Intrinsic anticancer drug resistance of malignant melanoma cells is abrogated by interferon-β and valproic acid

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ABSTRACT

Malignant melanoma, once metastasized, has a dismal prognosis because of intrinsic resistance to anticancer drugs. First line therapy includes the methylating agents dacarbazine and temozolomide. Although DNA mismatch repair and MGMT are key determinants of cellular resistance to these drugs, there is no correlation between these markers and the therapeutic response in melanoma, indicating as yet unknown mechanisms of drug resistance. We show that in malignant melanoma cells with wild-type p53, the temozolomide-induced DNA damage O\textsuperscript{6}-methylguanine triggered up-regulation of the Fas/CD95/Apo-1 receptor without activating the apoptosis cascade. This was due to silencing of pro-caspase-8. A single treatment with interferon-\(\beta\) (IFN-\(\beta\)) reactivated pro-caspase-8 and sensitized melanoma cells to temozolomide. The key role of pro-caspase-8 in melanoma cell sensitization was verified by experiments in which the death receptor pathway was either blocked by expression of dominant-negative FADD, siRNA knock-down of pro-caspase-8 or stimulation with Fas/CD95/Apo-1 activating antibody. The expression of pro-caspase-8 could further be enhanced by additional pre-treatment with the HDAC inhibitor valproic acid (VPA), which together with IFN-\(\beta\) caused significant sensitization of melanoma cells in vitro. Sensitization of melanoma cells to temozolomide by IFN-\(\beta\) and VPA was also shown in a xenograft mouse model. The data provide a plausible explanation why therapy of malignant melanomas with alkylating anticancer drugs failed even in trials where the repair of the critical toxic lesion O\textsuperscript{6}-methylguanine was blocked by MGMT inhibitors and suggests approaches to abrogate intrinsic drug resistance by interferon and VPA-mediated reactivation of the death receptor pathway.

PRECIS

The paper provides evidence that the intrinsic resistance of melanoma cells to the 1st line therapeutic anticancer drug temozolomide can be abrogated by interferon and VPA-mediated reactivation of the death receptor pathway.
Introduction

First line chemotherapy of malignant melanoma includes the alkylating drugs dacarbazine (DTIC), temozolomide (TMZ) and/or fotemustine (FM) (1). DTIC is the most commonly used drug. TMZ has the same efficacy and molecular mode of action as the active metabolite of DTIC. It can be taken orally, does not need metabolic activation to form the active metabolite 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC), is able to cross the blood brain barrier and can therefore also be used for the treatment of melanoma brain metastases (2). Still, the response rate of the patients suffering from melanoma treated with DTIC or TMZ remains poor, with median survival rates between 5.3 and 6.7 months (3, 4). Treatment combining TMZ with interferon-\(\alpha\) (IFN-\(\alpha\)) can increase median survival to 11.8 months (5). There is, however, no clear rationale for concomitant treatment with interferon and TMZ except the finding that interferons may cause immunosensitization of the tumor (6).

Agents like TMZ methylate DNA at 13 positions (7). Prominent methylation lesions are N3-methyladenine, N3-methylguanine and N7-methylguanine, which are repaired by base excision repair (BER) (8). The minor lesion O\(^6\)-methylguanine (O\(^6\)MeG) is repaired by O\(^6\)-methylguanine-DNA methyltransferase (MGMT) (9). MGMT is a key node in resistance of tumor cells to methylating agents (10), thus MGMT over-expression protects (11) and MGMT inhibition greatly sensitizes towards alkylation induced cell kill (12). Killing by TMZ of cancer cells is executed by apoptosis and it has been shown that both the Fas/CD95/Apo-1 extrinsic and the mitochondrial intrinsic pathway can be triggered (13). O\(^6\)MeG does not trigger apoptosis directly, it requires DNA mismatch repair (MMR) (14). A current model states that during DNA replication O\(^6\)MeG erroneously pairs with thymine (15). The O\(^6\)MeG/T mismatch is recognized by the heterodimer MSH2/MSH6 that recruits PMS2/MLH1 (16). Therefore, loss of MMR or changes in MMR expression leads to changes in resistance of tumor cells to methylating agents (17, 18). During DNA replication, MMR causes persistent single-stranded gaps in the DNA (19) that give rise to
DNA double-strand breaks (DSBs) (20). The cell tolerates these DSBs by homologous recombination whereas non-homologous end-joining only plays a minor role in protection (21). Interferons are cytokines (22) that are produced in response to infection. There are two groups of IFNs, type I (IFN-α and -β) and type II (IFN-γ). Type I IFNs bind to the same receptor, which leads to activation of the JAK-STAT signaling pathway and changes in gene expression (23). IFNs on their own have cytotoxic effects in cancer cells, including melanomas. The anti-cancer effect in melanoma cells has been ascribed to the inhibition of cell growth (24) and the induction of apoptosis (25). In one trial where IFN-α was administered concomitantly with TMZ the rational was that IFN would cause immunosensitization of the tumor (6). In addition to any effects IFN may have on the immune system, it may also have a direct impact on TMZ-induced cell kill, which has to date not been addressed in preclinical studies. Here we show that p53 wild-type (p53wt) melanoma cells are refractory in death receptor-induced apoptosis despite the induction of Fas/CD95/Apo-1 expression following TMZ, which is due to silencing of pro-caspase-8 in these cells. A single IFN-β pre-treatment was able to reactivate pro-caspase-8 expression and greatly ameliorated the response to O6MeG when the cells were treated with TMZ, in vitro and in melanoma xenografts. The expression of pro-caspase-8 by IFN-β could further be enhanced by the addition of the histone deacetylase (HDAC) inhibitor valproic acid (VPA), which leads to a significant sensitization of melanoma cells in vitro and in melanoma xenografts. The data provide a rational basis for the co-administration of the apoptosis modulators IFN and VPA together with methylating anticancer drugs and suggests new therapeutic interventions.

**Materials and Methods**

**Cell lines and culture conditions.** All cell lines (D05, D14, RPMI7951 and A375) were described previously (26, 27). A375 was from American Type Culture Collection (ATCC, Manassas, USA), RPMI7951 from The German Cell Culture Depository (DSMZ, Braunschweig,
Germany), D05 and D14 were from C.W. Schmidt (Queensland Institute of Medical Research, Queensland, Australia) and checked there (28) as well as in the laboratory of Dr. Wölfel (Mainz). All cell lines were checked before experimental use for mycoplasma contamination. Cells were cultivated at 5% CO₂, 37°C in humidified atmosphere. D05 and D14 were cultivated in RPMI-1640 while RPMI7951 and A375 were cultivated in DMEM. In all cases, 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin were present. D05 and A375 are p53wt while D14 and RPMI7951 are p53mt (26).

Drugs and Drug treatment. IFN-α, (Interferon alpha-2a, Roferon-A) was from Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany. IFN-β (Interferon-beta-1a, Rebiff 22) was from Merck Serono, Darmstadt, Germany. VPA (Sigma) stock solution (100 mM) was prepared in distilled sterile H₂O. TMZ (Schering-Plough, USA) and FM (Muphoran) was prepared and handled as described (13, 26). A pre-incubation with 300 I.U./ml IFNs for 24 h followed by medium change to remove IFNs was done before TMZ, FM or γ-ray (IR) treatment. Pre-treatment of cells with VPA (1 mM for 168 h, replacing the medium and adding fresh VPA approximately every 48h) was performed followed by medium change to remove VPA before TMZ addition. To deactivate the MGMT protein, O₆-benzylguanine (O₆BG) (29) was added to the cells (final concentration 10 µM) 1 h before drug treatment. Ionizing irradiation was performed using a Cs-137 source at 3.75 Gy/min. Fas/CD95/Apo-1 activating antibody (APO-1-3, Alexis Biochemicals, Axxora Deutschland GmbH, Lörrach, Germany) was used at a dose of 25 ng/ml along with 25 ng/ml Protein A. TRAIL (SuperKillerTRAIL™, Alexis Biochemicals, Axxora Deutschland GmbH, Lörrach, Germany) was used at a dose of 25 ng/ml.

Isolation and stimulation of primary human lymphocytes. The isolation of lymphocytes from buffy-coat blood and stimulation with anti-CD3 plus anti-CD28 (BD PharMingen, Heidelberg, Germany) has been described previously (30).
**Quantification of Apoptosis.** The analysis of the sub–G1 fraction as well as the analysis of annexin V/propidium iodide (PI) double-stained cells by flow cytometry has been described previously (13, 31).

**Transfection of melanoma cells with MGMT, DN-FADD and siRNA against pro-caspase-8.** Stable MGMT transfectants were generated by co-transfection of D05 cells with the mammalian expression vector (pSV2MGMT) harboring the MGMT gene described previously (11) and the pSV2neo plasmid for selection. Stable dominant-negative FADD (DN-FADD) transfectants were generated in D05 and A375 cells by using the pcDNA3-FADD-DN plasmid described previously (32). Stable transfectants with siRNA targeted towards pro-caspase-8 was generated by transfecting the previously described plasmid (33) in A375. Following selection surviving clones were tested for protein expression by western blot.

**Preparation of protein extracts and MGMT activity assay.** Protein extracts were prepared for western blot analysis and MGMT activity testing as described (13). MGMT activity was determined as described (34).

**Immunoblotting.** SDS-PAGE was performed as described (13, 31). Antibodies used were anti-caspase-3 (Cell Signaling, Frankfurt), anti-caspase-7 (Cell Signaling, Frankfurt), anti-caspase-8 (Cell Signaling, Frankfurt), anti-caspase-9 (Cell Signaling, Frankfurt), anti-FADD (Calbiochem, Merck, Darmstadt), anti-Fas (Santa Cruz Biotechnology, Heidelberg), anti-MSH2 (Oncogene, Cambridge), anti-MSH6 (BD Pharmingen, Heidelberg), anti-MLH1 (Santa Cruz, Heidelberg), anti-PMS2 (BD Pharmingen, Heidelberg), anti-MGMT (Chemicon International Inc.) and anti-ERK2 (Santa Cruz, Heidelberg).
**Immunofluorescence labeling and microscopy.** Immunofluorescence labeling of $\gamma$H2AX foci was performed as described (21). The antibodies used were anti-$\gamma$H2AX (Upstate) and Alexa Fluor 546 (Molecular probes). Foci were not scored in apoptotic cells.

**Animal experiments.** Immuno-deficient mice (NOD.CB17-Prkdcscid/J) were housed in a sterile environment and allowed free access to food and water. A375 human melanoma xenografts were initiated by injecting $8 \times 10^6$ cells in the right and left flank and the treatments began when tumor volumes reached a suitable value. Four animals each were assigned to the different groups and tumor volume was standardized across the groups. The mice received one dose i.p. of TMZ (150 mg/kg). One hour prior to TMZ injection, all mice received $O^6$BG 30 mg/kg i.p.. Animal weights and tumor volumes were measured at indicated times following TMZ injection. The two groups that received IFN-β and VPA pre-treatment were treated as follows. Six days preceding $O^6$BG/TMZ injection mice were injected i.p. once daily with 500 mg/kg VPA. One day preceding $O^6$BG/TMZ injection mice were injected i.p. with $2 \times 10^5$ I.U. IFN-β. The *in vivo* experiments were performed twice, once with female mice and once with male mice.

**Results and Discussion**

**IFN-β sensitizes melanoma cells to TMZ-induced apoptosis.** The studies were initiated with cell lines well characterized as to their p53 status (26). In the p53wt cell line D05, interferon-α (IFN-α) and IFN-β caused significant sensitization to TMZ ($p=0.03$ and $p<0.0001$ for IFN-α and IFN-β respectively) when MGMT was depleted with the specific MGMT inhibitor $O^6$-benzylguanine ($O^6$BG) (Fig. 1A). When $O^6$BG was omitted, D05 cells showed sensitization towards TMZ only following IFN-β pre-treatment ($p=0.0012$). In p53mt D14 cells, IFN-α and IFN-β had a very weak sensitization effect on TMZ, which was significant for IFN-β ($p=0.002$) when depleting MGMT with $O^6$BG. Activation of caspase-3 and -7, the apoptosis executing caspases,
was also enhanced following IFN-β pre-treatment in D05 cells (Fig. 1B). For the chloroethylating anticancer drug fotemustine (FM), a significant increase in sensitivity was observed only in p53wt D05, but not in p53mt D14 cells pre-treated with IFN-β and O⁶BG (p=0.024) (Fig. 2A). Interestingly, no sensitization was found following treatment with IR (4 or 10 Gy) in D05 and D14 cells (Fig. 2B). Comparing IFN-α and IFN-β, the data revealed that IFN-β is more effective than IFN-α in sensitizing melanoma cells to alkylating agents (Fig. 1A and B).

To determine whether sensitization to TMZ is specific for melanoma cells, we repeated the experiments with a non-malignant cell type known to undergo O⁶MeG triggered apoptosis effectively (30). We chose primary human lymphocytes (PBLCs) isolated from peripheral blood. These lymphocytes were not proliferating, or were stimulated to proliferate with anti-CD3 and anti-CD28. Methylating agent induced apoptosis was only observed in proliferating lymphocytes once MGMT was depleted, as observed previously (30). IFN-β had no sensitization effect in both stimulated and non-stimulated PBLCs (Fig. 2C). Collectively, the results show that IFN-β sensitizes melanoma cells, but not non-tumor cells such as PBLCs, to TMZ.

**IFN-β sensitizes p53wt melanoma cells to the apoptosis inducing TMZ lesion O⁶MeG.** In our previous work with brain tumor cells, we have shown that p53 has a significant impact on the cytotoxic response of glioma cells to TMZ by up-regulating the Fas/CD95/Apo-1 receptor (13). The gene encoding Fas/CD95/Apo-1 harbors a p53 binding site in its promoter and, therefore, is subject to regulation by p53 (35). Thus, in glioma cells the apoptotic response upon methylating agents is clearly p53 dependent (13, 36). In contrast, in melanoma cells TMZ-induced apoptosis was not stimulated by p53 (26). As the most significant sensitization of IFN-β to TMZ was observed in the p53wt melanoma cell line, we determined whether p53 is required for this effect. To this end, we compared the apoptotic response of a panel of melanoma cell lines differing in their p53 status treated with TMZ, with or without IFN-β pre-treatment. The p53wt cell lines were
D05 and A375 while the p53mt cell lines were D14 and RPMI7951 (27, 37). Data in Fig. 3A show the x-fold increase in TMZ-induced apoptosis with IFN-β pre-treatment compared to TMZ without IFN-β. A significant increase in apoptosis level in the p53wt cells (up to 4 fold) was observed for D05 and A375, but not for the p53mt cells D14 and RPMI7951 (Fig. 3A). The data are taken to indicate that the sensitization effect of IFN-β in TMZ treated melanoma cells is dependent on p53, which is of interest as most malignant melanomas express p53wt (38-40).

TMZ, similar to dacarbazine, is a genotoxic anticancer drug that induces a broad spectrum of DNA alkylation lesions. Theoretically, IFN-β may sensitize melanoma cells to different DNA methylation lesions. To determine whether sensitization to TMZ by IFN-β depends on the specific adduct O6MeG, we compared isogenic D05 cells expressing different levels of MGMT following transfection, i.e. ∼200 up to 800 fmol/mg protein (Fig. 3B; for MGMT protein in the stable MGMT transfectants see the blot in this figure). Under MGMT expressing conditions, the sensitization of D05 cells by IFN-β was completely abrogated, while inactivation of MGMT with O6BG in both parental and MGMT transfected cells greatly sensitized cells to the combination treatment with IFN-β and TMZ (Fig. 3C). These results obtained with MGMT transfected cells clearly show that sensitization of melanoma cells to TMZ by IFN-β is due to the TMZ-induced DNA damage O6MeG.

IFN-β does not influence MGMT and MMR protein expression or DSB formation in melanoma cells following TMZ treatment. The DNA adduct O6MeG is dependent on MMR for conversion into toxic DSBs and, therefore, MGMT, MMR and DSBs are determinants of O6MeG-induced cell kill (41). Due to this, we ascertained whether IFN-β has an influence on these parameters by determining MGMT and MMR protein expression as well as DSBs arising from the processing of O6MeG. Treatment of D05 and D14 cells with 300 I.U./ml IFN-β neither had an influence on the expression of MGMT nor the MMR proteins MSH2, MSH6, PMS2 and MLH1.
(Fig. 4A). Since the processing of O₆MeG gives rise to the formation of DSBs (20, 30), which was also recently shown for melanoma cells (26), we determined whether sensitization of melanoma cells by IFN-β is related to γH2AX foci formation. TMZ induced γH2AX foci, which was demonstrated 24 up to 72 h following treatment, and IFN-β had no significant effect on the γH2AX foci level (for representative example Fig. 4B, and for quantification Fig. 4C). We infer that IFN-β neither has an impact on the repair of O₆MeG lesions by MGMT nor the conversion of O₆MeG/T lesions into DSBs.

**IFN-β induces pro-caspase-8 expression and thereby reactivates the Fas/CD95/Apo-1 apoptotic pathway.** Since no difference in repair and O₆MeG processing could be detected in the presence or absence of IFN-β, we reasoned that IFN-β might increase the effectiveness of triggering the apoptotic pathway following O₆MeG induction. In p53wt glioma cells it is known that TMZ can activate the Fas/CD95/Apo-1 dependent death receptor along with the mitochondrial apoptosis pathway, while in p53mt cells only the mitochondrial pathway is activated (13). As shown in Fig. 5A, in the p53wt melanoma cell lines D05 and A375 TMZ caused up-regulation of the Fas/CD95/Apo-1 receptor, which is expected as the gene encoding Fas/CD95/Apo-1 harbors a p53 binding site in its promoter and is subject to regulation by p53 (35). In the p53mt cell lines D14 and RPMI7951 no increase in Fas/CD95/Apo-1 was detected. We also observed that cells exhibiting both p53wt (D05) and p53mt (D14) activated the mitochondrial apoptosis marker caspase-9 following TMZ treatment, as shown by the increase in the activated fragments of this caspase (Fig. 5A).

As the effect of IFN-β was different in p53wt and mutant cells, we surmised the death receptor associated pro-caspase-8 could be involved. Therefore we checked its expression following IFN-β treatment in both cell types. In the D05 and A375 cells IFN-β caused a clear up-regulation of pro-caspase-8, while in the p53mt lines D14 and RPMI7951 no pro-caspase-8 up-regulation was
observed (Fig. 5B). We should note that D14 and RPMI7951 cells already express pro-caspase-8 that could not be further enhanced by IFN-β. Obviously, the basal pro-caspase-8 level in these cells has no impact on TMZ-induced killing, which is explained by the lack of expression and up-regulation of the death receptor in these p53mt cells (see Fig. 5A). Epigenetic down-regulation of pro-caspase-8 gene expression has been shown in several tumors, which is presumably due to promoter methylation (42, 43). IFN-β may be effective in demethylating the pro-caspase-8 promoter or stimulate its expression. It has been shown that IFN-α (44) and IFN-γ stimulate pro-caspase-8 expression and that the promoter of pro-caspase-8 contains an IFN-response element (45).

If pro-caspase-8 is the limiting factor in preventing the effective activation of the Fas/CD95/Apo-1 apoptosis pathway, inactivating the pathway as a whole or knocking down pro-caspase-8 should abolish the sensitization to TMZ following IFN-β pre-treatment. Both options were tested: Fas/CD95/Apo-1 dependent activation of the death receptor pathway was prevented by stable transfection of D05 with dominant-negative FADD (DN-FADD) and pro-caspase-8 was knocked down in A375 cells. DN-FADD clearly decreased the effectiveness of IFN-β in sensitizing D05 cells to TMZ (Fig. 5C), while knockdown of pro-caspase-8 completely abolished the sensitization of A375 cells by IFN-β to TMZ (Fig. 5D). Collectively, the data provide evidence that IFN-β sensitizes melanoma cells to TMZ due to the up-regulation of pro-caspase-8, thereby reactivating the cells ability to utilize the death receptor apoptosis pathway following O6MeG induction.

*Both the induction of Fas/CD95/Apo-1 by TMZ and the induction of pro-caspase-8 by IFN-β are required for the sensitization observed during combination treatment.* Having shown that the silenced death receptor pathway can be reactivated in p53wt melanoma cells by IFN-β, we asked whether stimulation of the Fas/CD95/Apo-1 receptor by an external ligand would be
able to ameliorate the TMZ response in the presence or absence of IFN-β. We made use of non-
transfected and DN-FADD transfected D05 cells to substantiate the effects were brought about
by the death receptor. Treatment with Fas/CD95/Apo-1 activating antibody (AB) did not induce
apoptosis significantly, while treatment with AB plus IFN-β or AB plus TMZ was effective (Fig.
6A). The strongest response was observed when the AB was administered after TMZ in IFN-β
pre-treated cells. Similar results were obtained with A375 cells (Fig. 6C). We should note that in
A375 cells, treatment with IFN-β plus AB did not induce apoptosis significantly, whereas the
inclusion of TMZ caused a dramatic apoptotic response. This is likely due to the low basal
Fas/CD95/Apo-1 receptor status of A375 cells (Fig. 5A), which needs to be up-regulated by
TMZ. In p53mt D14 cells, sensitization by the activating antibody did not occur, neither in the
presence nor the absence of TMZ and IFN-β (Fig. 6B), showing that the slight sensitization of
D14 cells by IFN-β (see Fig. 1A) does not rely on Fas/CD95/Apo-1. Induction of pro-caspase-8
by IFN-β should also influence Fas/CD95/Apo-1 independent apoptosis, e.g. by administering
TRAIL, which activates a different group of receptors, DR4 and DR5. This was in fact observed:
pre-treatment with IFN-β sensitized all melanoma cell lines tested to TRAIL (Fig. 6D).
Collectively, the findings demonstrate that IFN-β greatly sensitizes p53wt melanoma cells to
TMZ due to reactivation of the Fas/CD95/Apo-1 pathway by up-regulation of pro-caspase-8.

**VPA greatly ameliorates the sensitization of melanoma cells by IFN-β in vitro.** Is it possible
to further increase the pro-caspase-8 level and TMZ sensitivity of melanoma cells? To address
this question, we used the HDAC inhibitor VPA, which is known to cause gene reactivation (46),
and D05 cells were pre-treated with VPA, IFN-β or both. As shown in Fig. 7A, IFN-β on its own
causd increase of pro-caspase-8 protein expression, while VPA on its own did not. When D05
cells were pre-treated with IFN-β and VPA a very strong increase in pro-caspase-8 expression
was observed (Fig. 7A; for quantification see the induction factor, I.F.). The combinations of IFN-
and VPA pre-treatment also greatly sensitized D05 cells to low dose TMZ (30 µM) (Fig. 7B). The data revealed that combining IFN-β and VPA was more effective than the single treatments in enhancing the killing effect of TMZ in melanoma cells.

**IFN-β and VPA ameliorate the anticancer effect of TMZ in vivo.** To ascertain whether the findings can be translated to the melanoma cell growth *in vivo*, we determined the effect of A375 cells grown subcutaneously in immuno-deficient NOD-Scid mice. First, we analyzed whether the enhanced sensitivity of IFN-β pretreated cells would have an influence on melanoma cell growth treated with TMZ *in vivo*. Tumor sizes were determined 20 days after TMZ treatment. IFN-β on its own had a slight effect on tumor growth. A single treatment with TMZ (150 mg/kg) delayed tumor growth while TMZ in combination with a single IFN-β pre-treatment showed improved tumor growth inhibition (Fig. 7C left panel). Thus, IFN-β sensitizes melanoma cells both *in vitro* and *in vivo*. Secondly, we determined the effect of combined treatment. IFN-β and VPA pre-treatment on its own had no effect on tumor growth, while TMZ (150 mg/kg) in combination with VPA and IFN-β pre-treatment showed significant tumor growth inhibition (Fig. 7C right panel). At the end of the investigation period, IFN-β improved the reduction of tumor size by ∼25% compared with animals treated with TMZ/O6BG only, while IFN-β plus VPA caused a significant improvement by ∼45%. Similar to what was observed *in vitro*, the treatments were most effective if VPA and IFN-β were applied concomitantly prior to TMZ compared to pre-treatment with IFN-β alone. A model is shown in Fig. 7D. We are aware that the pathways evoked by combining VPA and IFN-β are complex and, therefore, we cannot exclude that additional mechanisms become activated by concomitant treatment with the drugs, but it is reasonable to posit that reactivation of the Fas/CD95/Apo-1 pathway is most critical.

For TMZ and dacarbazine, MGMT is a key factor in drug resistance (10). MGMT can be inactivated nearly to completion by O6BG and O6-(4-bromothenyl)guanine (O6BTG), both
currently included in clinical trials (47). A recently published trial, however, showed no benefit for melanoma patients receiving TMZ concomitant with O\textsuperscript{6}BTG (48). In light of our current data it is pertinent to conclude that melanomas cannot respond to TMZ/dacarbazine based chemotherapy because the death receptor pathway is silenced due to lack of pro-caspase-8. This causes melanoma cells to undergo apoptosis in response to O\textsuperscript{6}MeG via the less accessible mitochondrial pathway, making them refractory to chemotherapy (13). The data presented here suggest a clinical trial in which reactivators of caspase-8, namely IFN-\textbeta and VPA, should be administered prior to O\textsuperscript{6}BG/O\textsuperscript{6}BTG and TMZ/dacarbazine.

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References


Figure legends

**Figure 1:** IFN-α and IFN-β sensitize melanoma cells to TMZ. (A) Apoptosis induced by 50 µM TMZ in p53wt D05 and p53mt D14 cells with or without IFN and O6BG pre-treatment. Apoptosis response was assayed using the sub-G1 method 144 h after TMZ addition. Experiments were performed at least three times. (B) Activation of caspase-3 and -7 in D05 cells treated with 10 µM O6BG and 50 µM TMZ with or without IFN-β pre-treatment.

**Figure 2:** Effect of IFN-α and IFN-β on melanoma cells and primary human lymphocytes. (A) Apoptosis induced by 32 µM FM in p53wt D05 and p53mt D14 cells with or without IFN and O6BG pre-treatment. Apoptosis was assayed using the sub-G1 method 144 h after FM addition. (B) Apoptosis induced by 4 and 10 Gy IR in p53wt D05 and p53mt D14 cells with or without IFN pre-treatment. Apoptosis was assayed using the sub-G1 method 72 h after IR. (C) Primary human lymphocytes were stimulated or not using anti-CD3 and anti-CD28, allowing for the comparison of TMZ-induced apoptosis in proliferating versus non-proliferating cells. IFN-β was not removed and cells were treated 100 µM TMZ in the presence or absence of O6BG. Apoptotic response was measured 96 h after TMZ addition by sub-G1 flow cytometry. All experiments were performed at least three times.

**Figure 3:** Influence of p53 and O6MeG on IFN-β sensitized melanoma cells. (A) Sensitization of the p53wt D05 and A375 and the p53mt D14 and RPMI7951 cell lines treated with O6BG and 50 µM TMZ by IFN-β. Apoptosis was assayed using the sub-G1 method 144 h after TMZ addition. Experiments were performed at least three times. (B) MGMT activity in D05, D14 and D05 MGMT-transfected cells. HeLa MR and HeLa S3 were used as negative and positive controls, respectively. Right panel, western blot analysis of D05 MGMT-transfected cells (the clones...
D05MGMT4 and D05MGMT30). ERK2 was used as loading control. (C) Induced apoptosis in D05 MGMT-transfected cells by 50 µM TMZ in the absence or presence of O6BG pre-treatment. Experiments were performed at least three times.

**Figure 4:** Expression of MGMT, MMR proteins and DSBs in IFN-β treated melanoma cells. (A) Western blot analysis of MGMT, MSH2, MSH6, PMS2 and MLH1 of cells pre-treated with IFN-β. ERK2 was used as loading control. (B) γ-H2AX foci formation in D05 cells untreated or treated with O6BG and 50 µM TMZ 72 h later. (C) Quantification of γ-H2AX foci in D05 cells at indicated times after O6BG and 50 µM TMZ addition, with or without IFN-β pre-treatment. A total number of at least 40 cells were scored for each condition and experiments were repeated three times.

**Figure 5:** IFN-β reactivates the Fas/CD95/Apo-1 apoptosis pathway by up-regulating pro-caspase-8, thereby sensitizing p53wt melanoma cells to TMZ. (A) FasCD95/Apo-1 receptor (Fas-R) expression in D05, A375, D14 and RPMI7951 cells. Caspase-9 activation in D05 and D14 cells at indicated times. Cells were treated with O6BG and 50 µM TMZ and analyzed by western blot. Fas-R expression was determined 72 h after TMZ. Caspase-9 activation was confirmed by the appearance of the active cleaved fragments. ERK2 was used as loading control. (B) Western blot analysis of IFN-β-induced pro-caspase-8 expression in D05, A375, D14 and RPMI7951 cells. The expression was quantified and set in relation to the control not treated with IFN-β. For quantification, ERK served as loading control. (C) Induced apoptosis in D05 and DN-FADD transfected D05 cells at indicated times following O6BG and 50 µM TMZ treatment in the absence or presence of IFN-β pre-treatment determined by the annexin V/PI method. Insert: western blot analysis of DN-FADD expression in D05 and the corresponding transfected cells. ERK2 was used as loading control. (D) Induced apoptosis in A375 and A375 pro-caspase-8 knockdown cells at indicated times following O6BG and 100 µM TMZ treatment in the absence
and presence of IFN-β pre-treatment determined by sub-G1 flow cytometry. Insert: western blot analysis of pro-caspase-8 knockdown in A375 cells. ERK2 was used as loading control. Experiments were repeated at least three times.

**Figure 6:** TMZ in combination with IFN-β triggers the Fas/CD95/Apo-1 dependent apoptosis pathway in melanoma cells. (A) Apoptosis triggered by IFN-β, Fas/CD95/Apo-1 receptor activating antibody (AB), TMZ and combination treatments in D05 and D05 DN-FADD transfected cells. Apoptosis was determined 96 h after TMZ addition by annexin V/PI flow cytometry. Fas/CD95/Apo-1 receptor activating antibody (AB) was added 24 h before apoptosis determination. Insert: western blot analysis of DN-FADD expression in D05 cells. ERK2 was used as loading control. (B) Apoptosis triggered by IFN-β, AB, TMZ and combination treatments in D14 cells. Cells were treated in the same manner as described in A. (C) Apoptosis triggered by IFN-β, AB, TMZ and combination treatments in A375 and A375 DN-FADD transfected cells. Cells were treated in the same manner as A. Insert: western blot analysis of DN-FADD expression in A375 cells. ERK2 was used as loading control. (D) Apoptosis triggered by IFN-β and TRAIL in D05, A375 and D14 cells. Cells were treated with IFN-β, TRAIL was added and apoptosis was assayed 6 h later using the annexin V/PI method. Experiments were repeated at least three times.

**Figure 7:** VPA in combination with IFN-β greatly sensitized melanoma cells and xenografts to low dose TMZ. (A) Western blot analysis of pro-caspase-8 in D05 cells treated with IFN-β, VPA or both. (B) Induced apoptosis in D05 cells treated with O6BG and 30 µM TMZ in the presence of IFN-β and/or VPA pre-treatment. Apoptosis was determined 120 h after TMZ addition by Sub-G1 flow cytometry; results are shown normalized to the appropriate controls. (C) Growth inhibition of A375 melanoma xenografts following O6BG/TMZ. Left, effect of IFN-β on TMZ
treated xenografts. Relative tumor volume was determined 20 days after O\textsuperscript{6}BG/TMZ treatment.

Right, effect of IFN-\(\beta\) and VPA co-pretreatment on O\textsuperscript{6}BG/TMZ treated xenografts. Relative tumor volume is shown as a function of time following implantation. O\textsuperscript{6}BG on its own had no effect on tumor growth. See materials and methods for treatment conditions. (D) Model for the sensitization of melanoma cells to TMZ by IFN-\(\beta\) and VPA. Following methylation of DNA by TMZ, O\textsuperscript{6}MeG is converted into DSBs in a DNA synthesis and MMR dependent manner. These DSBs then activate apoptosis via the intrinsic and extrinsic pathways. The Fas dependent extrinsic pathway relies on the p53 provoked expression of Fas-R. Fas-triggered apoptosis can, however, not be effectively activated because melanoma cells express low levels of pro-caspase-8. By pre-treating melanoma cells with IFN-\(\beta\) and valproic acid the pro-caspase-8 protein levels is restored and Fas can successfully activate the caspase cascade and apoptosis can progress successfully. In p53 mutant cells pre-existing pro-caspase-8 is not effective because these cells are impaired in Fas-R up-regulation.
Figure 1: Roos et al. 2011
Figure 2: Roos et al. 2011
Figure 3: Roos et al. 2011
Figure 5: Roos et al. 2011
Figure 6: Roos et al. 2011
Intrinsic anticancer drug resistance of malignant melanoma cells is abrogated by interferon-ß and valproic acid

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