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# The glutathione transferase inhibitor 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX) increases temozolomide efficacy against malignant melanoma

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ABSTRACT

First line treatment of metastatic melanoma includes the methylating agent dacarbazine or its analogue temozolomide (TMZ) with improved pharmacokinetics and tolerability. However, the prognosis of the metastatic disease is poor and several trials are evaluating TMZ in polychemotherapy protocols. The novel glutathione transferase P1-1 (GSTP1-1) inhibitor 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX) has recently shown activity against melanoma through c-Jun N-terminal kinase activation. In this study we have investigated the *in vitro* and *in vivo* efficacy of NBDHEX and TMZ combination against melanoma. The results indicated that NBDHEX and TMZ exerted *in vitro* synergistic antiproliferative effects in murine B16 and human A375 melanoma cells. In B16 cells TMZ as single agent caused cell accumulation at the $G_2$/M phase of cell cycle, whereas NBDHEX induced mainly apoptotic effects. NBDHEX provoked a higher level of p53 phosphorylation with respect to TMZ and the drug combination caused a more than additive increase of p53 activation.

The *in vivo* efficacy of NBDHEX and TMZ has been investigated in an orthotopic B16 model. Treatment with NBDHEX provoked a reduction of tumour growth comparable to that obtained with TMZ, whereas the drug combination significantly increased tumour growth inhibition with respect to the single agents, without worsening TMZ myelotoxicity. Immunohistochemical analysis of tumour grafts revealed a profound reduction of Cyclin D1 and CD31 in all treatment groups; VEGF expression was, instead, markedly decreased only in NBDHEX or NBDHEX and TMZ treated samples. These findings indicate that NBDHEX represents a good candidate for combination therapies including TMZ, offering new perspectives for the treatment of melanoma.



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## 1. Introduction

The incidence of malignant melanoma is increasing and, unfortunately, the survival of patients affected by metastatic melanoma with standard treatment has not changed in the last 30 years. In fact, the 1-year survival of patients with unresectable melanoma, treated with a variety of chemotherapeutic protocols is about 25%, as indicated by the meta-analysis of a large number of phase II trials.[1] The first-generation methylating agent dacarbazine is still the current reference drug; this agent requires enzymatic conversion to the active cytotoxic metabolite 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) in the liver and is characterized by a remarkable systemic toxicity. Temozolomide (TMZ) is, instead, a second-generation alkylating agent that spontaneously generates MTIC and has recently shown some efficacy in stage IV melanoma. In a phase III trial comparing intravenous dacarbazine versus oral TMZ for metastatic melanoma, TMZ was as effective as dacarbazine but with an improved quality of life.[2,3] Unlike dacarbazine, TMZ has an excellent oral bioavailability, possesses the ability to cross the blood–brain barrier and limited myelotoxicity. TMZ is presently licensed for the treatment of newly diagnosed glioblastoma multiforme concurrently with radiation followed by a maintenance treatment and for refractory anaplastic astrocytoma and is approved in several countries for the treatment of metastatic melanoma.[4] Due to its limited efficacy in monotherapy against melanoma, the drug is under evaluation in phase II trials in combination with classical chemotherapeutic agents (e.g. cisplatin), immunomodulating agents (e.g. thalidomide or lenalidomide, interleukin-2, interferon) or with targeted therapies (monoclonal antibodies or tyrosine kinase inhibitors) (www.clinicaltrials.gov).[5–9] However, the results of some completed phase II studies indicated that the overall survival is often not altered and that haematological toxicity is frequently higher with the combined treatment.[5–8]

The mechanism of action of TMZ involves DNA methylation with the generation of methyl adducts mainly represented by N7-methylguanine (70%) and, to a lesser extent, by N3-methyladenine (9%) or $O^6$-methylguanine (5%).[10] Despite the wide spectrum of methyl adducts, the antitumour activity of TMZ has been mainly attributed to $O^6$-methylguanine, since an inverse correlation exists between sensitivity to TMZ and the expression levels of the DNA repair protein $O^6$-methylguanine methyltransferase (MGMT), that transfers the alkyl adduct from the $O^6$ position of guanine to an internal cysteine. The toxicity induced by unrepaired $O^6$-methylguanine derives from the erroneous pairing of the methylated base with cytosine or with thymine during DNA synthesis. In fact, the resultant mismatches are recognised by the mismatch repair (MMR), which excises the mispaired base to reinsert cytosine or thymine again opposite to $O^6$-methylguanine. The reiterated futile cycles of MMR intervention eventually cause DNA strand breaks, growth arrest or apoptosis.[11] Thus, tumour sensitivity to TMZ requires both low MGMT levels and a functional MMR. In MGMT deficient and MMR proficient melanoma cells TMZ mainly causes growth arrest in $G_2$/M rather than apoptosis.[12]

Currently, the National Comprehensive Cancer Network recommends enrolment in a clinical trial, over other existing treatments, as either first line therapy or second-line therapy for patients with unresectable stage IV metastatic malignant melanoma. Due to the lack of standard therapy for patients with metastatic melanoma whose disease failed to respond to first-line systemic treatment, new therapies for recurrent or metastatic melanoma are urgently required.

Recently, it has been shown that the glutathione transferase P1-1 (GSTP1-1) inhibitor 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX) possesses a significant growth inhibitory effect against malignant melanoma and other tumour types including leukaemia, small cell lung cancer and osteosarcoma.[13–17] This compound disrupts the complex between GSTP1-1 and c-Jun N-terminal Kinase (JNK) inducing JNK activation, cell cycle arrest and cell death.[14] Since NBDHEX is capable of triggering apoptosis in melanoma cells and possesses a different mechanism of action with respect to TMZ, we investigated the *in vitro* and *in vivo* efficacy and toxicity of the combination of both drugs against malignant melanoma. The results indicated that NBDHEX and TMZ combination significantly reduced *in vivo* tumour growth with respect to each drug used individually without worsening the myelotoxicity of the methylating agent.

## 2. Materials and methods

### 2.1. Cell lines

The murine melanoma cell line B16 of C57BL/6 ($H\text{-}2^b/H\text{-}2^b$) origin and the human melanoma cell line A375 (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 containing 10% foetal calf serum (Sigma-Aldrich, Milan, Italy), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich), at 37 °C in a 5% $CO_2$ humidified atmosphere. PARP-1 silenced B16 melanoma cell line was obtained as previously described.[18]

### 2.2. Drugs

TMZ (Sigma-Aldrich) (100 mM) and NBDHEX (50 mM) stock solutions were prepared by dissolving the drugs in dimethyl sulfoxide. NBDHEX was synthesized as previously reported.[19]

### 2.3. Cell growth assays

The short term effects of drug treatment on cell growth were evaluated by the sulphorhodamine B (SRB, Sigma-Aldrich) assay. Briefly, cells ($2 \times 10^4$ cells/well) were seeded in sextuplicate in 96-well plates, exposed to graded concentrations of the drugs and allowed to incubate for 48 h. After cell-fixation, proteins were stained with SRB and plates were read at 540 nm with a Millipore Cytofluor 2350-microplate reader (Millipore, Milan, Italy). The long term effects on cell proliferation were evaluated by colony formation assay (CFA). Cells were seeded in triplicate into 6-well plates ($2 \times 10^2$/well) and, after overnight incubation, treated with TMZ or NBDHEX. After 10 d colonies were fixed, stained with 2% methylene blue in 95% ethanol and counted. Only colonies comprising >50 cells were scored as survival colonies.

Chemosensitivity was measured in terms of $IC_{50}$, i.e., the concentration of the drug capable of inhibiting cell growth

by 50%. To study whether the combination NBDHEX and TMZ was synergic against melanoma lines, cells were exposed to NBDHEX or TMZ alone or in combination at fixed equipotent ratios (corresponding to 1, 0.5, 0.2, 0.1 times the $IC_{50}$ for each drug). The dose-effect curves were analysed by the median-effect method of Chou and Talalay using the Calcusyn Software (Biosoft, Cambridge, UK). The combination index (CI) indicates a quantitative measure of the degree of drug interaction in terms of synergistic (CI < 1), additive (CI = 1) or antagonistic effect (CI > 1).

### 2.4. *Flow cytometry analysis*

Apoptosis or cell cycle perturbations were evaluated by flow-cytometry analysis of the DNA content. Untreated or drug treated cells ($1 \times 10^6$) were washed with PBS and fixed in 70% ethanol at –20 °C for at least 18 h. Cells were then centrifuged, suspended in 1 ml of a solution containing 0.1% (w/v) sodium citrate, 0.1% Triton-X (v/v), 50 μg/ml propidium iodide (PI), 10 μg/ml RNase, and incubated in the dark at 37 °C for 15 min. The PI fluorescence was measured on a linear scale using a FACScan flow cytometer and the CellQuest software. Data collection was gated using forward light scatter and side scatter to exclude cell debris and aggregates. Apoptotic cells were represented by a broad hypodiploid peak easily distinguishable from the diploid DNA content in the red fluorescence channel. For cell cycle analysis, the Mod-Fit software version 3.0 was used (Becton and Dickinson, San Jose, CA, USA).

### 2.5. *Western blot analysis*

For Western blot analysis the following primary antibodies were used: monoclonal anti-phospho-activated JNK isoform (Thr183, Tyr185) (Cell Signaling Technology, Milan, Italy; 1:1000), polyclonal anti-p53 and anti-phospho-p53 (Ser15) (Cell Signaling Technology; 1:1000 dilution), polyclonal anti-GSTP1-1 (MBL, Naka-ku, Japan; 1:1000), anti-β-tubulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:400) and anti-actin (Sigma-Aldrich; 1:5000). Anti-rabbit and

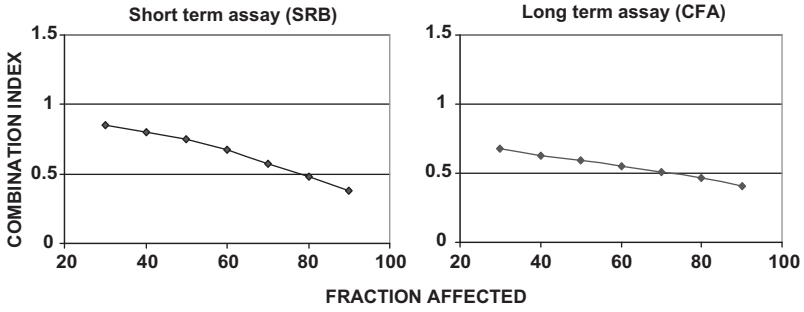


**Fig. 1 – *In vitro* synergistic effect of NBDHEX and TMZ combination in B16 melanoma cells. Chemosensitivity of B16 cells was assessed by SRB assay and colony-formation assay (CFA) 2 d or 10 d after treatment, respectively. Cells were treated with NBDHEX or TMZ as single agents to determine the $IC_{50}$ for each drug or with NBDHEX and TMZ combination using fixed molar ratios (1, 0.5, 0.2, 0.1 times the $IC_{50}$ for each drug). Combination index-fraction affected plots of the interactions between NBDHEX and TMZ were generated by computer analysis using CalcuSyn software Version 2.0. The CI indicates a synergistic effect when CI < 1. The results are representative of one out of three experiments with comparable results.**

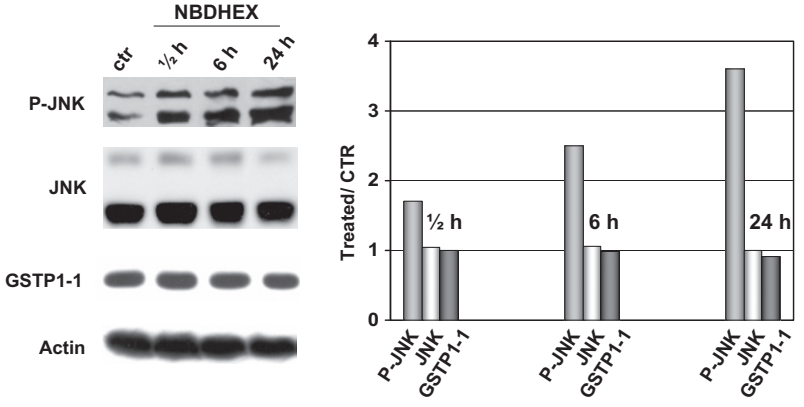


**Fig. 2 – JNK activation in B16 melanoma cells induced by NBDHEX. B16 cells were treated with NBDHEX (4 μM) for 30 min, 6 or 12 h. Cell lysates (50 μg) were then electrophoresed and analysed for the expression of phosphorylated and total JNK or GSTP1-1. The optical densities (OD) values were normalised according to the values of actin. Histograms represent the ratio between normalised OD values of NBDHEX treated samples and untreated control. The results are representative of one out of two experiments with comparable results.**

anti-mouse secondary antibodies (Sigma-Aldrich) were used at the appropriate dilutions. Signals were quantified using a Kodak densitometer (Rochester, NY, USA).

### 2.6. *In vivo studies*

The intra-muscular (i.m.) transplantation procedure was performed as previously described.[20] Briefly, B16 melanoma cells ($2.5 \times 10^5$ in 0.5 ml of PBS) were inoculated i.m. in the hind leg muscle of histocompatible male B6D2/F1 mice (Charles River, Calco, Milan, Italy).

Treatment started when tumour nodules were palpable. TMZ and NBDHEX were dissolved in 10% (v/v) and 20% (v/v) dimethyl sulfoxide, respectively and diluted in saline. TMZ was administered intraperitoneally (i.p.) at the dose of 100 mg/kg/d for 5 d, whereas NBDHEX was given *per os* at the dose of 8 mg/kg/d for 10 d. Control mice were always injected with drug vehicle. Drug toxicity was evaluated by treating tumour bearing mice (5/group) with the compounds under study, used as single agents or in combination. Body weight was measured three times weekly and survivals were recorded for 3 weeks after the last treatment. Toxicity was assessed on the basis of apparent drug-related deaths and net body weight loss {i.e., [(initial weight – lowest weight)/initial weight] × 100%}. Death was considered drug-related when it occurred within 7 d after the last treatment. Whole blood was collected from the retro-orbital sinus (5 mice/group) on day 7 during drug treatment or on day 13 (i.e., 3 d after NBDHEX discontinuation) and processed for complete blood count and routine haematochemistry. Animals were anesthetized with 2,2,2-tribromoethanol (0.25 mg/g of body weight, Sigma-Aldrich) administered i.p. before the blood drawing procedures.

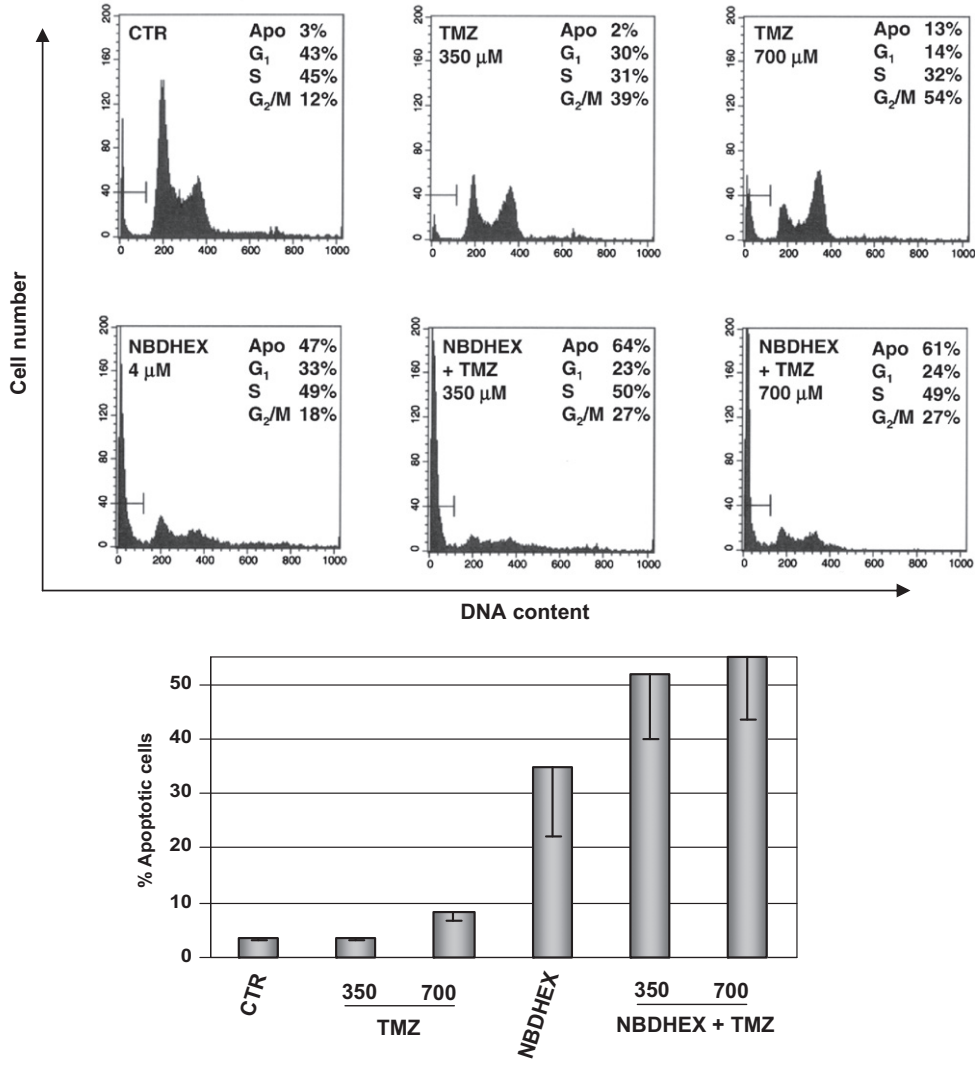


**Fig. 3 – Cell cycle and apoptosis analysis of cells treated with NBDHEX and TMZ. B16 cells were treated with TMZ (350 or 700 μM) and NBDHEX (4 μM) as single agents or in combination and analysed by flow cytometry after 48 h. *Upper panel*: Profiles were obtained using the CellQuest software (Becton and Dickinson). The percentages of apoptotic cells and of cells in the different phases of cell cycle are indicated. The data are representative of one out of three experiments with similar results. *Lower panel*: Histograms represent the mean percentage values (-standard deviation, SD) of apoptotic cells from three different experiments.**

Bone marrow specimens were obtained at autopsy through bone marrow sampling from the femurs of mice and used for cytological analysis. All aspirate smears were prepared according to standard procedures and routinely stained by May-Grunwald/Giemsa technique.

For the analysis of drug efficacy, tumours were measured with calliper and volumes calculated according to the formula: $[(\text{width})^2 \times \text{length}]/2$. Melanoma growth was monitored by measuring the volume of tumour nodules every 3 d for 3 weeks. The animals were euthanised when their tumours reached a volume of ~4000 $mm^3$. The results of *in vivo* studies were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni test for multiple comparisons. The results were considered to be statistically significant at $P < 0.05$ (two-sided).

All procedures, involving mice and care of laboratory animals, were performed in compliance with our institution guidelines and with national (D.L. No. 116, 251 G.U., Suppl. 40, 18th February 1992 and G.U. 163, July 1994) or international laws (EEC Council Directive 86/609, OJ L 358. 1, 12th December 1987; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996).

### 2.7. Histological and immunohistochemical analyses

Histological examination of the tumour nodules was performed using additional animals that were not considered for the monitoring of tumour growth. Tissues were fixed in 10% (w/v) phosphate-buffered formaldehyde and, after fixation for one week at room temperature, samples were dehydrated in graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Thereafter, 7-μm sections were deparaffinized with xylene, rehydrated, stained with haematoxylin-eosin and observed with light microscopy (Axostar Plus equipped with AxioCam MRc, Zeiss, Milan, Italy). Detection of VEGF, CD31 and Cyclin D1 was carried out after section deparaffinisation and boiling in 0.01 M citrate buffer for 4 min. Endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. Nonspecific adsorption was minimised by incubating the sections in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with mouse monoclonal anti-VEGF antibody (Santa Cruz Biotechnology; 1:100 in PBS), goat polyclonal anti-CD31 (Santa Cruz Biotechnology; 1:100 in PBS) and rabbit polyclonal anti Cyclin D1 (Abcam, Cambridge, UK; 1:100 in PBS). Sections were washed with PBS and incubated with the secondary antibody. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA). The counterstain was carried out with nuclear fast red (red background) (Sigma-Aldrich). All sections were analysed by light microscopy (see above). Immunocytochemistry photographs ($n = 5$) were analysed by densitometry using Imaging Densitometer (AxioVision, Zeiss) and a computer program.

## 3. Results

### 3.1. NBDHEX increases the in vitro anti-proliferative effect of TMZ against melanoma

To assess the role of GSTP1-1 inhibition mediated by NBDHEX in TMZ antitumour activity against B16 melanoma we performed SRB assay and CFA for the analysis of short and long term effects on cell proliferation, respectively. B16 cells were initially exposed to graded concentrations of NBDHEX and

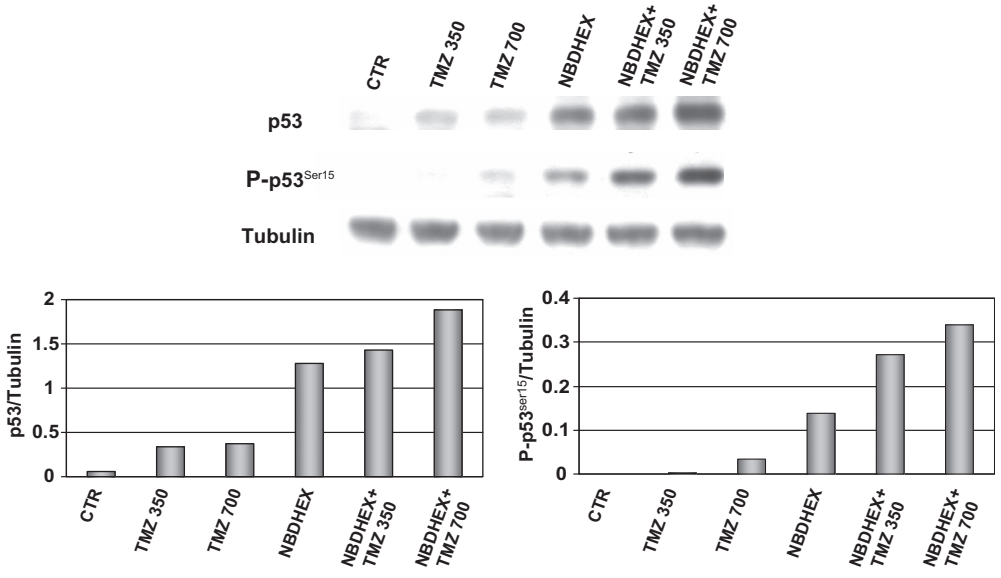


**Fig. 4 – Analysis of total and phosphorylated p53 expression in B16 cells treated with NBDHEX and TMZ. B16 cells were treated with TMZ (350 or 700 μM), NBDHEX (4 μM) as single agents or in combination for 3 h; cell lysates (50 μg) were then electrophoresed and analysed for the expression of total and phosphorylated p53 (P-p53$^{Ser15}$). Histograms represent the ratios between the optical densities (OD) of total or phosphorylated p53 protein and tubulin. The results are representative of one out of two experiments with comparable results.**

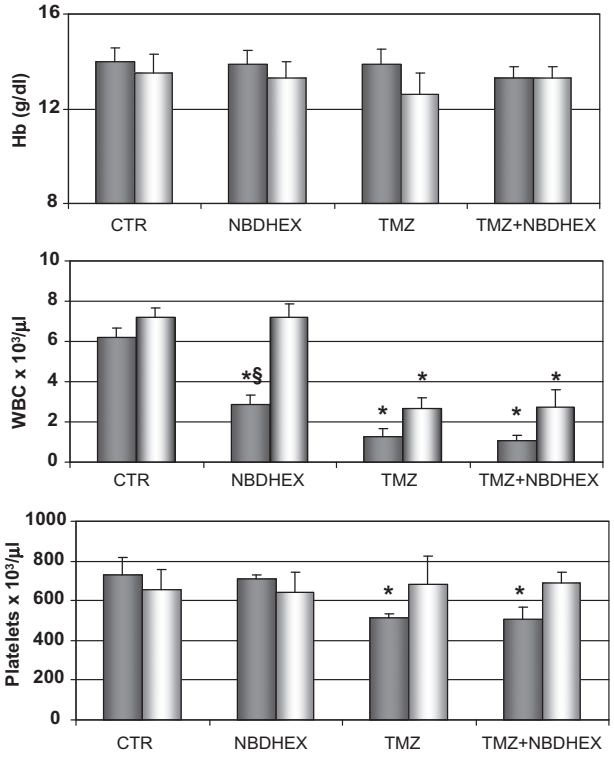


**Fig. 5 – NBDHEX does not exacerbate myelotoxicity induced by TMZ. Tumour bearing mice were treated with drug vehicles, NBDHEX (8 mg/kg/d for 10 d), TMZ (100 mg/kg/d for 5 d) or with the drug combination. Whole blood was collected from the retro-orbital sinus (5 mice/group) of tumour bearing mice on day 7 during drug treatment (*dark column*) or on day 13 (i.e., 3 d after NBDHEX discontinuation) (*light column*) and processed for complete blood counts. Values are expressed as the mean + SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni test for multiple comparisons. Differences in haemoglobin (Hb) levels between groups were not statistically significant (NS). During the first week, differences in the number of total white blood cells (WBC) were as follows: treated groups versus vehicle treated controls (CTR) were always statistically significant (*, $P < 0.05$); TMZ versus TMZ + NBDHEX, NS; NBDHEX versus TMZ or TMZ + NBDHEX, $P < 0.05$ (§). During the second week differences in the number of total WBC were as follows: CTR or NBDHEX versus TMZ or TMZ + NBDHEX, $P < 0.05$ (*); TMZ versus TMZ + NBDHEX or CTR versus NBDHEX, NS. During the first week, differences in the number of platelets were as follows: CTR or NBDHEX versus TMZ or TMZ + NBDHEX, $P < 0.05$ (*); TMZ versus TMZ + NBDHEX or CTR versus NBDHEX, NS. During the second week differences in the number of platelets between all groups were NS.**

the results indicated that the drug induced a dose-dependent reduction of cell growth with $IC_{50}$s of 2.3 ± 0.6 μM and 8 ± 1.7 nM by SRB assay (2 d after treatment) and CFA (10 d after treatment), respectively. TMZ $IC_{50}$s were 702 ± 5.6 μM and 115 ± 12 μM by SRB assay and CFA, respectively. Then, cells were treated with NBDHEX and TMZ in combination, at fixed equipotent ratios selected on the basis of the $IC_{50}$ values of each cell line, as described in Section 2. Fig. 1 shows the results of the median effect analysis using Calcusyn Software. The CI values indicate that the combination NBDHEX and TMZ was synergistic using both assays (CI < 0.9 and <0.7 at all fraction affected values in SRB and CFA, respectively; $n = 3$). Consistently with the results obtained with the murine melanoma, synergistic anti-proliferative effects of NBDHEX ($IC_{50}$ 6.9 ± 0.3 nM) and TMZ ($IC_{50}$ 110 ± 10 μM) combination were observed also in the A375 human melanoma cell line (CI < 0.8 at all fraction affected values in CFA; $n = 2$).

Interestingly, the anti-proliferative effect of TMZ in B16 cells was dramatically enhanced by silencing of poly(ADP-ribose) polymerase-1 (PARP-1),[18,21] which coordinates the repair of the N-methylpurines mediated by the base excision repair (TMZ $IC_{50}$ in PARP-1 silenced B16 cells: 70 ± 5 μM by CFA; $n = 3$). On the other hand, abrogation of PARP-1 expression did not substantially affect sensitivity of B16 cells to NBDHEX (NBDHEX $IC_{50}$ in PARP-1 silenced B16 cells: 8.9 ± 2 nM by CFA; $n = 3$). These data indicate that NBDHEX does not directly act on DNA, which is, instead, the main target of the methylating agent TMZ.

### 3.2. *Apoptosis and p53 induction in B16 melanoma cells treated with NBDHEX and TMZ*

We initially tested the ability of this compound to cause apoptosis in B16 melanoma cells. To this end cells were exposed to graded concentrations of NBDHEX as a single agent and the results indicated that this agent was endowed with apoptotic effects also in B16 cells and that 10 μM concentration induced apoptosis in almost 100% of cells at 48 h (data not shown). Thus, for *in vitro* studies on the combined effects of NBDHEX and TMZ, a concentration of NBDHEX capable of inducing a moderate level of apoptosis was chosen (i.e., 4 μM). Since JNK plays an important role in apoptosis and NBDHEX is able to trigger JNK activation by phosphorylation in a variety of tumour types including melanoma,[13–15,22] we performed a time course analysis of JNK phosphorylation in B16 cells treated with 4 μM NBDHEX. The results presented in Fig. 2 show that NBDHEX induced JNK activation by phosphorylation as early as 30 min after treatment, which increased at later time-points (6 and 24 h). A phosphorylated form of JNK was detected also in untreated cells in accordance with previous data showing constitutive activation of this kinase in melanoma.[14,23] Conversely, the expression of total JNK did not substantially change at all time-points (Fig. 2). Moreover, the levels of GSTP1-1 protein were not modulated by NBDHEX treatment (Fig. 2). These results are consistent with the ability of NBDHEX to trigger the release of GSTP1-1 from the complex leading to JNK activation without any relevant change in GSTP1-1 expression.

Treatment of B16 cells with 350 μM or 700 μM TMZ did not or only slightly affected the percentage of apoptotic cells, while the methylating agent mostly accumulated cells at the G2/M phase of cell cycle 48 h after treatment (Fig. 3), in accordance with previous studies.[12,21] On the other hand, 4 μM NBDHEX induced mainly apoptotic effects and when this compound was associated with TMZ apoptosis prevailed over the G2/M accumulation induced by TMZ (Fig. 3).

Western blot analysis of total and phosphorylated $p53^{Ser15}$ showed that NBDHEX (4 μM) provoked a higher level of p53

phosphorylation and stabilization with respect to TMZ treatment. Addition of NBDHEX to the methylating agent caused a more than additive increase of p53 activation (Fig. 4). The ratio of phospho-p53$^{Ser15}$ to total p53 protein was 0.10 with NBDHEX and 0.005–0.09 with 350 and 700 μM TMZ, respectively; these ratios increased to 0.19 after addition of NBDHEX to the methylating agent.

### 3.3. *Systemic administration of NBDHEX does not increase TMZ systemic toxicity*

The toxicity of TMZ or NBDHEX as single agents or in combination (TMZ 100 mg/kg/d/i.p. for 5 d and NBDHEX 8 mg/kg/d/*per os* for 10 d), was tested in B16 melanoma growing i.m. in B6D2/F1 mice. Animal body weight was not significantly

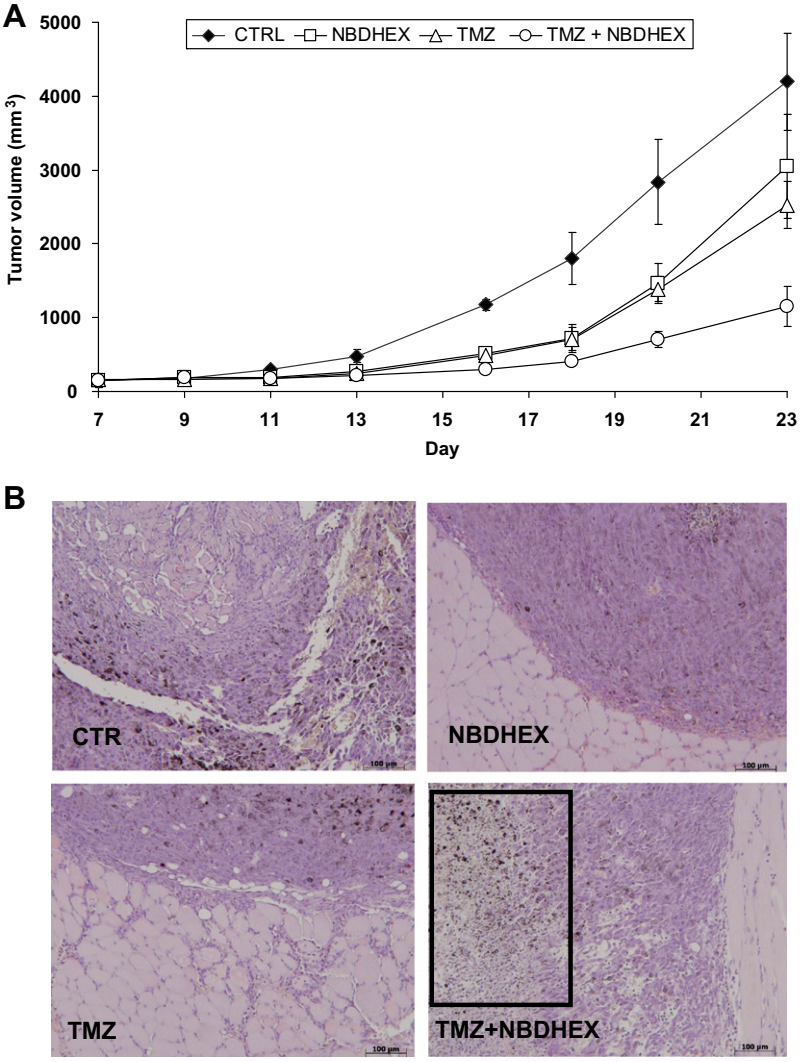


**Fig. 6 – NBDHEX increases the antitumour activity of TMZ against B16 melanoma *in vivo*. Treatment (TMZ 100 mg/kg/d/i.p. for 5 d or NBDHEX 8 mg/kg/d/*per os* for 10 d) started on day 7, when tumour nodules were palpable. Panel A. Symbols represent the means of tumour nodule volumes determined in eight animals for each group every 2–3 d. Bars: ±SD. Statistical analysis of tumour volume was performed by one-way analysis of variance (ANOVA) followed by Bonferroni test for multiple comparisons. Differences between TMZ + NBDHEX and TMZ or NBDHEX were statistically significant starting from day 20 onward ($P < 0.05$); differences between TMZ or NBDHEX and drug vehicle (control mice) were statistically significant starting from day 11 onward ($P < 0.05$); differences between TMZ and NBDHEX groups were NS at all time-points. Panel B. Histological analysis of the muscle hind-limb obtained from mice (3 mice/group) bearing B16 melanoma and treated with drug vehicle (control mice) or with the indicated antitumour agents was performed by H&E staining of samples collected on day 15. Representative histological sections of melanoma growing in the hind limb muscles are presented. Microscopic examination of the muscle showed a massive tumour infiltration of the tissue in the sections from control animals. Conversely, analysis of the muscle tissues collected from mice treated with TMZ or NBDHEX showed a reduction of tumour mass with no infiltration of the muscle in the perilesional area; in the drug combination necrotic regions (framed area) within the tumour were observed. Similar results were obtained in other two samples per group. Histological data were quantified and the values were categorised into 3 scores as follows: 0, absence of tumour cells; 1% to 10% of tumour cells; 2, from 11–50% of tumour cells; and 3, above 50% of tumour cells: CTR, 2.7 ± 0.1; NBDHEX, 1.2 ± 0.1; TMZ, 1.1 ± 0.2; TMZ and NBDHEX 1.5 ± 0.2.**

affected by drug treatment until day 5. At day 6, treatment with TMZ as a single agent or in combination with NBDHEX induced a weight loss of 6% and 9%, respectively, whereas NBDHEX as a single agent did not significantly reduce body weight. Afterwards all mice recovered the initial weight. To evaluate whether NBDHEX might exacerbate TMZ-induced myelosuppression, or might negatively affect kidney or liver functions, blood samples were processed for routine haematochemistry and complete blood count. The results indicated that TMZ treatment caused 80% and 62% decline of WBC on days 7 and 13, respectively (Fig. 5). NBDHEX provoked a 64% reduction of WBC only on day 7 with a prompt and complete recovery. Co-administration of NBDHEX and TMZ did not enhance the myelosuppressive effects of TMZ. The methylating agent induced a significant decrease (30%) in platelet count during the first week, while NBDHEX did not affect platelet counts and did not further enhance the effect of TMZ. No changes in haemoglobin levels were observed with both drugs (Fig. 5). The cytological analysis of bone marrow smears showed a higher percentage of myeloblasts and promyelocytes in the samples obtained from mice treated with NBDHEX as compared to mice treated with TMZ (myeloblasts and promyelocytes percentages: NBDHEX, 32% versus TMZ 17%). This finding might account for the prompt recovery of normal white blood cell counts observed in the NBDHEX group (Fig. 5).

The results of routine haematochemistry, that included glycaemia, azotaemia, creatinine, cholesterol, transaminase, alkaline phosphatase, amylase, bilirubin, plasma proteins, did not show significant differences between vehicle and drug treated groups (data not shown).

### 3.4. *In vivo treatment of melanoma with TMZ and NBDHEX is accompanied by a reduction of cell proliferation and tumour-associated vascularisation*

The efficacy of TMZ and NBDHEX combination was investigated in an orthotopic melanoma model. Treatment with NBDHEX as monotherapy induced a reduction of tumour growth comparable to that obtained with TMZ (Fig. 6A). Noteworthy, NBDHEX and TMZ combination significantly increased tumour growth inhibition with respect to treatment with the single agents ($P < 0.05$).

Histological examination of melanoma nodules performed 15 d after the beginning of treatment revealed a massive tumour infiltration of muscle tissue in H&E sections from animals treated with drug vehicles. Treatment of melanoma with NBDHEX or with TMZ as single agents induced a reduction of tumour mass with no infiltration of the muscle in the perilesional area; necrotic areas within the tumour were observed only in the group treated with the drug combination (TMZ and NBDHEX) (Fig. 6B). Staining of the tumour sections with the anti-Cyclin D1 antibody from control mice revealed an intense and diffuse positivity in the malignant cells and in muscle fibres of the perilesional area, whereas a marked decrease of Cyclin D1 staining was detected in tumour

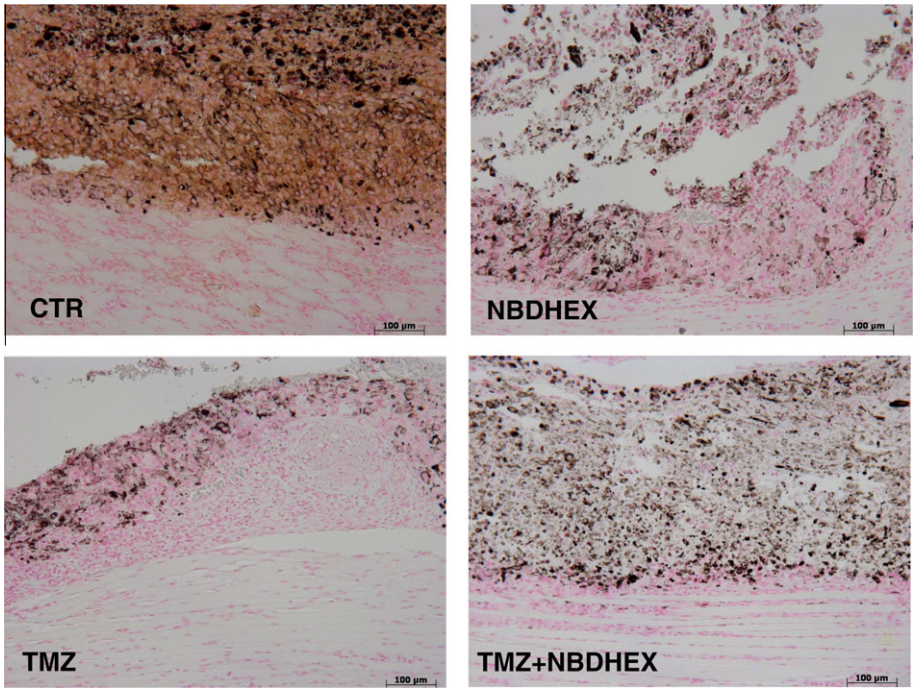


**Fig. 7 – Immunohistochemical of Cyclin D1 expression in B16 grafts from controls and from mice treated with TMZ and NBDHEX as single agents or in combination. Expression of Cyclin D1 was analysed by immunohistochemical staining performed in tumour samples, collected on day 15 (3 mice/group). Representative histological sections of melanoma growing in the muscle hind-limb are presented. Positive Cyclin D1 staining, an index of cell proliferation, was found only in the cytoplasm and nuclei of tumour sections from control mice. Similar immunohistochemical profiles were seen in other two samples per group. The results of densitometric analysis expressed as percentage of tissue area that stained positively for Cyclin D1 were as follows: CTR, 6.7 ± 0.2; NBDHEX, 1.4 ± 0.2; TMZ, 1.4 ± 0.1; TMZ and NBDHEX; 2.0 ± 0.3.**

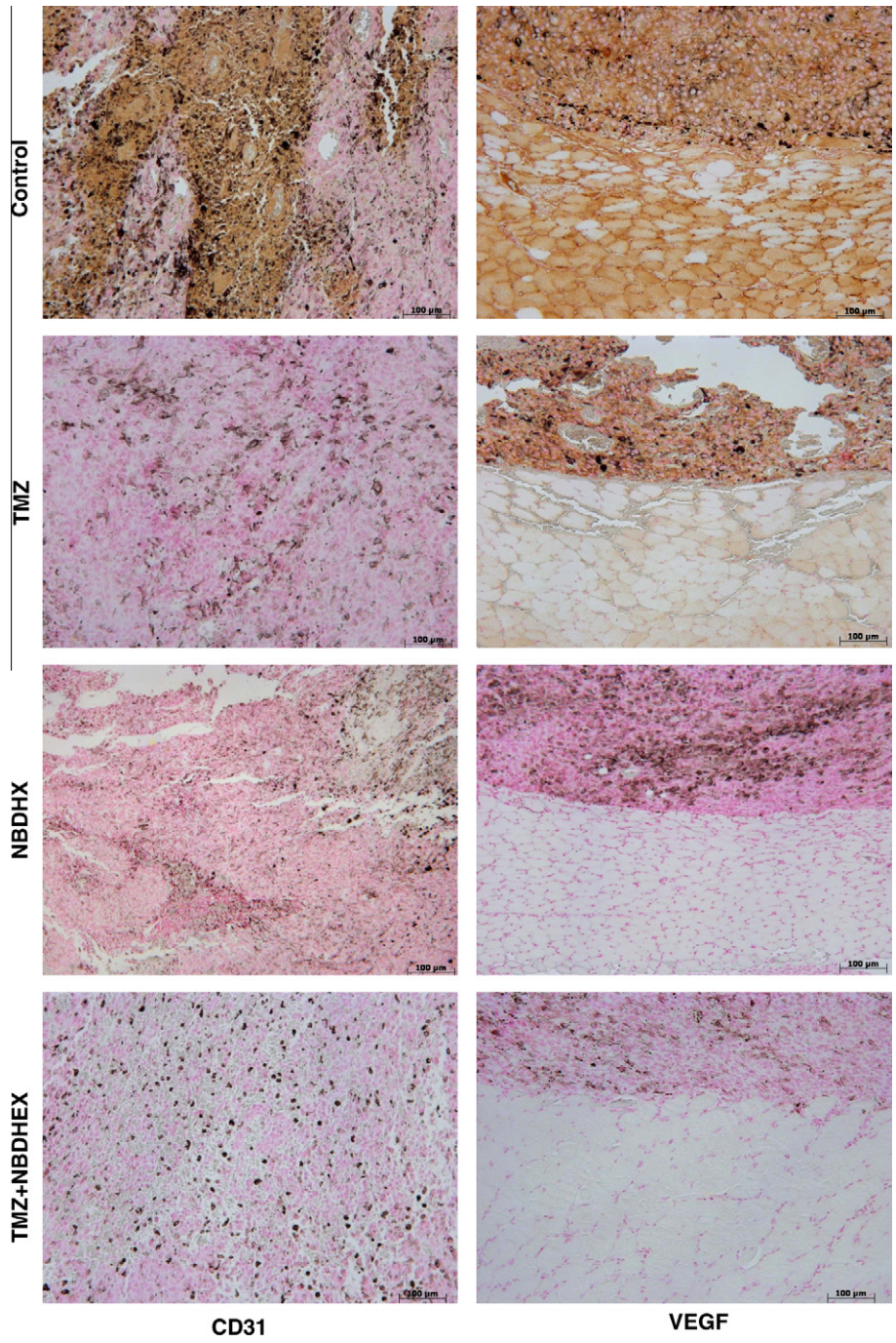


**Fig. 8 – Immunohistochemical of PECAM-1/CD-31 and VEGF expression in B16 grafts from controls and from mice treated with TMZ and NBDHEX as single agents or in combination. PECAM-1/CD31 (*left panels*) and VEGF (*right panels*) expression were analysed by immunohistochemical staining performed in tumour samples, collected on day 15 (3 mice/group). Representative histological sections of melanoma growing in the muscle hind-limb are presented. Positive PECAM-1/CD31 staining (6.1% ± 0.2), an index of highly vascularised tumour, was found in the tissues derived from control mice. On the contrary, melanoma samples from drug-treated mice presented barely detectable positive staining of vessels (NBDHEX, 1.3% ± 0.1; TMZ, 1% ± 0.1; TMZ + NBDHEX 1.2% ± 0.4). A positive VEGF staining in melanoma cells was observed (2.8% ± 0.4). On the contrary, no such positive staining for VEGF was detectable in tumour tissues from mice treated with NBDHEX or NBDHEX + TMZ. In the group treated with TMZ, positive VEGF staining was found only in tumour cells (1.1% ± 0.2). Similar immunohistochemical profiles were seen in other 2 samples per group.**

sections derived from drug treated mice (Fig. 7). The microvessel neo-formation within the tumour was analysed by immunohistochemical staining for platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD31), an indicator of tumour-associated angiogenesis. Melanoma samples from control mice were highly vascularised, since a positive staining

for CD31 was observed around the vessels (Fig. 8). On the other hand, sections from mice treated with NBDHEX or TMZ as single agents or in combination presented a barely detectable staining of vessels. Intense staining for VEGF was detected in the control tumour, and only in tumour cells derived from TMZ treated animals. In contrast, treatment with NBDHEX or with TMZ and NBDHEX completely inhibited the expression of VEGF in tumour mass (Fig. 8).

## 4. Discussion

The GSTP1-1 inhibitor NBDHEX is a novel antitumour agent with anti-proliferative and apoptotic effects in leukaemia and a variety of solid tumours including melanoma.[13–17] In this study we demonstrated, for the first time, that NBDHEX is capable of enhancing the *in vitro* and *in vivo* antitumour activity of TMZ, a methylating agent used for the treatment of metastatic melanoma.

NBDHEX is a novel inhibitor of the cytosolic GSTP1-1 with the interesting property of being a hydrophobic agent that easily penetrates through the cell membrane and is not a substrate of efflux transporter proteins, which represent a frequent mechanism of tumour resistance to a variety of anticancer drugs. Indeed, NBDHEX has been shown to overcome resistance due to over-expression of export pumps (i.e., *p*-glycoprotein and multidrug resistance protein 1) or to high levels of glutathione related detoxification systems.[13,15–17] In the present study, we show that abrogation of PARP-1 expression does not affect sensitivity of B16 cells to NBDHEX indicating that this compound does not directly act on DNA. In fact, this drug targets the active site of GSTP1-1 in complex with JNK leading to the activation of the JNK/c-Jun pathway.[24] This compound in combination with TMZ provoked synergistic anti-proliferative effects against melanoma cells, as indicated by the CI values evaluated in both murine (B16) and human (A375) melanoma cell lines. In B16 melanoma cells, NBDHEX caused marked apoptotic effects at micromolar concentrations capable of inducing apoptosis also in melanoma cell lines of human origin.[14] On the other hand, concentrations of TMZ (i.e., 700 μM) about 10-fold higher than that corresponding to plasma peak concentration in patients did not induce apoptosis in this tumour type, mainly causing growth arrest at the G2/M phase of the cell cycle, in accordance with previous studies.[12,21,25] When a NBDHEX concentration, which provoked a prolonged JNK activation and a moderate level of apoptosis, was combined with the methylating agent, marked apoptotic effects were observed. The role of JNK activation in increasing melanoma sensitivity to TMZ has been described also in combination studies with a natural product.[26] Conversely, in glioma cells JNK activation was suggested to play a cytoprotective role in response to TMZ since JNK inhibitors enhanced cytotoxicity of the methylating agent.[27] Indeed, JNK has been shown to exert opposite effects depending on cell type, anticancer drug or kinetics and stability of JNK phosphorylation.[27] In this regard it should be noted that while transient JNK phosphorylation has been associated with increased cell survival in response to anticancer drugs, a sustained JNK activation instead contributed to apoptosis signalling.[27] Moreover, treatment of B16 melanoma cells with NBDHEX was accompanied by induction of p53 phosphorylation on Ser-15 to a higher extent than that observed with TMZ as a single agent. Combined treatment with NBDHEX and TMZ caused more pronounced p53 stabilization with respect to NBDHEX or TMZ used as single agents. P53 is necessary for TMZ mediated cell growth arrest and Ser-15 is regarded as one of the principal sites on p53 that is phosphorylated by JNK in response to cellular stress.[28,29] The prolonged activation of JNK triggered by NBDHEX may justify the increase of p53 stability. Therefore, the increase of anti-tumour activity derived by the combination of NBDHEX and TMZ might be attributed to the independent mechanism of action of the two drugs that converge on the JNK/p53 axis accelerating apoptotic cell death. In addition, we cannot rule out the possibility that the inhibition of the GSTP1-1 catalytic activity by NBDHEX may contribute to the reported synergistic anti-proliferative effect. However, there are no experimental evidences that the methyldiazonium ion (the methylating species generated by TMZ decomposition) is conjugated to GSH by GSTP1-1 or effluxed out of cancer cells. Moreover, no correlation has been reported so far between the GST activity and sensitivity to TMZ in melanoma tumours.[30]

The results of the *in vivo* experiments indicated that NBDHEX at a dose of 8 mg/kg for 10 d showed an efficacy similar to that of the standard dosing of TMZ (100 mg/kg for 5 d) used in preclinical studies.[12,18,21] Interestingly, the drug combination significantly inhibited tumour growth with respect to the single agents, delaying the resuming of tumour growth. This effect was accompanied by the appearance of necrotic areas within the tumour which were not detected in treatments with the single agents. Immunohistochemical staining of the tumour sections for Cyclin D1, an indicator of tumour cell proliferation, and of PECAM/CD31, a marker of microvessel neo-formation within the tumour mass, resulted to be negative both in the single and combined treatments. Moreover, no expression of VEGF was detected in the sections of tumour grafts derived from mice treated with NBDHEX as a single agent or administered together with TMZ. This observation is of particular interest; in fact melanoma cells are a source of several angiogenic factors, including the members of the VEGF family of growth factors that are involved in autocrine and paracrine regulation of melanoma progression promoting not only neo-angiogenesis but also directly activating tumour growth. In fact, melanoma cells express VEGF receptors that, upon interaction with their ligands, can modulate the proliferative potential, apoptosis and invasiveness of melanoma cells.[31] Therefore, the down-modulation of VEGF expression detected in melanoma grafts exposed to the GSTP1-1 inhibitor might contribute to the *in vivo* antitumour effect and to its capability of enhancing TMZ efficacy.

The most common side effect of TMZ is myelotoxicity, primarily neutropenia and thrombocytopenia that may increase in combination therapies with inhibitors of MGMT mediated repair or anticancer agents with overlapping toxicities.[32,33] An interesting aspect of the methylating agent and GSTP1-1 inhibitor combination is that the addition of NBDHEX did not exacerbate TMZ myelotoxicity nor delayed recovery of bone marrow function. Actually, analysis of bone marrow smears showed a higher percentage of myeloblasts and

promyelocytes in the samples derived from mice treated with NBDHEX as a single agent, suggesting that this effect might account for the prompt recovery of normal white blood cell counts observed in the NBDHEX group and for the lack of exacerbation of TMZ neutropenia. This finding can be explained with the role of GSTP1-1 as a negative modulator of the maturation and growth of haematopoietic progenitors due to inhibition of JNK that in turn activates c-Jun, a key regulator of cellular proliferation, differentiation, and apoptosis. In fact, an increase of bone marrow progenitor proliferation has been detected in GSTP1-1 knock-out mice or in mice treated with the peptidomimetic GSTP1-1 inhibitor TLK199, which is currently in clinical trials for myelodysplastic syndromes.[34,35] In addition, neither TMZ nor NBDHEX, as single agents or in combination, significantly affected liver and kidney functions.

In conclusion, these findings indicate that NBDHEX might represent a good candidate for combination therapies including TMZ for the treatment of malignant melanoma, due to their different mechanisms of action and non-overlapping systemic toxicities.

## Conflict of interest statement

None declared.

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