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Poly(ADP-ribose) glycohydrolase inhibitor as chemosensitiser of malignant melanoma for temozolomide

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Abstract

Disruption of poly(ADP-ribose) polymerase (PARP) pathways by inhibitors of PARP catalytic domain has been shown to increase the anti-tumour activity of temozolomide (TMZ). Since PARP is inhibited by poly(ADP)ribosylation, herein we tested whether inhibition of poly(ADP-ribose) glycohydrolase (PARG) might enhance TMZ efficacy. The PARG inhibitor *N*-bis-(3-phenyl-propyl)9-oxo-fluorene-2,7-diamide (GPI 16552) was administered in combination with TMZ to mice injected subcutaneously or intracranially with B16 melanoma cells. The ability of treatment to reduce melanoma metastatic spreading and invasion of the extracellular matrix was also tested. The results indicated that combined treatment with GPI 16552 and TMZ significantly reduced melanoma growth, increased life-span of mice bearing tumour at the CNS site, and decreased the ability of melanoma cells to form lung metastases and to invade the extracellular matrix. In conclusion, PARG inhibition represents an alternative strategy to enhance TMZ efficacy against melanoma in peripheral as well as at CNS site.

Keywords: Poly(ADP-ribose) polymerase; (ADP-ribose) polymers; Poly(ADP-ribose) glycohydrolase; Temozolomide; Cancer; Metastasis; Brain tumours; Blood-brain barrier

1. Introduction

Poly(ADP)ribosylation of cellular proteins is an immediate cellular response to genotoxic damage induced by oxidative stress, ionizing radiation or alkylating agents. This post-translational modification is carried out mainly by poly(ADP-ribose) polymerase-1 (PARP-1), the founding member of the growing family of PARPs [1]. Upon binding to DNA strand breaks, PARP-1 uses NAD^+ as substrate to synthesize ADP-ribose polymers (PAR) that are targeted to itself or to other acceptor proteins including enzymes involved in DNA repair.

Elevated synthesis of PAR, deriving from extensive DNA injury and PARP-1 over-activation, may cause cell death due to ATP depletion consequent to NAD^+ consumption. Extensive poly(ADP)ribosylation has been involved in a number of diseases and its modulation through inhibition of PARP activity has been exploited to counteract PARP-1 mediated cell death

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in a broad spectrum of pathologies, such as stroke, diabetes, shock, inflammation, asthma or acute renal failure [2]. Moreover, due to the role of PARP-1 in co-ordinating DNA repair, PARP inhibitors have been exploited to enhance the efficacy of anti-cancer chemotherapy and radiotherapy [3]. In fact, a number of studies have recently demonstrated the *in vivo* efficacy of PARP inhibitors to increase the anti-tumour activity of methylating agents, topoisomerase I inhibitors and ionizing radiation [4–8].

Poly(ADP)ribosylation confers negative charges to proteins that promptly dissociate from DNA by electrostatic repulsion and PAR are degraded rapidly by poly (ADP-ribose) glycohydrolase (PARG), the only known enzyme capable of hydrolysing PAR to ADP-ribose via endo- and exo-glycosidic cleavage. Three isoforms of PARG have been described that are encoded by a single gene [9]. The full-length PARG protein ($PARG_{110}$) is located in the nucleus, while the most abundant splice variants PARG-99 and PARG-102 are cytoplasmic [10]. They are ubiquitously expressed in mammalian cells and involved in a number of cell processes including cell cycle regulation, mitotic spindle assembly, development, differentiation, cell death and DNA repair [11–14].

The importance of PARG in PAR metabolism and in cellular responses to genotoxic stress has been recently demonstrated in $PARG_{110}$ deficient mice. In particular, genetic ablation of $PARG_{110}$ sensitises cells to DNA damage induced by alkylating agents and ionizing radiations [15]. Moreover, the complete absence of PARG activity causes early embryonic lethality, indicating that the regulation of PAR levels is critical for cell survival and that there are no significant compensatory mechanisms for the catabolism of PAR. Embryonic stem cell lines derived from PARG null mice show increased sensitivity to genotoxic agents capable of activating PARP-1 and of further increasing PAR levels [16].

The studies on PARG knockout mice suggest that inhibition of PARG may represent a strategy to enhance the effectiveness of chemotherapeutic agents. Considering that over-activation of PARP-1 and PAR accumulation induced by oxidative, genotoxic, or radiation treatments can cause cell death in a dose-dependent manner [17], it is conceivable that inhibition of PAR degradation may have similar consequences as the stimulation of poly(ADP)ribosylation. Moreover, since PARP-1 is strongly inhibited by poly(ADP)ribosylation, PARG inhibition could maintain PARP-1 in an inactive state preventing the removal of the polymers from the enzyme. Therefore, PARG inhibitors might indirectly compromise PARP-1 function in the repair of DNA damage induced by certain chemotherapeutic agents.

In regard to pharmacological inhibitors of the enzyme, natural plant tannins like green tea polyphenols have been described to inhibit PARG activity, with consequent accumulation of PAR, and to induce growth inhibition and apoptosis in cancer cell lines [18–21]. However, the lack of specific, potent and permeable PARG inhibitors has hampered *in vivo* testing of the hypothesis that PARG inhibitors may support efficacy of anti-cancer treatments.

Recently, the non-tannin small molecule GPI 16552 has been shown to inhibit PARG activity and to reduce neuronal damage in an *in vivo* model of stroke and to exert a protective effect against intestinal injury induced by splanchnic artery occlusion shock [22,23]. Moreover, another PARG inhibitor, GPI 18214, besides reducing intestinal injury provided protection against septic shock-like syndrome [24]. This effect was attributed to reduced PAR degradation and consequent inhibition of PARP-1, which would be maintained in its auto-(ADP-ribosyl)ated and inactive form [22–24]. In this study we have investigated whether systemic administration of GPI 16552 might enhance the anti-tumour efficacy of the methylating agent temozolomide (TMZ), an anti-cancer drug currently used for the treatment of primary and secondary brain tumours. The results showed that the combination of GPI 16552 and TZM significantly reduced the growth of B16 melanoma and possessed anti-metastatic activity *in vivo*. Overall, our data provide the first evidence that pharmacological inhibition of PARG may sensitise melanoma cells to chemotherapy.

2. Materials and methods

2.1. Cell lines and culture conditions

The murine melanoma B16 cell line of C57BL/6J (H-2^b/H-2^b) origin (ATCC, Manassas, VA) and the human B lymphoblastoid MT-1 cell line were cultured in RPMI-1640 (Sigma–Aldrich, Milan, Italy) supplemented with 10% fetal calf serum (Sigma–Aldrich), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma–Aldrich), at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Drugs

The PARG inhibitor *N*-bis-(3-phenyl-propyl)9-oxo-fluorene-2,7-diamide, GPI 16552 was synthesized in Guilford laboratories (Guilford Pharmaceuticals Inc., Baltimore, MD, USA). TMZ was provided by Schering-Plough Research Institute (Kenilworth, NJ, USA). $MeOSO_2(CH_2)_2$–lexitropsin (Me-Lex) was prepared as previously described [25]. Drug stock solutions were prepared by dissolving TMZ or GPI 16552 in dimethyl sulfoxide (DMSO) and Me-Lex in 95% ethanol. The final concentration of ethanol or DMSO in drug treated cultures was always less than 0.1% (v/v) and did not contribute to toxicity (data not shown).

2.3. Chemosensitivity studies

Cells were treated with GPI 16552 (5–25 μM) or with TMZ (31–250 μM). For the experiments to assess the ability of GPI 16552 to enhance the anti-proliferative effects of TMZ, the PARG inhibitor was added to cell cultures 15 min before the methylating agent. Long-term survival was analysed by colony-formation assay [5].

Cell line chemosensitivity was evaluated in terms of IC_{50}, i.e. the concentration of the drug expressed in μM capable of inhibiting colony-forming ability by 50%.

2.4. Analysis of poly(ADP-ribose) modified proteins

For drug treatment, B16 and MT-1 cells (3×10^5 cells/ml) were cultured in flasks (Becton & Dickinson Italia, Milan, Italy) and 50 mM hydrogen peroxide (H_2O_2), or 25 μM Me-Lex was added to cell cultures, either alone or 15 min after PARG inhibitor (15 μM). Thirty minutes or 1 h after drug treatment, cells were washed three times with 5 ml ice-cold PBS with 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride and suspended at the concentration of 1×10^7/ml in a denaturing buffer, as previously described [26]. Briefly, cells were disrupted by sonication on ice (sonication buffer: Tris–HCl 62.5 mM pH 6.8, 4 M urea, 10% (v/v) glycerol, 2% (w/v) SDS, 5% β-mercaptoethanol, 0.003% (w/v) bromophenol blue) twice for 30 s (60 W) and heated for 15 min at 65 °C. Samples were separated by 7.5% SDS–PAGE and transferred onto a nitrocellulose filter. The membrane was then saturated overnight with 5% (w/v) blocking agent (Amersham Biosciences, Milan, Italy) in TBS and then incubated for 3 h with a rabbit polyclonal antibody against PAR (1/1000 dilution; Biomol, Plymouth Meeting, PA, USA). After several washings with TBS containing 0.2% Tween-20, the membrane was incubated for 1 h in the presence of donkey anti-rabbit antibody (1/15,000) conjugated to horseradish peroxidase. Visualisation of immunoreactive bands was performed by an ECL system (Amersham). The signal was quantified by bidimensional densitometry using a Kodak Imaging densitometer (Image Analysis Software, Rochester, NY, USA).

2.5. Extracellular matrix (ECM) invasion

The ability of B16 cells to invade the ECM was analysed by an *in vitro* test in Boyden chambers, using 8 μM pore diameter polycarbonate filters (Nuclepore, Whatman Incorporated, Clifton, NJ, USA) coated with 10 μg of matrigel, a commercial basement membrane matrix (BD Biosciences, Bedford, MA, USA). B16 cells (2×10^5) were loaded into the upper chamber. Migration medium (0.1% BSA, 1 μg/ml heparin in RPMI 1640) was always used to determine the basal invasion value. After 4 h of incubation at 37 °C in a CO_2 incubator, the filter was removed from the camera and cells were fixed in 4% paraformaldehyde in PBS and stained in 0.5% (w/v) crystal violet. Cells from the upper surface were removed by wiping with a cotton swab, and the ability of the B16 cells to invade the ECM was determined by counting the migrating cells attached to the lower surface of the filter in 12 randomly selected microscopic fields (200× magnification) per experimental condition. The results are expressed as an invasion index, that is the ratio between the number of cells/microscopic field in the experimental condition analysed and the number of cells/microscopic field in the basal conditions (untreated cells). The invasion index at basal conditions corresponds to 1.

2.6. In vivo studies

For *in vivo* treatment, GPI 16552 (40 mg/kg) was injected intra peritoneum (i.p.), 15 min before TMZ administration. TMZ was administered i.p. at 100 mg/kg, a dose commonly used for *in vivo* preclinical studies [4,5]. Control mice were treated with vehicles only. Drug toxicity was evaluated by treating healthy B6D2F1 (C57BL/6 × DBA/2) male 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 days after the last treatment. Experiments were performed to determine the maximal tolerated dose of the drug combination.

The efficacy of TMZ ± GPI 16552 treatment was initially evaluated on melanoma growing subcutaneously (s.c.) in B6D2F1 mice (8/group). For this purpose B16 cells (2.5×10^5) were inoculated in the flank of the animal. Tumours were measured with calipers and volume calculated according to the formula: $[(\text{width})^2 \times \text{length}]/2$. Treatment with 40 mg/kg/i.p. GPI 16552 + 100 mg/kg/i.p. TMZ for 3 days started 6 days after challenge, when the volume of tumour nodules reached 100–150 mm^3. Melanoma growth was monitored by measuring tumour nodules every 2–4 days for 3 weeks.

For the analysis of the influence of PARG inhibitor and TMZ combination on spontaneous metastases, B16 cells (2.5×10^5) were injected in the hind leg and treatment (see above) started after three days. To evaluate the influence of the drugs under study on generation of artificial metastases, B16 cells (2.5×10^5 in 0.2 ml) were injected into the tail vein of mice and treatment started 24 h after tumour challenge.

Three weeks after tumour challenge, animals were sacrificed and lungs removed and fixed in Bouin's

solution to distinguish tumour nodules from lung tissue. The number of metastases was determined using a dissecting microscope [5].

The intracranial (i.c.) transplantation procedure was performed as previously described [4]. Briefly, cells (1×10^4 in 0.03 ml of PBS) were injected i.c. through the center-middle area of the frontal bone to a 2 mm depth, using a 0.1 ml glass microsyringe and a 27-gauge disposable needle into mice. Before tumour challenge, animals were anesthetized with tiletamine hydrochloride plus zolazepam hydrochloride (Zbletil 100, Virbac, Carros, France) at 20 mg/kg/intramuscularly (i.m.). in a volume of 50 μl. In mice bearing tumour in the brain, treatment started on day 2 after challenge, when tumour infiltration in the surrounding brain tissue was histologically evident [4]. Mice were treated daily with the 3-days schedule and monitored for mortality. Median survival times (MST) were determined and the percentage increase in lifespan (ILS) was calculated as: {[MST (days) of treated mice/MST (days) of control mice] − 1} × 100. Efficacy of treatments was evaluated by comparing survival curves between treated and control groups.

All procedures involving mice and care were performed in compliance with national and international guidelines (European Economy Community Council Directive 86/109, OLJ318, December 1, 1987).

2.7. Statistical analysis

For statistical analysis of the growth of s.c. melanoma nodules or metastasis number in the lungs, the significance of the differences between experimental groups (8 animals/group) was evaluated by *t*-test. *P* values are two-sided (software Microsoft Excel).

Survival curves were generated by Kaplan–Meier product-limit estimate and statistical differences between the various groups (8 animals/group) were evaluated by log-rank analysis with Yates correction (software Primer of Biostatistics, McGraw-Hill, New York, NY, USA).

Statistical significance was determined at an $\alpha = 0.05$ level. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. GPI 16552 reduces PAR turn-over

It has been previously demonstrated that GPI 16552 inhibits PARG activity with an IC_{50} of 1.7 μM [22]. Herein we tested the ability of GPI 16552 to reduce degradation of PAR generated by cell treatment with genotoxic agents that induce PARP-1 over-activation and poly(ADP)ribosylation of cellular proteins. To this end, B16 cells were treated with the oxidant hydrogen peroxide (H_2O_2) that is known to generate DNA strand breaks through the formation of hydroxyl radicals and to induce PARP-1 activation. Moreover, leukaemia MT-1 cells were exposed to Me-Lex, a selective N3-adenine methylating agent which we have recently shown to induce PARP-1 over-activation and cell death in sensitive cells with low levels of *N*-methylpurine glycosylase [26,27]. Western blot analysis was performed using anti-PAR antibody 30 and 60 min after treatment. The results indicate that Me-Lex or H_2O_2 induced a transient increase in protein poly(ADP)ribosylation, as evidenced by the diffuse increase of PAR immunoreactivity on proteins of high molecular weight. After 1 h, the level of PAR proteins was only slightly higher than that of untreated control. Treatment with Me-Lex + GPI 16552 reduced PAR turn-over resulting in a higher level and a longer persistence of PAR. Treatment with GPI 16552 delayed degradation of PAR also in B16 cells treated with H_2O_2 (Fig. 1).

3.2. In vitro treatment with the PARG inhibitor GPI 16552 in combination with TMZ significantly reduces clonogenic survival of melanoma cells

B16 melanoma cells were exposed to graded concentrations of GPI 16552 as single agent, and cell growth was analysed by colony-formation assay. The results indicated that GPI 16552 exhibited intrinsic growth inhibition. Thereafter, 6 μM GPI 16552, a concentration of corresponding to IC_{20}, was tested for its ability to enhance the anti-proliferative effect of TMZ. The results of colony formation assay indicated that 6 μM GPI 16552 enhanced growth inhibition induced by TMZ by about threefold (Table 1).

3.3. In vivo treatment with GPI 16552 increases the efficacy of TMZ against malignant melanoma including CNS localisations

To monitor the toxicity of treatment, intact animals were treated with graded doses of GPI 16552 in combination with TMZ. The administration of GPI 16552 (40 mg/kg/i.p.) + TMZ (100 mg/kg/i.p.) for three days was tolerated with a maximal weight loss of 12%. All mice recovered the initial body weight two weeks after treatment. This schedule was used for the studies on anti-tumour activity.

The anti-tumour activity of GPI 16552 and TMZ combination was tested in B16 melanoma growing s.c. in B6D2F1 mice and compared with the effects induced by TMZ or GPI 16552, used as single agents. The results show that GPI 16552 significantly enhanced the anti-tumour effect of TMZ (Fig. 2). The drug combination markedly inhibited tumour growth with respect to vehicle treated control or to animals treated with the single agents ($P < 0.0001$ starting from day 15). At day 23, animals were sacrificed to avoid unacceptable suffering and according to ethical guidelines.

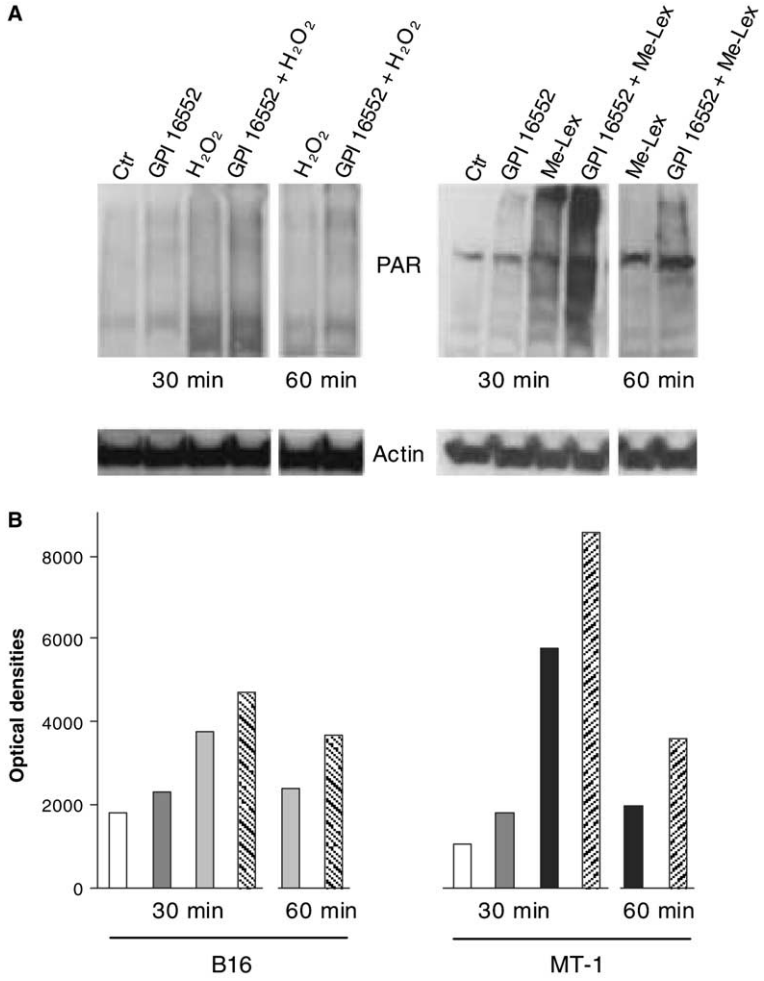

Fig. 1. GPI 16552 increases formation of PAR in response to genotoxic damage. (A) B16 (left panel) or MT-1 (right panel) cells were left untreated (Ctr), treated with 50 mM H_2O_2 or 25 μM Me-Lex, as single agents or in combination with 15 μM GPI 16552, and then analysed by immunoblot for PAR formation or actin expression at the indicated time points. Poly(ADP)ribosylated proteins (PAR) comprised between 250 and 105 kDa are shown. Results are representative of two independent experiments. (B) Histograms represent densitometric values corresponding to the above shown immunoblot. Values are as follows: B16 untreated control (white column), 1788; 30 min GPI 16552 (dark grey column), 2300; 30 min H_2O_2 (light grey column), 3750; 30 min GPI 16552 + H_2O_2 (dashed column), 4700; 60 min H_2O_2 (light grey column), 2400; 60 min GPI 16552 + H_2O_2 (dashed column), 3650; MT-1 untreated control (white column), 1050; 30 min GPI 16552 (dark grey column), 1813; 30 min Me-Lex (black column), 5750; 30 min GPI 16552 + Me-Lex (dashed column), 8590; 60 min Me-Lex (black column), 1952; 60 min GPI 16552 + Me-Lex (dashed column), 3600.

Then, it was investigated whether systemic administration of GPI 16552, might increase the efficacy of TMZ against B16 melanoma growing at the CNS site. The results indicated that survival times of mice bearing B16 melanoma treated with GPI 16552 + TMZ were significantly higher than that observed in animals receiving vehicle only ($P < 0.0001$) or TMZ as single agent ($P < 0.006$). No significant differences in survival times were observed between vehicle treated control and the groups treated with the single agents (Fig. 3 and Table 2).

Table 1
In vitro chemosensitivity of B16 melanoma

Treatment	IC_{50} (μM)
GPI 16552	12 ± 1
TMZ	120 ± 20
TMZ + 6 μM GPI 16552	30 ± 6

Chemosensitivity was assessed by colony-formation assay. Data represent the mean values of three independent experiments ± SE.

3.4. Treatment with GPI 16552 in combination with TMZ significantly reduces metastasis formation in vivo and the ability of melanoma cells to invade the ECM

To assess the influence of drug treatment on the ability of melanoma to give rise to spontaneous metastases, B16 cells were injected i.m. and three weeks after tumour challenge animals were sacrificed and the lungs of

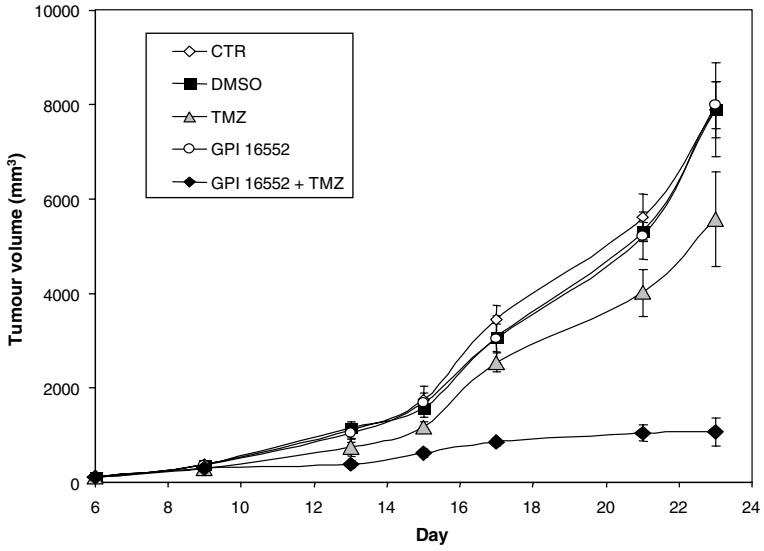

Fig. 2. *In vivo* treatment with GPI 16552 enhances the anti-tumour activity of TMZ against B16 melanoma growing subcutaneously. Treatment (GPI 16552 40 mg/kg/day/i.p. ± TMZ 100 mg/kg/day/i.p. for 3 days) started on day 6, when the volume of tumour nodules reached 100–150 mm^3. Symbols represent the means of tumour nodule volumes determined in the animals of each group every 2–4 days. Bars: ±SE. The results of statistical analysis (starting from day 13) are as follows: GPI 16552 + TMZ *vs.* CTR or *vs.* GPI 16552, $P < 0.0001$ at all time points; GPI 16552 + TMZ *vs.* TMZ, $P = 0.0004$ at day 13 and $P < 0.0001$ thereafter; TMZ *vs.* CTR, $P < 0.005$.

vehicle or drug treated animals were analysed. The results showed that combined treatment markedly reduced the number of spontaneous metastases with respect to treatment with the single agents (Fig. 4).

Moreover, the PARG inhibitor GPI 16552 in combination with TMZ significantly reduced the ability of B16 melanoma cells, injected i.v., to colonize the lung giving rise to artificial metastases. In fact, the number of melanoma nodules in the lungs observed after treatment with GPI 16552 + TMZ was significantly lower ($P < 0.0001$) than that detected in mice treated with TMZ used as single agent (Fig. 5). Treatment with GPI 16552 did not significantly affect development of metastases with respect to control animals.

To analyze whether the PARG inhibitor might affect the ability of B16 melanoma to invade the ECM, cells were treated with 10 μM GPI 16552 as single agent or in combination with 125 μM TMZ for 24 h. Cells were then detached, counted and plated in Boyden chamber equipped with filters coated with matrigel. At this time

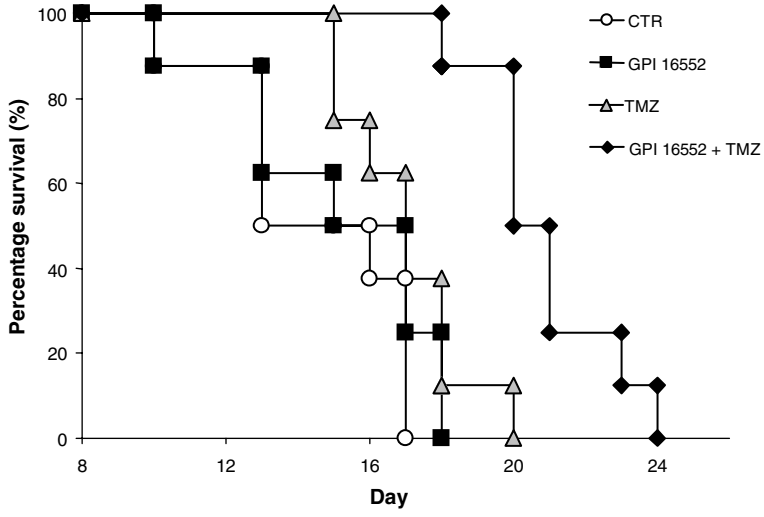

Fig. 3. Systemic administration of the PARG inhibitor GPI 16552 in combination with TMZ enhances survival of mice bearing B16 melanoma at the CNS site. Animals (8 per group) were inoculated i.c. with B16 cells (day 0) and treated with the 3-days schedule, described in legend to Fig. 2, on day 2 after tumour challenge. Control animals (CTR) were treated with drug vehicle only. Survival curves of tumour bearing mice are presented. A significant increase ($P < 0.0001$) in survival was observed when survival curves of mice treated with GPI 16552 + TMZ were compared to those of controls or of animals treated with each single drug. Treatment with GPI 16552 or with TMZ as single agents was ineffective.

Table 2
Influence of GPI 16552 and TMZ on survival of animals bearing B16 melanoma at the CNS site

Treatment	MST (range)	ILS[a] (*vs.* CTR)	P_1[b] (*vs.* CTR)	ILS[c] (*vs.* TMZ)	P_2[b] (*vs.* TMZ)
Vehicle (CTR)	15 (10–17)				
GPI 16552	16 (10–18)	6%	0.34		
TMZ	17 (15–20)	13%	0.064		
GPI 16552 + TMZ	21 (20–24)	40%	<0.0001	23%	0.006

[a] Increase in lifespan (ILS) of drug-treated mice was calculated comparing their median survival time (MST) with those of control animals injected with drug vehicles only (CTR).
[b] *P* was calculated comparing survival curves of drug-treated groups *vs.* CTR (P_1), or comparing survival curves of mice treated with the drug combination *vs.* mice treated with TMZ (P_2).
[c] ILS of mice treated with GPI 16552 + TMZ was calculated comparing their MST with those of mice treated with TMZ only.

point, no difference in viability and percentage of apoptotic cells were detected among the different experimental groups (data not shown). The results, expressed as invasion index, indicated that combined treatment with GPI 16552 and TMZ significantly reduced the ability of melanoma cells to invade matrigel with respect to vehicle ($P < 0.0001$) or to treatement with the single agents ($P = 0.0005$) (Fig. 6).

4. Discussion

In the present report we have demonstrated for the first time that PARG inhibition significantly increased the anti-tumour activity of the methylating agent TMZ against malignant melanoma. In fact, *in vivo* treatment of melanoma bearing mice with the PARG inhibitor GPI 16552 in combination with TMZ markedly inhibited tumour growth and its metastatic spreading with respect to treatment with the single agents.

The *in vivo* efficacy of GPI 16552 to counteract the damage induced by PARP-1 over-activation has been recently reported [22,23]. In fact, this compound was capable of reducing intestinal injury induced by splanchnic artery occlusion and brain infarct volume in a rat model of focal cerebral ischemia [22]. The neuroprotective effect afforded by GPI 16552 at the dose of 40 mg/kg indirectly indicated that this compound can cross the blood-brain barrier reaching therapeutic concentrations at the CNS site. The *in vivo* efficacy of GPI 16552 as PARG inhibitor together with its ability to cross the blood-brain barrier encouraged us to test its potential therapeutic utility as chemosensitiser for tumours located in the brain. Herein, we demonstrate that GPI 16552 at the same dose is able to enhance the anti-tumour activity of TMZ against intracerebral melanoma, since mice treated with the drug combination survived longer than animals receiving the single agents. On the basis of these data it is tempting to speculate that the advantage deriving from the use of PARG inhibitors in combination with chemotherapy for CNS primary tumours or metastases is twofold: enhancement of the growth inhibitory activity of chemotherapeutic agents and protection of the surrounding brain tissue from ischemic damage.

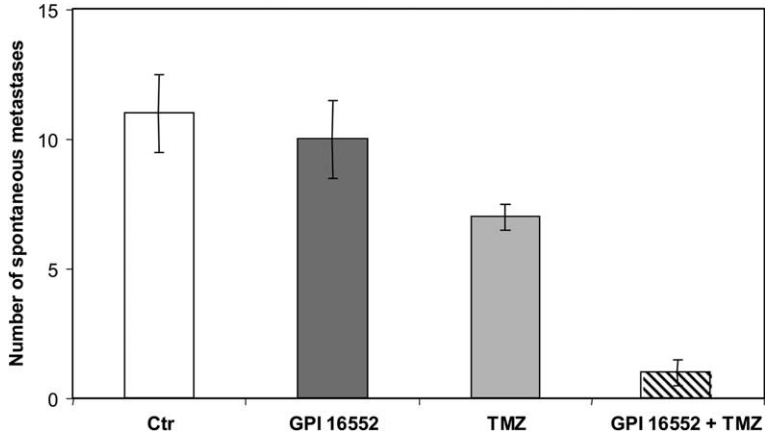

Fig. 4. *In vivo* treatment with GPI 16552 in combination with TMZ reduces generation of spontaneous metastases from B16 melanoma. Animals were injected i.m. with B16 melanoma cells and treated with the 3-day schedule as described in legend to Fig. 2. After three weeks, mice were sacrificed and the number of spontaneous metastases recorded by counting tumour nodules on lung surface. Histograms represent the number of spontaneous metastases. Bars: ±SE. Statistical significant differences are as follows GPI 16552 + TMZ *vs.* CTR, *vs.* GPI 16552 or *vs.* TMZ, $P < 0.0001$.

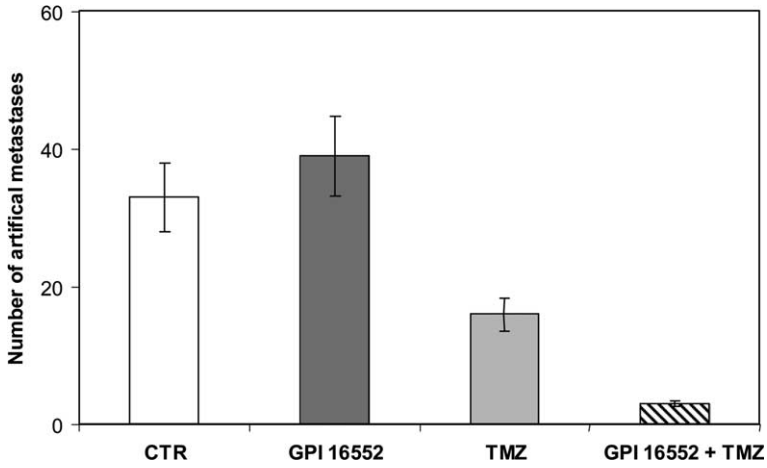

Fig. 5. Influence of GPI 16552 treatment as single agent or in combination with TMZ on B16 pulmonary artificial metastases. Animals were injected i.v. with B16 melanoma cells and treated with the 3-day schedule described in the legend to Fig. 2. After three weeks, mice were sacrificed and the number of artificial metastases recorded by counting tumour nodules on lung surface. Histograms represent the mean values of the number of metastases detected in the different treatment groups. Bars: ±SE. Statistically significant differences are as follows: GPI 16552 + TMZ *vs.* CTR or *vs.* TMZ, $P < 0.0001$; TMZ *vs.* CTR, $P = 0.001$; GPI 16552 *vs.* CTR, $P = 0.19$.

In the experimental model of B16 malignant melanoma, besides providing local tumour control, the combination of the PARG inhibitor with TMZ markedly affected the ability of melanoma cells to detach from the primary tumour and spread to the lung giving rise to metastasis generation. The reduced metastatic potential of the primary tumour may reflect the significant decrease in the ability of the melanoma cell to invade the ECM observed upon exposure to GPI 16552 + TMZ with respect to vehicle treated control or to cells exposed to the single agents. Moreover, when B16 cells were injected i.v. to assess the efficacy of drug treatment against artificial metastases, the PARG inhibitor in combination with TMZ significantly reduced the ability of melanoma cells to colonize the lungs as compared to treatment with TMZ alone.

It is not clear how PARG inhibition may mediate the metastatic pathway but PARP-1 is known to be involved in regulating gene expression. For example, in rodents treated with PARP inhibitors, the expression of ICAM-1 and cytokines are down-regulated [28]. It is conceivable that PARG inhibition affects the poly(ADP-ribosyl)ation pathway similar to PARP inhibition to limit expression of key mediators of metastasis.

The mechanism underlying the chemosensitising effect afforded by PARG inhibition likely involves disruption of PAR pathway as treatment of tumour cells with GPI 16552 slows down degradation of PAR generated upon

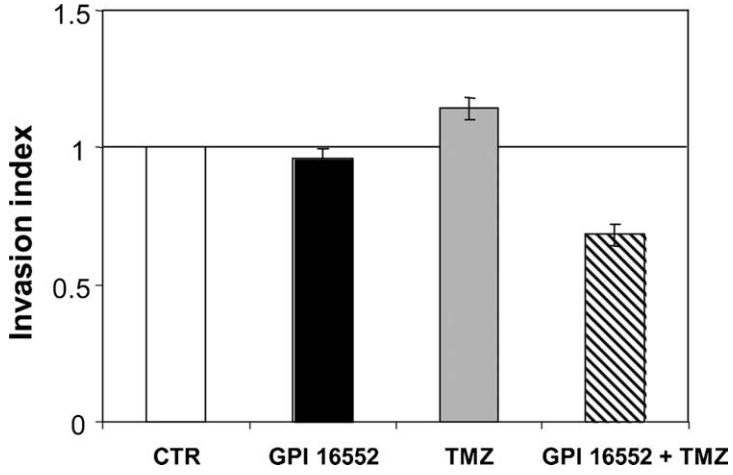

Fig. 6. Treatment with PARG inhibitor GPI 16552 in combination with TMZ reduces the ability of B16 melanoma cells to invade the ECM. The ability of B16 melanoma cells to invade the ECM was analysed in Boyden chambers using filters coated with matrigel, as described in Section 2. The results are expressed as invasion index in response to matrigel and histograms represent the mean of four independent experiments. Bars: ±SE. The results of statistical analysis are as follows: GPI 16552 + TMZ *vs.* CTR, or *vs.* TMZ, $P < 0.0001$; GPI 16552 + TMZ *vs.* GPI 16552, $P = 0.0005$.

genotoxic injury (i.e. oxidation or N3-adenine methylation) which provokes PARP-1 over-activation. PARP-1 activation results in the synthesis of poly(ADP-ribose) that modifies itself, and other nuclear proteins. Auto-(ADP-ribosyl)ation of PARP-1 causes inhibition of its catalytic activity; degradation of poly(ADP-ribose) by PARG releases PARP from such inhibition and permits it to continuously produce the polymer.

Our data are in contrast with those reported by Falsig and colleagues showing that pre-treatment with either GPI 16552 or gallotannin did not enhance PAR accumulation or delay the time-dependent loss of PAR immunostaining in astrocytes upon exposure to hydrogen peroxide [29]. Even though GPI 16552 concentrations were comparable to those tested in the present report, discrepancies in the results may be attributed to the different assay conditions, length of the incubation period with the PARG inhibitor and/or different cell types tested.

However, the enhanced anti-tumour effect of TMZ resulting from PARG inhibition cannot be simply attributed to PAR accumulation, which has been previously shown to be toxic to cells [16]. In fact, when melanoma cells were treated with TMZ concentrations capable of inhibiting cell growth no PARP-1 over-activation was detected, nor could PAR accumulation be observed upon cell exposure to TMZ + GPI 16552 within 1 h (data not shown). On the other hand, toxic concentrations of GPI 16552 ($\geqslant 30\,\mu M$) indeed increased cellular PAR content (data not shown). However, such high concentrations are unlikely to be reached *in vivo* since GPI 16552 as single agent was devoid of intrinsic anti-tumour activity.

Lack of evidence of high levels of PAR in TMZ treated B16 melanoma cells, does not exclude a transient activation of PARP-1 in response to the damage provoked by this methylating agent. TMZ gives rise to a wide spectrum of methyl adducts mainly represented by *N*-methylpurines. Indeed, PARP-1 is involved in the repair of N7-methylguanine and N3-methyladenine provoked by DNA interaction with 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide, the reactive intermediate that forms from the spontaneous hydrolysis of TMZ. These methylated bases do not generally contribute to TMZ toxicity in most cells because they are promptly removed by the short-patch base excision repair (BER) system. The intervention of PARP-1 takes places at early steps of the repair process downstream from the action of 3-methylpurine DNA glycosylase and apurinic-endonucleases, and is likely triggered by the DNA nicks resulting from the single strand breaks. Upon poly(ADP)ribosylation, X-ray cross complementing factor 1 (XRCC-1) is recruited to the damaged site, where it acts as scaffold protein co-ordinating the correct handling of damaged DNA by the other BER components. The chemosensitising effect deriving from the combination of TMZ with inhibitors of PARP-1 catalytic subunit, would derive from inhibition of XRCC1 recruitment to DNA breaks, which hampers strand rejoining and generates unrepaired single strand breaks. Since PARP-1 also interacts with DNA polymerase β and ligase III, treatment with PARP inhibitors would hinder completion of the repair process avoiding poly(ADP)ribosylation of these BER components [2,3].

In the case of the combination of TMZ and PARG inhibitor the resulting enhancement of anti-tumour activity of the methylating agent may also derive from altered PARP-1 function, even though GPI 16552 did not directly inhibit PARP-1 activity (data not shown). Previous studies have shown that auto-poly (ADP)ribosylated PARP-1 possesses markedly reduced activity [30]. On this basis, PARG inhibition would lead to perturbation of the reversal of the auto-poly (ADP)ribosylated PARP-1 to the unmodified form that might have similar consequences to PARP-1 functional inactivation.

It has been reported that PARG deficiency sensitises cells to apoptosis induced by DNA damaging agents [16]. In particular, the failure to remove PAR from proteins bound to ADP polymers leads to growth arrest and cell death, because many PAR modified proteins are essential for cell survival and DNA repair. On this basis it is tempting to speculate that inhibition of PARG activity obtained by the use of chemical modulators might indirectly impair the function of proteins involved in cell death, or in the repair of DNA damage induced by alkylating agents.

In conclusion, our results indicate PARG as novel target to enhance the efficacy of anti-tumour agents and confirm that disruption of the PAR pathway is an efficacious strategy for chemosensitisation of resistant tumours.

Conflict of interest statement

None declared.

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