Original Articles

A novel temozolomide analog, NEO212, with enhanced activity against MGMT-positive melanoma in vitro and in vivo

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A B S T R A C T

The alkylating agent temozolomide (TMZ) represents an important component of current melanoma therapy, but overexpression of O6-methyl-guanine DNA methyltransferase (MGMT) in tumor cells confers resistance to TMZ and impairs therapeutic outcome. We investigated a novel perillyl alcohol (POH)-conjugated analog of TMZ, NEO212, for its ability to exert anticancer activity against MGMT-positive melanoma cells. Human melanoma cells with variable MGMT expression levels were treated with NEO212, TMZ, or perillyl alcohol in vitro and in vivo, and markers of DNA damage and apoptosis, and tumor cell growth were investigated. NEO212 displayed substantially greater anticancer activity than any of the other treatments. It reduced colony formation of MGMT-positive cells up to eight times more effectively than TMZ, and much more potently induced DNA damage and cell death. In a nude mouse tumor model, NEO212 showed significant activity against MGMT-positive melanoma, whereas TMZ, or a mix of TMZ plus POH, was ineffective. At the same time, NEO212 was well tolerated. NEO212 may have potential as a more effective therapy for advanced melanoma, and should become particularly suitable for the treatment of patients with MGMT-positive tumors.

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Introduction

Melanoma incidence is increasing and, despite recent therapeutic advances, the prognosis for patients with metastatic disease remains poor [1,2]. Traditionally, first-line treatment of metastatic melanoma has included the methylating agent dacarbazine or its oral analog temozolomide (TMZ) [3]. Newer agents have been approved recently, such as B-RAF inhibitors vemurafenib and dabrafenib, anti-CTLA-4 (cytotoxic T lymphocyte-associated antigen 4) monoclonal antibody ipilimumab, MEK (mitogen-activated protein kinase kinase) inhibitor trametinib, and others (see details in review [4]). However, therapeutic responses to these new drugs frequently are short-lived, and many patients with advanced melanoma do not obtain long-lasting clinical benefit. In view of these limitations, and despite its limited clinical efficacy, TMZ has remained an

important part of current treatment regimens for patients with metastatic melanoma [5].

TMZ is an alkylating agent that is able to cross the BBB after oral dosing. Besides its use for metastatic melanoma, it is also part of treatment regimens for patients with glioblastoma multiforme (GBM) [6]. In both applications however, the emergence of treatment resistance is common and is frequently linked to the overexpression of MGMT (O6-methyl-guanine DNA methyltransferase), a DNA repair protein that removes alkyl groups located at the O6-position of guanine [7,8]. Because the primary toxic DNA lesion set by TMZ is alkylation of O6-guanine, high expression levels of MGMT protect tumor cells from the cytotoxic impact of TMZ and contribute to treatment resistance [7,9,10].

MGMT activity is unusual in that it represents a "suicide" mechanism, whereby acceptance of the alkyl group from DNA irreversibly inactivates the enzyme and leads to its rapid degradation [7]. This feature is exploited by the use of specific MGMT inhibitors, such as O6-benzylguanine (O6-BG) or lomeguatrib, which act as pseudosubstrates and also lead to rapid degradation of the protein [11,12]. Ablation of MGMT activity after treatment of MGMT-positive cells with MGMT inhibitors generally increases their sensitivity to killing by TMZ, and this has been well established in numerous in vitro and in vivo tumor models (see detailed refs. in Ref. 11). However, a recent phase-II clinical trial yielded mixed

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outcomes when O6-BG and TMZ were administered to brain cancer patients with TMZ-resistant tumors: while the addition of an MGMT inhibitor restored TMZ-sensitivity in a fraction (16%) of patients with anaplastic glioma, there was no significant effect (3%) in patients with GBM [13]. While the underlying reasons for this disappointing outcome remain to be established, similar approaches are being pursued in melanoma patients as well [14,15]. As an alternative to combining TMZ with MGMT inhibitors, we have focused our research on possibilities to improve the inherent anticancer activity of TMZ against MGMT-positive tumor cells. Toward this goal, we have generated a novel TMZ analog by covalently conjugating TMZ and perillyl alcohol.

Perillyl alcohol (POH) is a monoterpene and a natural constituent of caraway, lavender and lilac oil, cherries, cranberries, sage, spearmint, celery seeds, and certain other plants [16]. Although this compound had shown promising activity in several preclinical cancer models [17,18], it did not enter clinical practice, primarily because dose-limiting intestinal toxicity became evident in clinical trials [19–21]. However, recent phase I/II clinical studies in Brazil demonstrated that simple intranasal inhalation of POH was effective against recurrent GBM, in the absence of detectable toxic events [22,23]. Based on these promising results, we hypothesized that covalently linking POH to TMZ might yield a novel therapeutic compound with inherently increased anticancer activity that perhaps might also be applicable to TMZ-resistant cancers, such as MGMT-positive melanoma. Here, we present our results validating this prediction in melanoma cells in vitro and in vivo.

Materials and methods

Pharmacological agents

TMZ was obtained from the pharmacy at the University of Southern California (USC) and dissolved in DMSO to a concentration of 50 mM. NEO212 was provided by NeOnc Technologies (Los Angeles, CA) and was dissolved in DMSO at 100 mM. POH and O6-BG were purchased from Sigma-Aldrich (St. Louis, MO). DMSO was obtained from Sigma-Aldrich as well. In all cases of cell treatment, the final DMSO concentration in the culture medium never exceeded 1%, and was much lower in most cases. Stock solutions of all drugs were stored at –20 °C.

Cell lines

Human melanoma cell lines A375, A2058, M238, and M249 were propagated in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified incubator at 37 °C and a 5% CO_2 atmosphere. Human melanoma cell lines M21, M24, Colo-38, C-8161 and CaCl 74-36 were propagated under similar conditions, except that RPMI was used instead of DMEM. All cell culture reagents were provided by the Cell Culture Core Lab of the USC/Norris Comprehensive Cancer Center and prepared with raw materials from Cellgro/MediaTech (Manassas, VA); FBS was obtained from Omega Scientific (Tarzana, CA). Cell lines were kindly provided by the laboratories of Alan Epstein (USC) [24], Yves DeClerck (USC) [25], and Ali Jazirehi (UCLA) [26].

Primary human melanoma cells, derived from an intracranial lesion of a melanoma patient, were kindly provided by the laboratory of Michael Wong (USC). The cells were cultured in EGM-2 medium (BulletKit from Lonza, Walkersville, MD) containing basal endothelial growth medium plus supplements (Lonza cat# CC-3162) and 10% FBS.

Colony formation assay

Depending on the cell line (and plating efficiency), 200–1000 cells were seeded into each well of a 6-well plate and treated as described in detail previously [27]. In the case of primary melanoma cells, 1000 cells were seeded and let grow for 24 days in the presence or absence of drug treatment; because only small colonies formed, the stained colonies were counted under the microscope.

Immunoblots

Total cell lysates were analyzed by Western blot analysis as described earlier [28]. The primary antibodies were purchased from Cell Signaling Technology (Beverly, MA) or Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used according to the manufacturers' recommendations. All immunoblots were repeated at least once to confirm the results.

Animal model

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of USC, and all rules and regulations were followed during experimentation on animals. Athymic mice (Harlan, Inc., Indianapolis, IN) were implanted with 2×10^6 cells into the right flank. About ten days later, once palpable tumors had developed, animals were assigned to different treatment groups. The control group received vehicle only (45% glycerol, 45% ethanol, 10% DMSO), whereas the treatment groups received drug dissolved in vehicle. Tumor volume was measured with calipers every 2–3 days. In parallel, body weight was recorded.

Tissue analysis

When animals were euthanized, organs and tumor tissues were collected and stored frozen or fixed in formalin. For the detection of MGMT protein by immunohistochemistry, tumor tissues were processed as described earlier [29]. The primary antibody was a mouse monoclonal anti-human MGMT antibody (Abcam, Cambridge, MA; cat #ab39253). The secondary antibody was biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), followed by incubation with the Vectastain Elite avidin–biotin–peroxidase complex kit (Vector Laboratories). Positive staining was visualized with amino-ethylcarbazole substrate (red). Hematoxylin was used as the counterstain to mark nuclei (blue).

For histopathological analysis, organs were placed in 10% formalin for 24–48 hours. Thereafter, tissues were embedded in paraffin and cut into sections of 5 microns thickness. Slides were then stained using standard hematoxylin–eosin (H&E) stains, and evaluated under a light microscope.

Analytical diagnostics/blood analysis

For the investigation of NEO212's impact on general health and specific body functions of treated mice, two laboratory test panels were performed: Superchem (comprehensive blood chemistry test, including liver and kidney values) and complete blood count (CBC) with differential. Both tests were performed by Antech Diagnostics (Irvine, CA). Animals received 30 mg/kg NEO212, or vehicle only, once daily for 28 continuous days. Twelve hours after the final dosing, blood samples were collected from each mouse and shipped to Antech Diagnostics.

Statistical analysis

All parametric data were analyzed using the Student t-test to calculate the significance values. A probability value (p) < 0.05 was considered statistically significant.

Results

NEO212 is more cytotoxic than its individual constituents

A novel analog of temozolomide (TMZ) was created by covalently linking perillyl alcohol (POH) to TMZ's amide functionality (Suppl. Fig. S1). The cytotoxic potency of this new compound, called NEO212, was analyzed by colony formation assay (CFA) in five different human melanoma cell lines (A2058, A375, M238, M21, M249) and compared to the cytotoxicity of TMZ. As shown in Fig. 1, NEO212 suppressed colony formation much more potently than TMZ in all cell lines. Table 1 summarizes this outcome and presents IC50 values, as well as the differential toxicity between the two drugs. The IC50 (i.e., the concentration of drug that reduced colony formation by 50%) of NEO212 ranged from 3.5 to 39 µM, whereas the IC50 of TMZ ranged from 12 to 265 µM, and NEO212 was from 3.4 to 8.0 times more potent than TMZ. Increased toxicity of NEO212 could also be observed in primary melanoma cells from a human tumor specimen (Fig. 1, Table 1).

Previous studies showed that POH is able to exert cytotoxic effects in cancer cells, although concentrations approaching the millimolar range were required [30,31]. We therefore tested whether simply mixing the two compounds TMZ and POH could mimic the effects of the NEO212 conjugate. First, we treated four of the melanoma cell lines (A2058, A375, M238, M249) with POH alone and analyzed colony-forming ability. Consistent with earlier studies with other tumor cell types [30,31], millimolar concentrations of POH were required in order to achieve pronounced growth inhibition (Fig. 2A). Second, we determined survival of M249 cells in response to mixing equimolar concentrations of TMZ with POH. However, as shown in Fig. 2B, mixing TMZ with POH was unable
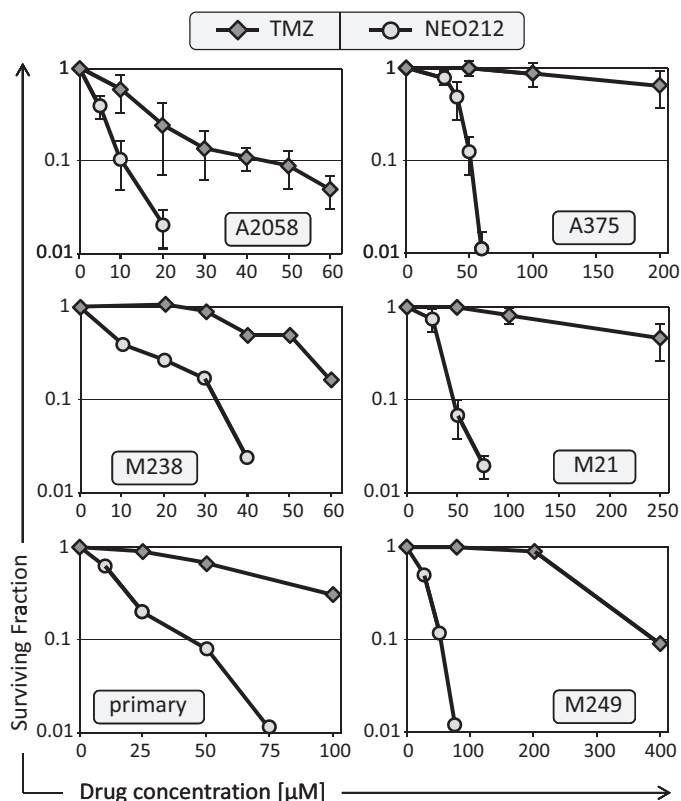
Fig. 1. Survival of melanoma cells after drug treatment. Five different melanoma cell lines (as indicated), as well as a culture of primary melanoma tissue cells (labeled 'primary') were exposed to increasing concentrations of TMZ (diamonds) or NEO212 (circles) for 48 hours, and long-term survival was determined via colony formation assay (CFA). In all graphs, colony formation by control cells (treated with vehicle only) was set at 1. Graphs with error bars display mean (±SD) from ≥3 independent experiments; graphs without error bars show the average from two independent experiments.

Fig. 2. Survival of melanoma cells after drug treatment. (A) Four different melanoma cell lines (as indicated) were exposed to increasing concentrations of POH for 48 hours, and long-term survival was determined by CFA two weeks later. Data points display mean (±SD; n = 3). (B) M249 cells were exposed for 48 hours to increasing concentrations of TMZ (diamonds), NEO212 (circles), or equimolar concentrations of TMZ plus POH (squares), followed by CFA. (C) A375 cells were treated with 50 μM each of NEO212, TMZ, POH, TMZ plus POH mix, or remained untreated, and colony survival was documented by photographing 6-well plates of treated cells. Shown is a highly representative outcome (selected from several repeats with nearly identical outcome).

to achieve the superior toxicity of NEO212, and in fact adding POH did not at all further increase the toxicity of TMZ alone. Similar results were obtained with other melanoma cell lines as well, and a representative colony formation assay is shown in Fig. 2C, where cells were exposed to 50 μM each of NEO212, TMZ, POH, or TMZ plus POH mix; as shown, mixing 50 μM TMZ with 50 μM POH was unable to mimic the strong cytotoxic activity of 50 μM NEO212. Altogether, these results present NEO212 as a novel compound with increased potency over TMZ that cannot be matched by merely mixing its individual parts, TMZ and POH.

NEO212 causes DNA damage and apoptosis

In order to investigate the effects of NEO212 in greater detail, we characterized its impact on DNA damage and cell death at the molecular level. Lysates from drug-treated cells were subjected to
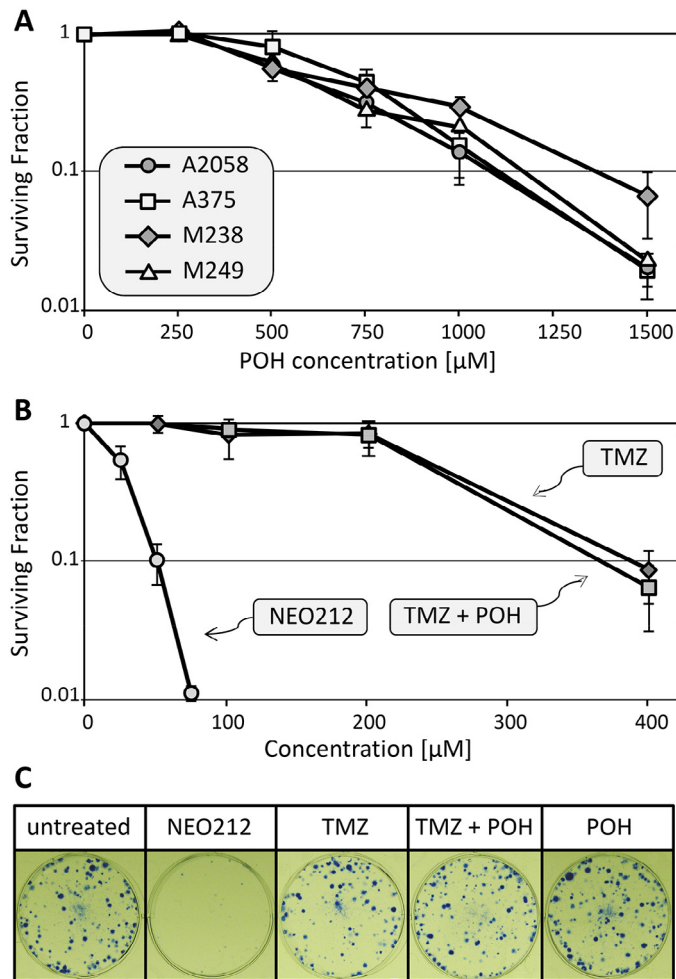
Western blot analysis for the DNA strand breakage indicator γ-H2AX (phosphorylated histone H2A), and the presence of two apoptosis markers, cleaved (i.e. activated) caspase 7 and cleaved PARP-1 (poly ADP-ribose polymerase-1).

It is well known that tumor cells treated with physiological concentrations of TMZ (<100 μM) in vitro survive for several (5–7) days seemingly unaffected before substantial cell death becomes apparent [32,33]. We observed a similar phenotype when melanoma cell

Table 1
Shown are IC50 values (i.e., drug concentrations that reduce colony forming ability by 50%) and differential toxicity between NEO212 and TMZ (i.e., fold increased potency of NEO212 over TMZ). The last column indicates MGMT protein levels in each cell line, as determined by Western blot analysis.

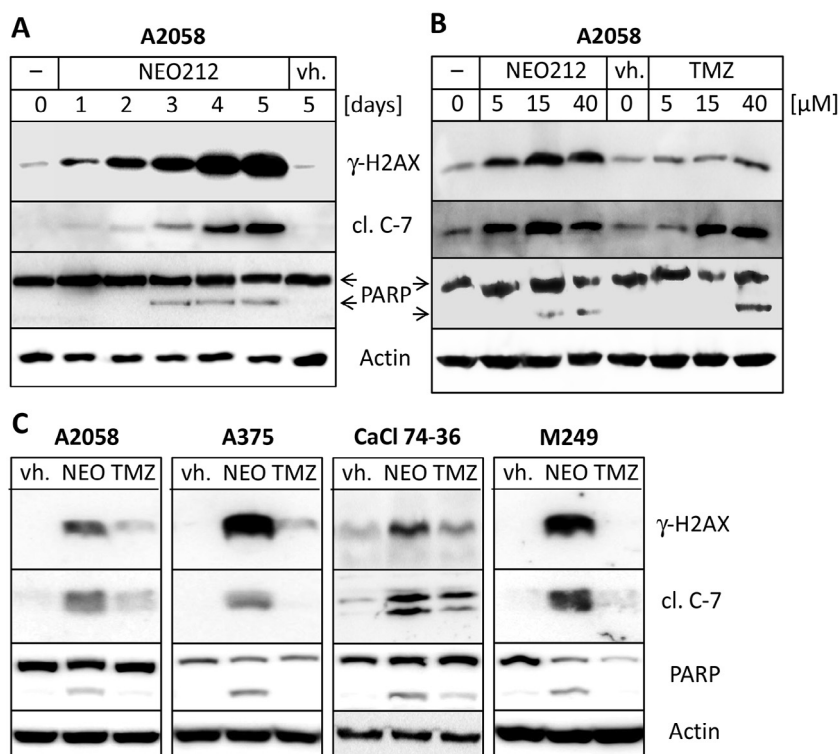| Cell line	TMZ (μM)	NEO212 (μM)	Differential (-fold)	MGMT status
A2058	12	3.5	3.4	—
M238	40	8.5	4.7	+/−
Primary melanoma cells	70	13	5.4	+
A375	265	39	6.8	++
M21	228	29	7.9	++
M249	254	32	7.9	++

Fig. 3. Drug effects on markers of DNA damage and apoptosis. (A) A2058 cells were treated with a single dose of 15 μM NEO212, or vehicle (vh.) only, and cells were harvested every day for up to 5 days thereafter. Analysis was by Western blot with antibodies against γ-H2AX (a marker for double-strand DNA damage), and the apoptosis markers cleaved caspase 7 (cl. C-7) and PARP (faster migrating band of PARP represents proteolytically cleaved fragment). Actin was used as a loading control. (B) A2058 cells were treated with increasing concentrations of NEO212 or TMZ, or vehicle alone. After 4 days, cells were harvested and analyzed as in (A). (C) Various melanoma cells were treated with equimolar concentrations of NEO212 (NEO) or TMZ, or vehicle (vh.) alone, and analyzed as in (A). A2058 cells received 15 μM for 72 h; A375 cells received 65 μM for 80 h; CaCl 74-36 cells received 80 μM for 50 h; M249 cells received 80 μM for 96 h.

lines were treated with NEO212, i.e., cell cultures only began to deteriorate approximately a week after the onset of drug treatment. For this reason, it was possible to collect lysates from drug-treated cells over the course of several days. As shown in Fig. 3, treatment of A2058 melanoma cells with NEO212 resulted in greatly increased levels of γ-H2AX protein, indicating accumulation of DNA strand breaks over the course of 1–5 days. Markers of apoptotic cell death (cleaved caspase 7 and PARP1) emerged as well, starting at day 3. In comparison, TMZ required higher dosages to cause similar outcomes (Fig. 3B). When several melanoma cell lines (A2058, A375, CaCl 74-36, M249) were treated in parallel with equimolar concentrations of either NEO212 or TMZ, NEO212 caused more DNA damage (i.e., higher γ-H2AX protein levels) and more cell death (cleaved caspase 7 and PARP) than TMZ in all cases (Fig. 3C).

Because in Fig. 2 we had shown that an equimolar combination of TMZ plus POH was unable to achieve the same potency in blocking colony survival as the NEO212 conjugate, we next investigated whether this cellular outcome would also be reflected at the molecular level of DNA damage and apoptosis marker proteins. We therefore treated A2058 cells with the same concentration (20 μM each) of NEO212, TMZ, POH, or TMZ plus POH mix, and analyzed the induction of γ-H2AX and activated caspase 7 after 24 hours and 5 days. As shown in Fig. 4, both indicator proteins were induced quite prominently by NEO212, whereas induction by TMZ was noticeably weaker, and mixing TMZ with POH (TMZ + POH) did not increase TMZ potency any further. The greater effect of NEO212 over a mix of TMZ + POH was also established in A375 cells (Fig. 4). Thus, the results from the cell survival assay (Fig. 2) correlated closely with the effects of these compounds on DNA damage and apoptosis markers (Fig. 4), and in all cases NEO212 clearly generated the stron-
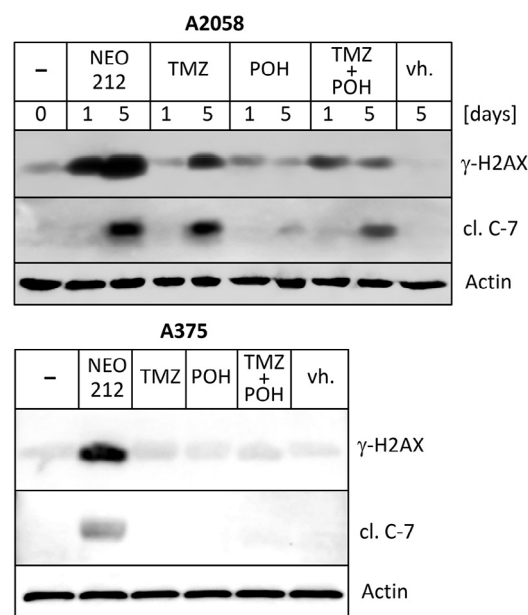

Fig. 4. Effects of equimolar TMZ plus POH mix. A2058 cells received 20 μM of each NEO212, TMZ, POH, TMZ plus POH mix, or vehicle (vh.) alone, and were harvested after 1 and 5 days. A375 cells received 60 μM of each drug and were harvested after 5 days. Western blot analysis was performed for DNA damage marker γ-H2AX and apoptosis marker cleaved caspase 7 (cl. C-7). Actin was used as a loading control.

gest anticancer impact that could not be mimicked by merely mixing its individual components.

Cytotoxic effect of NEO212 involves O6-guanine methylation

The DNA repair protein MGMT is known to play a key role in cellular resistance to TMZ; we therefore investigated how it would impact the cytotoxic potency of NEO212. We first determined its basal level of expression by Western blot analysis of 10 different melanoma cell lines. As shown in Fig. 5A, four of the cell lines (A2058, M24, M24met, C8161) were negative for MGMT protein expression, two (M238, Colo38) displayed very low levels, and four (A375, M21, M249, CaCl 74-36) were strongly positive. For comparison purposes, we also assessed MGMT protein levels in a commonly used glioblastoma cell line known to be MGMT positive (T98G), and in an MGMT-positive breast cancer cell line (MDA-MB-468). This side-by-side evaluation revealed that MGMT protein levels in the strongly positive melanoma cell lines were similar to the levels found in other, well-characterized, MGMT-positive tumor types (Fig. 5A).

We next aligned MGMT expression with the cytotoxic potency of NEO212 in comparison to TMZ. As summarized in Table 1, the IC50 of both NEO212 and TMZ clearly increased with increasing levels of MGMT expression. The IC50 was lowest in MGMT-negative cells, moderate in weakly MGMT-positive cells, and highest in strongly MGMT-positive cells. Intriguingly however, resistance to TMZ increased more sharply than resistance to NEO212; i.e., the fold
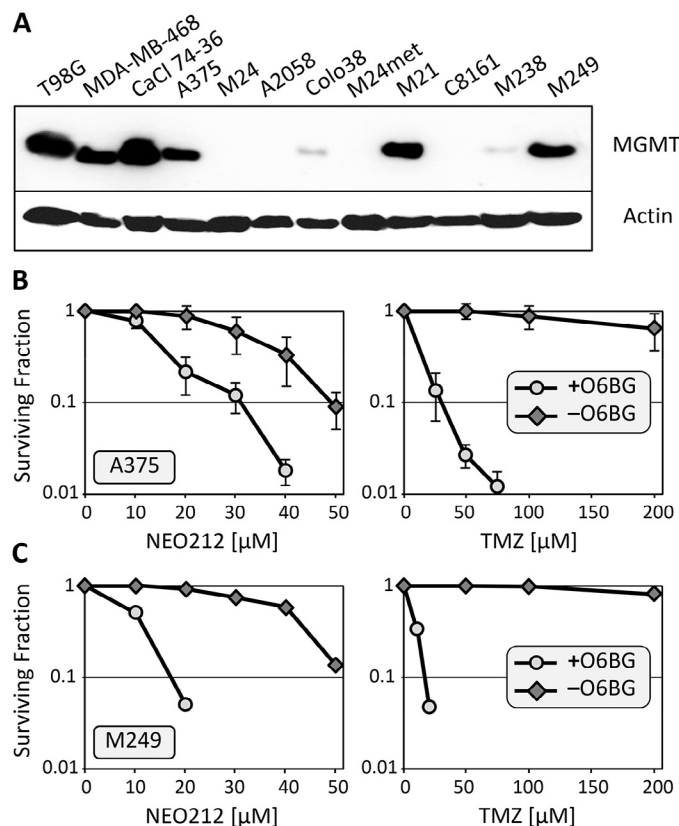

Fig. 5. MGMT expression levels and effect of O6BG on colony formation. (A) Lysates from 10 melanoma cell lines were probed for basal level expression of MGMT protein and compared to a glioblastoma (T98G) and breast carcinoma (MDA-MB-468) cell line. (B) A375 and (C) M249 cells were pre-incubated with or without 15 μM O6-BG for 30 minutes, followed by the addition of increasing concentrations of NEO212 or TMZ. Ten to twelve days later, colonies were stained and counted. In all graphs, colony formation by control cells (treated with vehicle or O6-BG only) was set at 1. Graphs with error bars display mean (±SD) from 3 independent experiments; graphs without error bars show the average from two independent experiments.
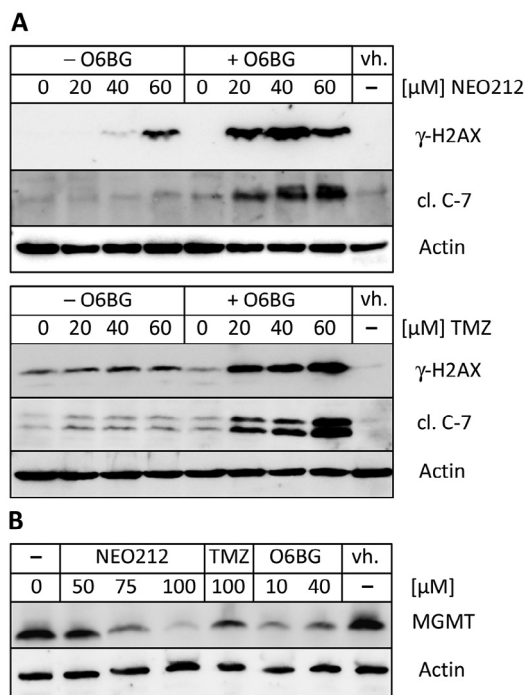

Fig. 6. Enhancement of DNA damage and downregulation of MGMT. (A) A375 cells were pre-incubated with or without 15 μM O6-BG for 30 minutes, followed by the addition of increasing concentrations of NEO212 (top panel) or TMZ (lower panel). Control cells received vehicle (vh.) only. Four days later, cellular lysates were prepared and analyzed by Western blot for DNA damage marker γ-H2AX and apoptosis marker cleaved caspase 7 (cl. C-7). (B) M249 cells were treated with NEO212, TMZ, O6-BG, or vehicle only at the concentrations indicated. Twenty hours later, cell lysates were prepared and analyzed for the presence of MGMT protein by Western blot. Actin was used as a loading control in all blots.

differential between NEO212 toxicity and TMZ toxicity increased from 3.4-fold in MGMT-negative cells to 8.0-fold in MGMT-positive cells (Table 1). As well, the average IC50 of NEO212 remained much lower (up to 39 μM) than the IC50 of TMZ, which increased up to 265 μM. These latter findings suggest that the increased potency of NEO212 over TMZ, although apparent in all cell lines analyzed, might become particularly advantageous in the context of therapeutically targeting MGMT-positive cells.

We further characterized the relevance of MGMT in NEO212's cytotoxic effects by applying O6-BG, an inhibitor of MGMT activity. We treated the MGMT-positive melanoma cell lines A375 and M249 with TMZ or NEO212 in the presence or absence of O6-BG, and measured cell survival by colony formation assay. As shown in Fig. 5B and C, inclusion of O6-BG significantly increased toxicity of NEO212 and TMZ in both cell lines, with somewhat stronger enhancement in the M249 cell line. Intriguingly, the presence of O6-BG seemed to narrow the differential potency of NEO212 and TMZ, consistent with our observation from Table 1 that the fold difference in drug potency increases with higher MGMT expression levels. We also performed the same treatment with MGMT-negative A2058 cells, but here the inclusion of O6-BG did not enhance the toxicity of either drug, as would be expected in the absence of MGMT protein (not shown).

The impact of O6-BG was also studied with regard to DNA damage and apoptosis. MGMT-positive A375 cells were treated with increasing concentrations of NEO212 (or TMZ) in the presence or absence of the inhibitor and analyzed for γ-H2AX protein levels and caspase 7 cleavage. As shown in Fig. 6A, inclusion of O6-BG greatly lowered the threshold of NEO212 concentrations that caused DNA damage and apoptosis; for instance, 20 μM NEO212 triggered a

strong increase in γ-H2AX expression and appearance of cleaved caspase 7 when O6-BG was present, but had no such effect in the absence of the inhibitor. The use of TMZ generated comparable results (Fig. 6A), and quantitative similar outcomes were also obtained with MGMT-positive M249 cells (not shown). Altogether, these results indicate that methylation of O6-guanine is among the key triggers for cell death caused by NEO212, and this DNA lesion appears to be set more effectively by NEO212 as compared to TMZ.

Potent methylation of O6-guanine by NEO212 was also indicated by our observation presented in Fig. 6B, where treatment of MGMT-positive cells with NEO212 resulted in a concentration-dependent depletion of cellular MGMT protein levels. This effect was quite pronounced and mimicked the depletion of MGMT by optimized concentrations of O6-BG. In comparison, 100 μM TMZ had a noticeably weaker effect. The results shown in Fig. 6B are from M249 cells, but a repetition with A375 cells yielded essentially the same outcome (not shown). It is conceivable that depletion of MGMT by NEO212 might at least in part contribute to the agent's potent anticancer effect in MGMT-positive melanoma cells.

NEO212 is active in vivo

Lastly, we asked whether NEO212 would be able to exert its anticancer effects in vivo as well. For these experiments, MGMT-negative A2058 or MGMT-positive A375 cells were implanted into the flanks of nude mice, and after palpable tumors had developed the animals received either vehicle or drug treatments. Because little is known about the bioavailability of NEO212, the first in vivo experiment tested two different modes of administration; i.e., the drug was given either via oral gavage or via subcutaneous injection into the neck area (i.e., removed from the tumor site) and compared to vehicle only, which was injected. Treatment was once daily and continued for 10 days. As presented in Fig. 7A, both groups of NEO212-treated animals displayed significantly ($p < 0.01$) reduced tumor growth as compared to animals that received vehicle only. NEO212 given via subcutaneous injection appeared slightly more effective than oral NEO212, but the difference was not statistically significant, indicating high bioavailability of the compound. Three days after the final dose of NEO212, all animals were euthanized because tumor volume in most control animals had reached its maximum allowable size. At this time, the average tumor volume of NEO212-treated animals was only 20–35% in comparison to controls.

Animals implanted with A375 cells were divided into 4 groups and treated with vehicle, NEO212, TMZ, or a mix of TMZ plus POH. All treatments were given via subcutaneous injection once daily for 21 consecutive days. Fig. 7B presents tumor growth in these animals during and after cessation of treatment. As shown, tumor growth of NEO212-treated animals was significantly ($p < 0.01$) delayed as compared to all the other groups. The therapeutic effect of TMZ, although marginally better than vehicle only, clearly was inferior to NEO212 treatment ($p < 0.01$). A mix of TMZ plus POH in essence yielded the same outcome as TMZ alone, i.e., the addition of POH did not further enhance the very modest potency of TMZ. We also confirmed that the tumors grown from A375 cells had maintained their MGMT-positive status in vivo (Suppl. Fig. S2), thus verifying that superior therapeutic activity of NEO212 indeed unfolded within MGMT-positive tumor tissue.

In all in vivo experiments, the body weight of animals was closely monitored. There was no major loss of weight during the drug treatment period in any of the groups (Suppl. Fig. S3). We also performed histopathological analysis on organs from vehicle-treated and NEO212-treated animals. However, no signs of potentially toxic impact of NEO212 on intestines, liver, kidney, spleen, lung, or brain could be detected (Suppl. Fig. S4A,B). As well, a few of the NEO212-treated animals were kept for an additional month after cessation
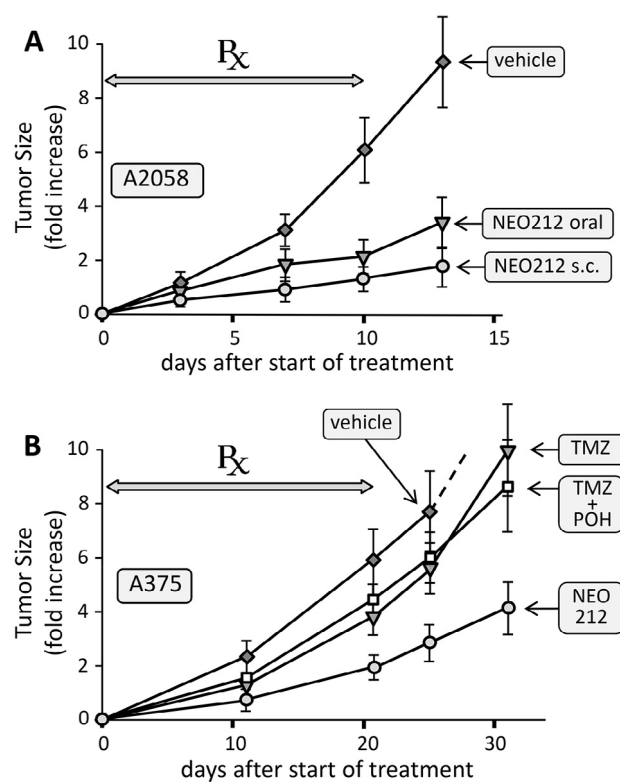

Fig. 7. Drug effects on subcutaneous tumor growth. (A) Mice carrying subcutaneously implanted A2058 cells were separated into three treatment groups (six animals each): (i) control (vehicle only, via subcutaneous injection), (ii) 30 mg/kg NEO212 via subcutaneous (s.c.) injection, and (iii) 30 mg/kg NEO212 via oral gavage. Once-daily treatment was initiated after palpable tumors had developed and continued for 10 days. Statistical difference between vehicle-treated and both groups of NEO212-treated animals: $p < 0.01$. (B) Mice carrying subcutaneously implanted A375 cells were separated into four treatment groups (six animals each): (i) control (vehicle only), (ii) 50 mg/kg NEO212, (iii) 50 mg/kg TMZ, (iv) 22 mg/kg POH mixed with 28 mg/kg TMZ (mimicking the dosage of the individual components contained in 50 mg/kg NEO212), all given via subcutaneous injection. Once-daily treatment was initiated after palpable tumors had developed and continued for 21 days. The difference between NEO212-treated animals and every other group was statistically significant ($p > 0.01$). Both graphs show fold increase in tumor size over time (mean ±SE). Two-headed arrow labeled Rx denotes treatment period.

of treatment under continuous observation, but no noticeable long-term toxic drug effects were noted.

For further analysis of the drug's impact on animal health, we treated groups of mice with vehicle or NEO212 once daily for 28 days, and then collected blood for detailed analysis by a comprehensive blood chemistry panel, as well as complete blood count (CBC) with differential. Blood from non-treated animals was included as well. As shown in Suppl. Table S1A, there was no significant difference in the number of red or total white blood cells, hemoglobin, or hematocrit. Differential analysis revealed no substantial differences in neutrophils, eosinophils, or monocytes, although the number of lymphocytes was slightly lower in some of the NEO212-treated mice (Suppl. Table S1B). Analysis of liver markers showed no difference for bilirubin levels or alkaline phosphatase levels, but there was a minor increase in alanine transaminase and aspartate transaminase levels (Suppl. Table S2), which seemed inconsequential compared to the more substantial hepatotoxic effects that are occasionally seen in a small number of patients treated with temozolomide [34]. Altogether, the outcome of our detailed analysis of tissues, blood cells and blood chemistry indicates that NEO212 was very well tolerated by these experimental animals.

Discussion

The incidence of melanoma is increasing worldwide, but management of disseminated disease has remained one of the most challenging problems in clinical oncology. The most commonly used melanoma drugs are dacarbazine and TMZ, which result in response rates of about 15% when used as single agents. In direct comparison to dacarbazine, TMZ yielded greater systemic exposure, longer progression-free survival, and improved quality of life due to oral administration [14,35]. It is well established that failure to respond to TMZ is largely due to both inherent and acquired chemoresistance, and a key contributing factor is elevated expression of the DNA repair protein MGMT. MGMT detoxifies DNA lesions set by alkylating agents by directly removing alkyl groups from O6-methylguanine, thus conferring resistance to TMZ and similar agents, such as dacarbazine, fotemustine, or carmustine/BCNU [2,3,6,10,11].

We have covalently conjugated POH to TMZ, thereby generating a novel TMZ analog, TMZ-POH or NEO212, which displays greater anticancer potency than each of its parental molecules, especially in melanoma cells with high levels of MGMT expression. Superior efficacy of NEO212 was confirmed in vitro in several melanoma cell lines, and was established through cell survival assays (Figs. 1 and 2) and by molecular analysis of markers of DNA damage and cell death (Figs. 3 and 4). Similarly, NEO212 displayed greater therapeutic efficacy than TMZ in MGMT-negative and MGMT-positive in vivo models (Fig. 7). Intriguingly, a mere mix of NEO212's constituents, TMZ and POH, was unable to achieve NEO212's superior efficacy, neither in vitro (Figs. 2 and 4) nor in vivo (Fig. 7B), indicating that NEO212 is a novel chemical entity with inherently increased potency that is greater than the sum of its parts.

At least part of NEO212's cytotoxic function is based on the deleterious methylation of DNA, revealing that this key characteristic of TMZ has been preserved in the conjugated NEO212 molecule. This conclusion is based on several observations: (i) overexpression of MGMT, which selectively repairs O6-methylguanine and provides profound protection against TMZ [7,8], also impinges on cellular responses to NEO212 (although to a much lesser extent than to TMZ; Table 1); (ii) the addition of the MGMT-inhibitor O6-BG increases NEO212's cytotoxic potency (Figs. 5B and 6A); and (iii) treatment with NEO212 down-regulates MGMT protein levels (Fig. 6B), which is consistent with the DNA repair enzyme's "suicide" mechanism of action, whereby acceptance of the alkyl group from O6-methylguanine leads to the protein's rapid degradation [36].

It remains unclear however, why NEO212 is so much more cytotoxic than TMZ and maintains its potency in MGMT-positive tumor cells. In a recent study with preclinical breast cancer and glioblastoma models, we have shown that in vitro the half-life of NEO212 appears to be somewhat longer than that of TMZ [27,37]. While this observation could serve to explain greater potency of NEO212 in general, it is unclear how this aspect would unfold preferentially in MGMT-positive cells. As an alternative, one could surmise that down-regulation of cellular MGMT levels, which takes place more effectively in response to NEO212 as compared to TMZ (Fig. 6B), might play a role; although this process will have to be investigated in greater detail, it is tempting to speculate that effective depletion of MGMT by NEO212 might prime the cells toward greater chemosensitivity.

Is it possible that the POH moiety of NEO212 may contribute additional functions to establish the superior cytotoxic potency of the conjugated molecule? POH is known to affect several intracellular processes. For instance, this monoterpene was shown to inhibit the activity of telomerase [38] and of sodium–potassium pump (Na^+/K^+-ATPase) [39], and to trigger pro-apoptotic endoplasmic reticulum (ER) stress [30]. As well, it was reported as a farnesyl-transferase inhibitor that results in the blockage of ras oncoprotein activity [40,41], although this has been challenged [42,43]. However, in all

these cases relatively high concentrations of POH (well above 100 μM) are required to achieve 50% inhibition of target activity (see also Fig. 2A). In comparison, NEO212 is active in the range of 4–40 μM in melanoma cells (Table 1). Intriguingly as well, when POH is mixed with TMZ and applied as a separate agent, this combination is unable to replicate the high potency of conjugated NEO212 (Figs. 2, 4 and 7B), indicating that the mere presence of non-conjugated POH is unable to provide additional potency over TMZ.

While the precise contribution of POH to NEO212's increased activity remains to be investigated further, our data clearly demonstrate NEO212's superior therapeutic activity over TMZ in every aspect investigated. NEO212 is up to 8-fold more potent against melanoma cell lines in vitro (Table 1), and it exerts its cytotoxic potency in the range of 4–40 μM. These values are very interesting for the following reason: The peak concentrations of TMZ measured in the plasma of human cancer patients are 50–70 μM [44,45]. In comparison, the IC50 of TMZ against MGMT-positive melanoma cells measured in vitro is well above 200 μM (Table 1). Considering this large differential, it is therefore not surprising that MGMT-positivity of tumor tissue is known to predict poor response to TMZ therapy [46,47], and indeed our own in vivo experiment with TMZ confirmed this detriment as well (Fig. 7B). In comparison, NEO212 displayed in vitro IC50s from 13–39 μM in MGMT-positive cells (Table 1) and did exert significant ($p < 0.01$) therapeutic activity in vivo against strongly MGMT-positive melanoma (Fig. 7B). Although NEO212 is not yet approved for human investigation and its peak plasma concentrations are unknown, it is conceivable, based on its chemical structure and its predicted physicochemical properties, that it might be possible to achieve plasma concentrations that are similar to TMZ. If that were the case, it would provide good support to predict that NEO212 will exert therapeutic activity against MGMT-positive melanoma in patients as well. We therefore propose that NEO212 should be investigated further as a potentially effective therapy for advanced melanoma, in particular for patients with MGMT-positive tumors.

Conflict of interest

TCC is founder and stakeholder of NeOnc Technologies, Woodland Hills, CA.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.12.021.

References

[1] J.R. Goodman, D. Grossman, Aspirin and other NSAIDs as chemoprevention agents in melanoma, Cancer Prev. Res. (Phila.) 7 (2014) 557–564.
[2] L. Tentori, P.M. Lacal, G. Graziani, Challenging resistance mechanisms to therapies for metastatic melanoma, Trends Pharmacol. Sci. 34 (2013) 656–666.
[3] T.R. Velho, Metastatic melanoma – a review of current and future drugs, Drugs Context 2012 (2012) 212242.

[4] C. Karimkhani, R. Gonzalez, R.P. Dellavalle, A review of novel therapies for melanoma, Am. J. Clin. Dermatol. 15 (2014) 323–337.

[5] I. Quirbt, S. Verma, T. Petrella, K. Bak, M. Charette, Temozolomide for the treatment of metastatic melanoma, Curr. Oncol. 14 (2007) 27–33.

[6] J. Zhang, M.F. Stevens, T.D. Bradshaw, Temozolomide: mechanisms of action, repair and resistance, Curr. Mol. Pharmacol. 5 (2012) 102–114.

[7] A.E. Pegg, Multifaceted roles of alkyltransferase and related proteins in DNA repair, DNA damage, resistance to chemotherapy, and research tools, Chem. Res. Toxicol. 24 (2011) 618–639.

[8] M. Christmann, B. Verbeek, W.P. Roos, B. Kaina, O(6)-Methylguanine-DNA methyltransferase (MGMT) in normal tissues and tumors: enzyme activity, promoter methylation and immunohistochemistry, Biochim. Biophys. Acta 1816 (2011) 179–190.

[9] B. Kaina, M. Christmann, S. Naumann, W.P. Roos, MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents, DNA Repair (Amst) 6 (2007) 1079–1099.

[10] J.R. Silber, M.S. Bobola, A. Blank, M.C. Chamberlain, O(6)-Methylguanine-DNA methyltransferase in glioma therapy: promise and problems, Biochim. Biophys. Acta 1826 (2012) 71–82.

[11] B. Kaina, G.P. Margison, M. Christmann, Targeting O(6)-methylguanine-DNA methyltransferase with specific inhibitors as a strategy in cancer therapy, Cell. Mol. Life Sci. 67 (2010) 3663–3681.

[12] A.E. Pegg, L. Wiest, C. Mummert, L. Stine, R.C. Moschel, M.E. Dolan, Use of antibodies to human O6-alkylguanine-DNA alkyltransferase to study the content of this protein in cells treated with O6-benzylguanine or N-methyl-N'-nitro-N-nitrosoguanidine, Carcinogenesis 12 (1991) 1679–1683.

[13] J.A. Quinn, S.X. Jiang, D.A. Reardon, A. Desjardins, J.J. Vredenburgh, J.N. Rich, et al., Phase II trial of temozolomide plus o6-benzylguanine in adults with recurrent, temozolomide-resistant malignant glioma, J. Clin. Oncol. 27 (2009) 1262–1267.

[14] M. Ranson, P. Hersey, D. Thompson, J. Beith, G.A. McArthur, A. Haydon, et al., Randomized trial of the combination of lomeguatrib and temozolomide compared with temozolomide alone in chemotherapy naive patients with metastatic cutaneous melanoma, J. Clin. Oncol. 25 (2007) 2540–2545.

[15] A.J. Watson, M.R. Middleton, G. McGown, M. Thorncroft, M. Ranson, P. Hersey, et al., O(6)-Methylguanine-DNA methyltransferase depletion and DNA damage in patients with melanoma treated with temozolomide alone or with lomeguatrib, Br. J. Cancer 100 (2009) 1250–1256.

[16] P.L. Crowell, C.E. Elson, Isoprenoids, health and disease, in: R.E.C. Wildman (Ed.), Neutraceuticals and Functional Foods, CRC Press, Boca Raton, FL, 2001.

[17] J.D. Haag, M.N. Gould, Mammary carcinoma regression induced by perillyl alcohol, a hydroxylated analog of limonene, Cancer Chemother. Pharmacol. 34 (1994) 477–483.

[18] J.J. Mills, R.S. Chari, I.J. Boyer, M.N. Gould, R.L. Jirtle, Induction of apoptosis in liver tumors by the monoterpene perillyl alcohol, Cancer Res. 55 (1995) 979–983.

[19] G. Liu, K. Oettel, H. Bailey, L.V. Ummersen, K. Tutsch, M.J. Staab, et al., Phase II trial of perillyl alcohol (NSC 641066) administered daily in patients with metastatic androgen independent prostate cancer, Invest. New Drugs 21 (2003) 367–372.

[20] H.H. Bailey, S. Attia, R.R. Love, T. Fass, R. Chappell, K. Tutsch, et al., Phase II trial of daily oral perillyl alcohol (NSC 641066) in treatment-refractory metastatic breast cancer, Cancer Chemother. Pharmacol. 62 (2008) 149–157.

[21] S.M. Meadows, D. Mulkerin, J. Berlin, H. Bailey, J. Kolesar, D. Warren, et al., Phase II trial of perillyl alcohol in patients with metastatic colorectal cancer, Int. J. Gastrointest. Cancer 32 (2002) 125–128.

[22] C.O. da Fonseca, M. Simao, I.R. Lins, R.O. Caetano, D. Futuro, T. Quirico-Santos, Efficacy of monoterpene perillyl alcohol upon survival rate of patients with recurrent glioblastoma, J. Cancer Res. Clin. Oncol. 137 (2011) 287–293.

[23] C.O. da Fonseca, G. Schwartsmann, J. Fischer, J. Nagel, D. Futuro, T. Quirico-Santos, et al., Preliminary results from a phase I/II study of perillyl alcohol intranasal administration in adults with recurrent malignant gliomas, Surg. Neurol. 70 (2008) 259–266.

[24] A.L. Epstein, A.O. Martin, R. Kempson, Use of a newly established human cell line (SU-CCS-1) to demonstrate the relationship of clear cell sarcoma to malignant melanoma, Cancer Res. 44 (1984) 1265–1274.

[25] P. Henriet, Z.D. Zhong, P.C. Brooks, K.I. Weinberg, Y.A. DeClerck, Contact with fibrillar collagen inhibits melanoma cell proliferation by up-regulating p27KIP1, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 10026–10031.

[26] A.R. Jazirehi, R. Nazarian, A.X. Torres-Collado, J.S. Economou, Aberrant apoptotic machinery confers melanoma dual resistance to BRAF(V600E) inhibitor and immune effector cells: immunosensitization by a histone deacetylase inhibitor, Am. J. Clin. Exp. Immunol. 3 (2014) 43–56.

[27] T.C. Chen, H.Y. Cho, W. Wang, M. Barath, N. Sharma, F.M. Hofman, et al., A novel temozolomide-perillyl alcohol conjugate exhibits superior activity against breast cancer cells in vitro and intracranial triple-negative tumor growth in vivo, Mol. Cancer Ther. 13 (2014) 1181–1193.

[28] P. Pyrko, N. Soriano, A. Kardosh, Y.T. Liu, J. Uddin, N.A. Petasis, et al., Downregulation of survivin expression and concomitant induction of apoptosis by celecoxib and its non-cyclooxygenase-2-inhibitory analog, dimethyl-celecoxib (DMC), in tumor cells in vitro and in vivo, Mol. Cancer 5 (2006) 19.

[29] F.M. Hofman, C.R. Taylor, Immunohistochemistry, Curr. Protoc. Immunol. 103 (2013) Unit 21.4.

[30] H.Y. Cho, W. Wang, N. Jhaveri, S. Torres, J. Tseng, M.N. Leong, et al., Perillyl alcohol for the treatment of temozolomide-resistant gliomas, Mol. Cancer Ther. 11 (2012) 2462–2472.

[31] T. Yuri, N. Danbara, M. Tsujita-Kyutoku, Y. Kiyozuka, H. Senzaki, N. Shikata, et al., Perillyl alcohol inhibits human breast cancer cell growth in vitro and in vivo, Breast Cancer Res. 84 (2004) 251–260.

[32] Y. Hirose, M.S. Berger, R.O. Pieper, p53 effects both the duration of G2/M arrest and the fate of temozolomide-treated human glioblastoma cells, Cancer Res. 61 (2001) 1957–1963.

[33] A.V. Knizhnik, W.P. Roos, T. Nikolova, S. Quiros, K.H. Tomaszowski, M. Christmann, et al., Survival and death strategies in glioma cells: autophagy, senescence and apoptosis triggered by a single type of temozolomide-induced DNA damage, PLoS ONE 8 (2013) e55665.

[34] L.M. Grant, D.E. Kleiner, H.S. Conjeevaram, R. Vuppalanchi, W.M. Lee, Clinical and histological features of idiosyncratic acute liver injury caused by temozolomide, Dig. Dis. Sci. 58 (2013) 1415–1421.

[35] S. Danson, P. Lorigan, A. Arance, A. Clamp, M. Ranson, J. Hodgetts, et al., Randomized phase II study of temozolomide given every 8 hours or daily with either interferon alfa-2b or thalidomide in metastatic malignant melanoma, J. Clin. Oncol. 21 (2003) 2551–2557.

[36] M. Xu-Welliver, A.E. Pegg, Degradation of the alkylated form of the DNA repair protein, O(6)-alkylguanine-DNA alkyltransferase, Carcinogenesis 23 (2002) 823–830.

[37] H.Y. Cho, W. Wang, N. Jhaveri, D.J. Lee, N. Sharma, L. Dubeau, et al., NEO212, temozolomide conjugated to perillyl alcohol, is a novel drug for effective treatment of a broad range of temozolomide-resistant gliomas, Mol. Cancer Ther. 13 (2014) 2004–2017.

[38] T. Sundin, D.M. Peffley, D. Gauthier, P. Hentosh, The isoprenoid perillyl alcohol inhibits telomerase activity in prostate cancer cells, Biochimie 94 (2012) 2639–2648.

[39] D.G. Garcia, L.M. Amorim, M.V. de Castro Faria, A.S. Freire, R.E. Santelli, C.O. Da Fonseca, et al., The anticancer drug perillyl alcohol is a Na/K-ATPase inhibitor, Mol. Cell. Biochem. 345 (2010) 29–34.

[40] I.R. Hardcastle, M.G. Rowlands, A.M. Barber, R.M. Grimshaw, M.K. Mohan, B.P. Nutley, et al., Inhibition of protein prenylation by metabolites of limonene, Biochem. Pharmacol. 57 (1999) 801–809.

[41] P.L. Crowell, R.R. Chang, Z.B. Ren, C.E. Elson, M.N. Gould, Selective inhibition of isoprenylation of 21–26-kDa proteins by the anticarcinogen d-limonene and its metabolites, J. Biol. Chem. 266 (1991) 17679–17685.

[42] J. Karlson, A.K. Borg-Karlson, R. Unelius, M.C. Shoshan, N. Wilking, U. Ringborg, et al., Inhibition of tumor cell growth by monoterpenes in vitro: evidence of a Ras-independent mechanism of action, Anticancer Drugs 7 (1996) 422–429.

[43] R.J. Ruch, K. Sigler, Growth inhibition of rat liver epithelial tumor cells by monoterpenes does not involve Ras plasma membrane association, Carcinogenesis 15 (1994) 787–789.

[44] L.A. Hammond, J.R. Eckardt, S.D. Baker, S.G. Eckhardt, M. Dugan, K. Forral, et al., Phase I and pharmacokinetic study of temozolomide on a daily-for-5-days schedule in patients with advanced solid malignancies, J. Clin. Oncol. 17 (1999) 2604–2613.

[45] M. Brada, I. Judson, P. Beale, S. Moore, P. Reidenberg, P. Statkevich, et al., Phase I dose-escalation and pharmacokinetic study of temozolomide (SCH 52365) for refractory or relapsing malignancies, Br. J. Cancer 81 (1999) 1022–1030.

[46] M.E. Hegi, A.C. Diserens, S. Godard, P.Y. Dietrich, L. Regli, S. Ostermann, et al., Clinical trial substantiates the predictive value of O-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide, Clin. Cancer Res. 10 (2004) 1871–1874.

[47] P. Schraml, A. von Teichman, D. Mihic-Probst, M. Simcock, A. Ochsenbein, R. Dummer, et al., Predictive value of the MGMT promoter methylation status in metastatic melanoma patients receiving first-line temozolomide plus bevacizumab in the trial SAKK 50/07, Oncol. Rep. 28 (2012) 654–658.