in MRC-5. For combination experiments, cell lines were first exposed to amifostine or WR-1065 for 15 min and then exposed to fotemustine for two cell doubling times. Non-cytotoxic amifostine and WR-1065 concentrations used (0.2 and 0.6 and 0.1 and 0.3 mmol/l, respectively) were deduced from clinically achieved plasma values. Interactions were analyzed from the variations in $IC_{50}$ of fotemustine induced by pre-exposure of the cells to amifostine or WR-1065. In all melanoma cell lines, amifostine enhanced the cytotoxic activity of fotemustine as a significant decrease in $IC_{50}$ was observed. No significant difference was found between synergistic effects achieved with amifostine and WR-1065 given at half concentrations. No differential effect was found in the MRC-5 cell line as compared with the melanoma cell lines. Expression variation of $O^6$-methylguanine methyltransferase was not found to be implicated in the interaction. The present results demonstrating that amifostine or its main active metabolite do not impair the cytotoxicity of fotemustine justify an extensive clinical evaluation of this combination in metastatic melanoma. [© 2002 Lippincott Williams & Wilkins.]**

*Key words:* Amifostine, fotemustine, melanoma cells.

## Introduction

Fotemustine (Muphoran®, S10036; Servier, Courbevoie, France) is a lipophilic chloroethylnitrosourea derivative whose therapeutic activity was demonstrated in disseminated melanoma including those with brain metastases[1–3] as well as primary brain tumors.[4] Its mechanism of action consists mainly in inducing DNA strand breaks and cross-linking, including DNA–protein cross-linking.[5] More recently,[6] fotemustine was found to be active in pediatric medulloblastoma xenografts in relation to the expression level of $O^6$-methylguanine methyltransferase (MGMT). Clinical trials investigated fotemustine in association with temozolomide, which can deplete MGMT, in patients with malignant melanomas and gliomas,[7] and it was found not to modify the pharmacokinetics of temozolomide. The dose-limiting toxicity of fotemustine is a delayed, cumulative and dose-related thrombocytopenia and leukopenia.[9] Additional side effects of fotemustine consist of liver toxicity with a transient increase in transaminases and/or alkaline phosphatase (AP). The cytoprotective agent amifostine (Ethyol®, WR-2721; Schering Plough, Levallois-Perret, France), able to modulate the toxicity of alkylating agent-based chemotherapy,[9] offers new therapeutic alternatives for the management of fotemustine hematological and non-hematological toxicities.

Amifostine is an organic ethylphosphorothioic acid derivative, which was originally developed as a radioprotectant and used in oncology as a protective agent against the toxic effects of platinum derivatives, alkylating agents and radiation therapy.[10] *In vivo*, amifostine is rapidly dephosphorylated by APs into the free-thiol compound WR-1065, serving as a target for nucleophilic attack by activated alkylating agent intermediates.[11] Amifostine-induced cytoprotection appeared to be selective for normal tissues because of higher vascularization and a higher metabolization rate related to higher AP expression.[9] Clinically, amifostine was reported to protect melanoma

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patients from the toxic hematological and non-hematological side effects of fotemustine in a pilot phase I study.[12]

Furthermore, amifostine was reported to potentiate the *in vivo* antitumor activity of several drugs such as nitrogen mustard derivatives,[13,14] cisplatin combined with vinblastine[15] and carboplatin, alone[16] or combined with 5-fluorouracil.[17] A recent *in vitro* study[18] highlighted that amifostine and its metabolite WR-1065 could interfere with cell cycle progression at the $G_1$/S check-point. The present study was designed to investigate the influence on fotemustine cytotoxicity of *in vitro* pre-treatment of human melanoma cell lines and normal lung fibroblasts by amifostine or its metabolite WR-1065.

## Materials and methods

### Drugs and chemicals

Fotemustine (Muphoran®, S10036) was kindly provided by Institut de Recherches Internationales Servier. Amifostine (Ethyol®, WR-2721) and its AP-generated metabolite WR-1065 were kindly obtained from Schering Plough Laboratories. All culture media and reagents were purchased from Life Technologies (Cergy-Pontoise, France). Unless indicated, all reagents were obtained from Sigma (St Quentin Fallavier, France).

### Cell lines

Four human melanoma cell lines (RPMI-7950, SK-MEL2, SK-MEL5 and WM-115) were purchased from ATCC (Rockville, MD), and lung fibroblast (MRC-5) cell line from Eurobio (Les Ulis, France). All cell lines were cultured at 37°C, in phenol red-free RPMI 1640 medium supplemented with 10% fetal calf serum and in a 5% $CO_2$ atmosphere.

### AP expression

AP expression was analyzed using the *p*-nitrophenol colorimetric assay (Sigma) and normalized to protein content in the cell extracts determined using the bicinchoninic acid colorimetric assay (Pierce, Interchim, Montluçon, France). As positive control, phorbol ester-differentiated HCT-8 colon carcinoma cell line was used.

### MGMT expression

Exponentially growing cells ($10^6$) were collected and stored as cell pellets in dry ice until analyzed.

MGMT constitutive expression was analyzed as previously described.[19,20] Briefly, supernatants from cell extracts prepared by sonication were incubated with [$^3$H]methylnitrosourea methylated calf serum DNA. MGMT expression was determined by measuring the transfer of tritiated methyl groups from DNA to the protein fraction containing MGMT in cell extracts. Results are expressed as fmol/mg protein. MGMT mRNA expression was analyzed according to Rohlion *et al.*[21] using semi-quantitative RT-PCR with $\beta_2$-microglobulin as reference gene. Briefly, total RNA was extracted using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed at 42°C for 30 min using Superscript II reverse transcriptase (Life Technologies).

Primers used were: MGMT, 5′-CCTGG CTGAATGCCTATTTC-3′ and 5′-CAGCTTCCATAACA CCTGTCTG-3′; $\beta_2$-microglobulin, 5′-CATCCAGCGTACTCCAAAGA-3′ and 5′-GACAAGTCTGAATGCTCCAC-3′. PCR reactions were performed as followed 94°C for 1 min, then 35 cycles 94°C for 50 s, 57°C for 50 s and 72°C for 20 s using a PHC3 thermocycler (Techne, Osi, Paris, France). PCR products were then electrophoresed on 2% agarose gel (Metaphor agar; TEBU, Le Perray en Yvelines, France) stained with ethidium bromide and analyzed using image analysis (Geldoc1000; BioRad, Ivry sur Seine, France). Results were expressed as mean MGMT/$\beta_2$-microglobulin mRNA expression ratio (RER).

### Cell proliferation assays

Cell proliferation assays were performed using MTT (Sigma) as previously described.[22] Briefly, cell suspensions containing $2 \times 10^4$ viable cells/ml were plated into 96-well dishes and allowed to attach for 48 h at 37°C in a 5% $CO_2$ atmosphere. The culture medium was then removed and the cells were incubated for two doubling times at 37°C in the culture medium containing fotemustine ($10^{-3}$ to 10 mmol/l). When tested, amifostine or WR-1065 ($10^{-4}$ to 10 mmol/l) was added before fotemustine for 15 min. Control cultures were exposed to saline for 15 min and then allowed to grow for the same duration in complete culture medium.

MTT (final concentration 5 $\mu$mol/l) was then added into each well and the dishes were incubated for 3 h at 37°C to allow MTT metabolization into formazan

crystals. The crystals were finally solubilized by adding 50 $\mu$l of 25% sodium dodecylsulfate solution to each well. Blank control wells with no cells also received the same reagents. Absorbance was finally measured at 540 nm using a Multiskan MCC340 plate reader (Flow Laboratories, Les Ulis, France). Absorbance of blank control was subtracted from the absorbance values in the other wells. Fotemustine concentrations inhibiting 50% ($IC_{50}$) of the growth of cells were calculated using the median-effect analysis.[23]

### Doubling time

Cell doubling times were estimated from 8-day MTT assay growth curves of exponentially growing cell cultures seeded with $5 \times 10^3$ and $10^4$ cells/ml.

### Statistical analysis

Each experiment was repeated at least 3 times and, for cytotoxicity assays, the results expressed as mean values of a minimum of eight wells in triplicated assays. Student's *t*-test was employed to determine the statistical significance with a limit set to $p < 0.05$ using Statview 4.02 software (Abacus Concepts, Berkeley, CA).

## Results

### Cell lines characteristics

No significant difference was found between the AP activity (Table 1) in all melanoma cell lines and in the cell-free culture medium (negative control). In the differentiated HCT-8 cell line slight AP activity, approximately 2-fold the background noise value, was observed. In MRC-5 fibroblasts, AP activity was significantly higher and found to reach approximately 20-fold the background noise value.

The doubling times of the tumor cell lines (Table 2) varied from 18 h for WM115 cell lines to 72 h for SKMEL-2 cell lines. In MRC-5 cells, the doubling time was 27 h.

Constitutive MGMT expression was determined in MRC-5, SKMEL-2, SKMEL-5 and WM115 cells (Table 2). Melanoma cells expressed MGMT at similar levels (80–160 fmol/mg protein), while MRC-5 cells expressed at higher level (approximately 240 fmol/mg protein). No relationship was found between MGMT expression and the doubling time.

### Drug cytotoxicity

In all cell lines (Table 3), no significant cytotoxicity (less than 10% growth inhibition) was achieved with either amifostine or its metabolite WR-1065 at concentrations ranging from $10^{-4}$ to 10 mmol/l. In cell lines exposed to fotemustine ($10^{-3}$ to 10 mmol/l for 6 days) cytotoxicity was detected in all cell lines with $IC_{50}$ ranging from 0.05 to 0.18 mmol/l. No difference in $IC_{50}$ was observed between normal MRC-5 cells (0.05 mmol/l) and the most sensitive melanoma cell line WM-115 (0.05 mmol/l), having

**Table 1.** Alkaline phosphatase expression

| Specimen | AP ($U \times 10^3$/mg protein)[a] |
|---|---|
| Culture medium | 1.1 |
| HCT-8 | 1.3 |
| Differentiated HCT-8[b] | 2.7 |
| MRC-5 | 25.9 |
| RPMI-7951 | 0.8 |
| SKMEL-2 | 1.7 |
| SKMEL-5 | 1.1 |
| WM-115 | not determined |

[a]Exponentially growing cell cultures were analyzed for AP expression using the *p*-nitrophenol colorimetric assay and protein content using the bicinchoninic acid colorimetric assay.
[b]Phorbol ester-differentiated human HCT-8 colorectal cell line was used as positive control.

**Table 2.** Doubling time and constitutive MGMT mRNA and protein expression

| Specimen | Doubling time (h) | MGMT/$\beta_2$-microglobulin RER | MGMT (fmol/mg protein) |
|---|---|---|---|
| MRC-5 | 27 (3)[a] | 1.25 (0.22)[b] | 238 (28) |
| RPMI-7951 | 56 (6) | 0.82 (0.15) | 185 (22) |
| SKMEL-2 | 72 (8) | 0.84 (0.19) | 86 (12) |
| SKMEL-5 | 64 (6) | 1.02 (0.23) | 154 (18) |
| WM-115 | 18 (3) | 1.21 (0.25) | 127 (15) |

[a]Results are expressed as mean values (standard deviation) calculated from three independent experiments.
[b]Results are expressed as mean relative MGMT/$\beta_2$-microglobulin RER (standard deviation) calculated from three independent experiments.

**Table 3.** Cytotoxicity ($IC_{50}$) of fotemustine, amifostine (WR-2721) and its metabolite (WR-1065)

| Cell line | Fotemustine (mmol/l)[a] | WR-2721 (mmol/l)[b] | WR-1065 (mmol/l)[b] |
|---|---|---|---|
| MRC-5 | 0.05 (0.01) | NA[c] | NA |
| RPMI-7951 | 0.15 (0.01) | NA | NA |
| SKMEL-2 | 0.18 (0.02) | NA | NA |
| SKMEL-5 | 0.18 (0.02) | NA | NA |
| WM-115 | 0.05 (0.01) | NA | NA |

[a]Cells were continuously exposed for 6 days to fotemustine ($10^{-3}$ to 10 mmol/l) and then submitted to MTT assay. Results are mean $IC_{50}$ values (standard deviations) calculated from at least three independent determinations.
[b]Cells were exposed for 15 min to amifostine (WR-2721) or its metabolite (WR-1065) at concentrations ranging from $10^{-4}$ to 10 mmol/l and then submitted to MTT assay.
[c]Not achieved within the concentration range evaluated ($IC_{10} > 10$ mmol/l)

**Table 4.** Potentiation factors achieved when combining amifostine (WR-2721) or its metabolite (WR-1065) at non-cytotoxic concentrations with fotemustine

| | WR-2721 (mmol/l)[a] | | WR-1065 (mmol/l) | |
|---|---|---|---|---|
| | 0.2 | 0.6 | 0.1 | 0.3 |
| MRC-5 | 17 (2.0)[b] | 11 (1.8) | 25 (3.2) | 12.5 (1.5) |
| RPMI-7951 | 4.2 (0.5) | 5.5 (0.6) | 3.6 (0.5) | 2 (0.3) |
| SK-MEL2 | 20 (2.2) | 14 (1.6) | 17 (1.9) | 12.5 (2.0) |
| SK-MEL5 | 3.5 (0.5) | 4.8 (0.7) | 5.9 (0.7) | 3.6 (0.5) |
| WM-115 | 3 (0.6) | 2.9 (0.5) | 1.4 (0.2) | 1.2 (0.9) |

[a]Cells were exposed for 15 min to amifostine (WR-2721) or its metabolite (WR-1065), rinsed and then continuously exposed for 6 days to fotemustine ($10^{-3}$ to 10 mmol/l).
[b]Potentiation factors were calculated as the ratio of the $IC_{50}$ of fotemustine alone with the $IC_{50}$ of fotemustine combined with the cytoprotective agent. Results are mean values (standard deviations) calculated from at least three independent determinations using MTT assays.

the shortest doubling times (27 and 18 h, respectively). RPMI-7951, SKMEL-2 and SKMEL-5, having approximately 3-fold longer doubling times, were found to be approximately 3-fold less sensitive to fotemustine with $IC_{50}$ of 0.15, 0.18 and 0.18 mmol/l, respectively.

No relationship was found between fotemustine cytotoxicity and MGMT constitutive expression.

## Drug combinations

Due to the lack of cytotoxicity of WR-2721 and WR-1065 used alone in all cell lines, the concentrations to be combined with fotemustine were chosen from peak plasma concentrations ($C_{max}$) achieved in patients receiving amifostine at 815 mg/m$^2$ in 15 min i.v. infusion,[24] being 0.4 and 0.2 mmol/l, respectively, for WR-2721 and WR-1065. Therefore, in the following experiments, all cell lines were exposed to WR-2721 and WR-1065 concentrations of 0.2 and 0.6 and 0.1 and 0.3 mmol/l, respectively, for 15 min followed by fotemustine ($10^{-3}$ to 10 mmol/l) for two doubling times. WR-2721 and WR-1065 concentrations were considered to be non-cytotoxic in the cell line panel.

The results achieved with the drug combinations showed that neither WR-2721 nor WR-1065 exerted any antagonistic effect on fotemustine cytotoxicity. In all cases, whatever the cell line, exposure of the cells to WR-2721 or WR-1065 induced a statistically significant decrease in fotemustine $IC_{50}$. The interaction observed varied from additivity to a clear synergism with potentiation factors ranging from 1.2 to 25 (Table 4).

Regarding the potentiation factor, no statistical difference was found between WR-2721 and WR-1065, suggesting that the interaction could be independent of WR-2721 biotransformation.

No relationship was found between the potentiation observed and any of the biological parameter measured (AP activity, MGMT expression, doubling time).

## MGMT mRNA expression variation

No variation in MGMT expression was detected in RPMI-7951 cells exposed to WR-2721 (0.6 mmol/l) or WR-1065 (0.3 mmol/l) for 15 min, to fotemustine (0.15 mmol/l) for 2 to 18 h and to the drug combinations (Table 5) (Fig. 1).

**Table 5.** MGMT mRNA expression in the RPMI-7951 cell line exposed to fotemustine alone or preceded by exposure to WR-2721 or WR-1065

| Fotemustine (0.15 mmol/l) | Cytoprotective agent-free control | WR-2721 (0.2 mmol/l, 15 min) | WR-1065 (0.1 mmol/l, 15 min) |
|---|---|---|---|
| Drug-free control | 0.88 (0.13)[a] | 1.05 (0.16) | 1.02 (0.12) |
| 2 h | 0.81 (0.12) | 0.96 (0.16) | 0.96 (0.10) |
| 4 h | 0.83 (0.13) | 0.92 (0.13) | 0.91 (0.09) |
| 6 h | 0.92 (0.17) | 0.95 (0.11) | 0.88 (0.08) |
| 18 h | 0.89 (0.12) | 1.02 (0.17) | 0.90 (0.13) |

[a]Results are expressed as mean relative MGMT/$\beta_2$-microglobulin RER (standard deviation) calculated from three independent experiments.

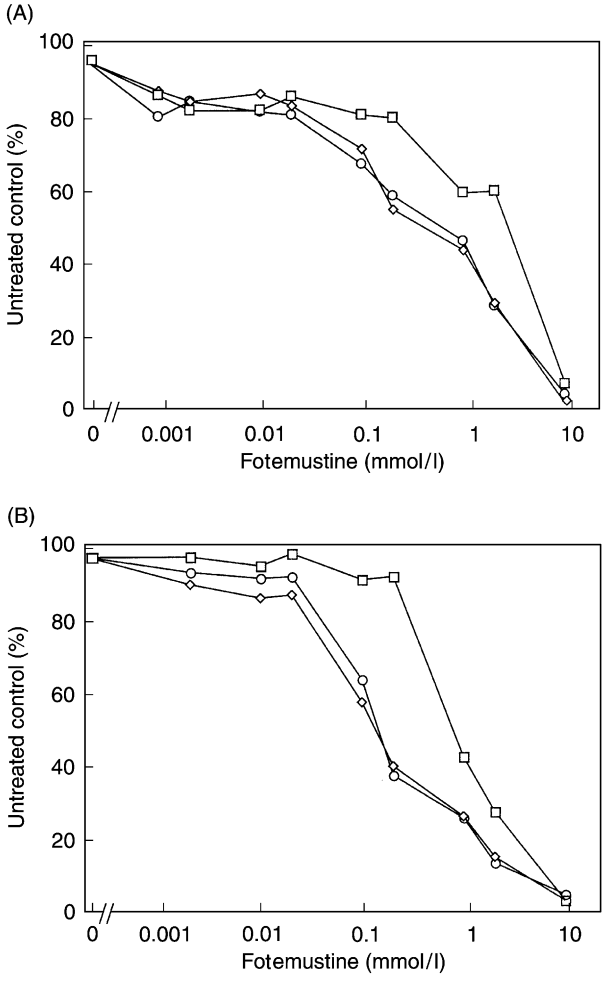


**Figure 1.** Representative dose–response curves of drug combinations. MRC-5 (A) and RPMI-7951 (B) cells were exposed to fotemustine alone (squares) or incubated for 15 min with non-cytotoxic concentrations of the cytoprotective agents amifostine (WR-2721, 0.6 mmol/l; diamonds) or its metabolite (WR-1065, 0.3 mmol/l; circles) and then exposed to fotemustine. Each point is the mean value of at least three experiments performed 6 times. Error bars were always lower than 15% of the mean.

## Discussion

Fotemustine is a nitrosourea derivative whose activity has been demonstrated in disseminated melanoma and adult primary brain tumors in humans.[1–3] Its mode of action has been reported to involve DNA alkylation of $O^6$-guanine and interstrand cross-linking[5] resulting in cell cycle perturbation with $G_2$/M block.[25,26] Amifostine (WR-2721) is a chemoprotective compound acting *in vivo* after metabolization by APs into its dephosphorylated free-thiol derivative WR-1065. Chemoprotection occurs through the inactivation by WR-1065 of charged carbonium ions of activated alkylating agents such as platinum derivatives and fotemustine. Amifostine has been reported to enhance the activity of alkylating agents *in vivo*.[13–17] More recently, a clinical study in melanoma strongly suggested that amifostine could be of interest to reduce myelosuppression induced by fotemustine.[12] Based on these reports the present study was designed to evaluate *in vitro* the nature of the interaction of amifostine with fotemustine in melanoma and normal fibroblast cell lines.

The present results first showed that AP activity was higher in normal cells than in neoplastic cells. These results are fully consistent with previous studies reporting that amifostine chemoprotection was higher in normal cells than in tumor cells through a difference in cellular metabolization into WR-1065 by APs.[9]

Consistently with its DNA alkylating activity, fotemustine cytotoxicity was found to be related to the cell proliferation, MRC-5 lung fibroblasts and WM-115 cell lines having the shortest doubling time being the most sensitive to fotemustine. Neither MGMT protein nor mRNA expression correlated with fotemustine cytotoxicity. This finding is not consistent with results reported from *in vivo* experiments in brain tumors treated with fotemustine.[6] This could be due to the narrow range of MGMT activity values

found in the cell line panel used in the present study which differs dramatically from the wide range of values (6–892 fmol/mg protein) found in brain tumors.

As in the present study, no correlation was found between MGMT expression and temozolomide response in melanoma clinical specimens.[27] This discrepancy might be related to the fact that other factors should influence cell death after unrepaired methylation of DNA such as mismatch repair and p53 status. As far as p53 status is concerned, our set of cell lines included both wild-type (MRC-5)[28] and mutant p53 (SK-MEL2)[29] cell lines. The SK-MEL5 cell line was reported to display wild-type p53 status but to express functionally aberrant P53 protein.[30] In the present study, all cell lines displaying p53 mutations or functional alterations appeared to be more resistant to fotemustine than cells displaying wild-type p53 status. In these cell lines, the present study could confirm the results recently reported by Zhang *et al*.[31] showing that amifostine could potentiate radiation response of cells displaying wild-type p53 status by increasing P53-dependent apoptosis induction which was reported to induced by alkylating agents.[32,33]

On the other hand, alterations of expression of genes involved in DNA mismatch repair alterations were recently reported to be involved in fotemustine resistance in melanoma cell lines.[34] Recent results further demonstrated that no relationship exists between the cytoprotective activity of the amifostine metabolite WR-1065 and cellular ability to repair DNA strand breaks,[35,36] and that both WR-1065 and its pro-drug WR-2721 induce the expression of $p21^{waf\text{-}1}$, resulting in a delay in $G_1$/S transition in breast cancer cells and mouse fibroblasts.[18] This is consistent with cell cycle analysis showing that exposure of melanoma cells to WR-2721 or WR-1065 and then to fotemustine led to a slight but reproducible increase in the $G_1$/S fraction (unpublished results).

More likely than interference with DNA repair mechanism, the interaction of WR-2721 and WR-1065 with the cell cycle could therefore explain the potentiation of fotemustine cytotoxicity observed in the present study.

In conclusion, the present study demonstrated that amifostine or its main metabolite had no antagonistic effect on fotemustine cytotoxicity in human melanoma cell lines. These results associated with the excellent tolerance of amifostine and fotemustine combination observed in the pilot clinical trial including 10 patients[15] are in favor of a larger clinical trial in disseminated metastatic melanoma evaluating increasing doses of fotemustine in combination with a fixed dose of amifostine.

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