Molecular Pharmacology Fast Forward. Published on July 29, 2009 as DOI: 10.1124/mol.109.058131
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The translesion polymerase Rev3L in the tolerance of alkylating anticancer drugs

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Molecular Pharmacology Fast Forward. Published on July 29, 2009 as DOI: 10.1124/mol.109.058131
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Running title:

Rev3L protects against temozolomide and fotemustine

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Document statistics:

Number of text pages – 20

Tables – 0

Figures – 4

References – 29

Number of words in abstract – 196

Non-standard abbreviations:

Base excision repair (BER), DNA double-strand break (DSB), single-strand breaks (ssb), O^6-methylguanine-DNA methyltransferase (MGMT), translesion DNA synthesis (TLS), mismatch repair (MMR), O^6-benzylguanine (O^6BG), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), mitomycin C (MMC), methyl methanesulfonate (MMS), poly(ADP-ribose)polymerase (PARP), polymerase β (Polβ) and polymerase ζ (Polζ).

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Abstract

Temozolomide and fotemustine, representing methylating and chloroethylating agents respectively, are used in the treatment of glioma and malignant melanoma. Since chemoresistance of these tumors is a common phenomenon, identification of the underlying mechanisms is needed. Here we show that Rev3L, the catalytic subunit of the translesion DNA polymerase ζ, mediates resistance to both temozolomide and fotemustine. *Rev3L* knockout cells are hypersensitive to both agents. Remarkably, cells heterozygous for *Rev3L* showed an intermediate sensitivity. Rev3L is not involved in the tolerance of the toxic O^6-methylguanine lesion. However, a possible role of Rev3L in the tolerance of O^6-chloroethylguanine or the subsequently formed N1-guanine-N3-cytosine interstrand crosslink is shown. Rev3L had no influence on base excision repair (BER) of the N-alkylation lesions, but is likely involved in the tolerance of N-alkylations or apurinic/apyrimidinic sites originating from them. We also show that Rev3L exerts its protective effect in replicating cells and that loss of Rev3L leads to a significant increase in DNA double-strand breaks following temozolomide and fotemustine treatment. The data show that Rev3L contributes to temozolomide and fotemustine resistance, thus acting in concert with O^6-methylguanine-DNA methyltransferase, BER, mismatch repair and DSB repair in defense against simple alkylating anticancer drugs.

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INTRODUCTION

Alkylating agents are widely used in cancer therapy and chemotherapy often fails because of development of a drug resistant tumor phenotype. Since alkylating agents primarily target DNA, DNA repair and damage tolerance mechanisms are on the forefront in the development of drug resistance. The chemotherapeutics temozolomide and fotemustine belong to the groups of methylating and chloroethylating agents, respectively. They are used for the treatment of brain tumors (astrocytomas, glioblastoma multiforme) and malignant melanoma as well as brain metastasis of other tumors. Chloroethylating agents such as carmustine are also part of the combination therapy for the treatment of Hodgkin's lymphomas, lung cancer and breast cancer.

Methylating and chloroethylating drugs induce a broad spectrum of DNA adducts. One of them, O^6-alkylguanine, has been identified to be a major killing lesion that has a high propensity in activating the apoptotic cell death pathway [for review see (Roos and Kaina, 2006)]. This damage is repaired by the suicide repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT), which attenuates or even completely abolishes the toxic effects provoked by O^6-alkylguanine. Therefore, MGMT is considered to be a key node in the defense against O^6-alkylating agents [for review see (Kaina et al., 2007)].

Other targets of O^6-alkylating agents are the N-residues of purines and pyrimidines in DNA. Most of the N-alkylpurines such as N7-alkylguanine, N3-alkyladenine and N3-alkylguanine are repaired by base excision repair (BER). Defects in BER sensitize cells to the killing effects of O^6-alkylating agents. Thus, cells lacking DNA polymerase β (Polβ) are hypersensitive to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), temozolomide and CNUs (Sobol et al., 1996), and this hypersensitivity is executed by apoptosis (Ochs et al., 2002). Cells defective in poly(ADP-ribose)polymerase (PARP) (de Murcia et al., 1997) or XRCC1 (Zdzienicka et al., 1992), which coordinate the last steps of BER, are also hypersensitive,

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and pharmacological inhibition of BER by methoxyamine, that blocks the repair of apurinic/apyrimidinic (AP) sites, enhances the killing effects of simple alkylating agents (Liu et al., 2003; Taverna et al., 2001). All this supports the view that deficiency or down-regulation of BER, or imbalance in BER enzymatic steps, contribute to alkylating drug sensitization.

Non-repaired DNA alkylation damage interferes with DNA replication and, thus, gives rise to critical secondary DNA damage. The primary lesions can, however, also be tolerated by translesion DNA synthesis (TLS), which prevents cells from becoming blocked irreversibly in the S- or G2-phase of the cell cycle. One of the key translesion polymerases is DNA polymerase ζ (Polζ), the catalytic subunit of which is Rev3L (Rattray and Strathern, 2003). Rev3L has recently been shown to operate along with other translesion DNA polymerases to bypass different DNA lesions, including AP sites (Shachar et al., 2009). Although Rev3L is expressed to similar levels in lung, gastric, colon and renal tumors compared to normal tissue (Kawamura et al., 2001) it has recently been found to be over-expressed in human gliomas compared to normal brain tissue (Wang et al., 2009).

Here we studied the response of cells lacking Rev3L to the cytotoxic effects of temozolomide and fotemustine. We show that *Rev3L* knockout cells are remarkably sensitive to these agents, undergoing apoptosis at high level. We also show that BER and MGMT is not involved in this phenotype, which suggests that Rev3L is a new player in alkylating drug resistance, and a target for new potential anticancer drugs. In addition we hypothesize that drug-induced overexpression of Rev3L may mediate alkylating drug resistance in human gliomas.

MATERIALS AND METHODS

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Drugs and drug treatment. Temozolomide (TMZ; Schering-Plough, Kenilworth, NJ, USA) stocks (35 mM) were prepared by dissolving the drug in dimethyl sulphoxide (DMSO) and diluting it with sterile dH_2O (2-fold dilution). The temozolomide stocks were aliquoted and stored at −80°C until use. Fotemustine (FM; diethyl1-[3-(2-chloroethyl)-3-nitrosoureido]ethylphosphate; Muphoran, Servier Research International, Neuilly sur Seine, France) was prepared fresh for each treatment at a concentration of 1 µg/µl in ethanol. In all cases, exponentially growing cells were treated with fotemustine or temozolomide. Once added to the medium, temozolomide has a half life of 1.81 h, while fotemustine has an even shorter half life (Denny et al., 1994; Tapiero et al., 1989). Therefore the treatment of cells can be seen as a pulse treatment. Time points indicated in figures refer to the time following addition of the drug to the medium unless stated otherwise. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG; Sigma, Munich, Germany) stocks were prepared by dissolving the drug in DMSO and then diluting with sterile dH_2O to a 10 mM concentration (100-fold dilution). To deactivate the MGMT protein, 10 µM O^6-benzylguanine (O^6BG) was added to the cells 1h before drug treatment.

Cells and culture conditions. *Rev3L* wild-type, *Rev3L* heterozygote, *Rev3L* knockout mice were obtained as follows. Rev3L+/- p53+/- C57Bl/6 mice (Van Sloun et al., 2002) were mated to Rev3+/- 129/OLA mice. 13.5 d.p.c. Rev3L-/- p53+/- embryos were trypsinized and cultured until spontaneous immortalization, at very low frequency, occurred. Immortalized clones were isolated and displayed loss of the wild type p53 allele, as determined by allele-specific PCR. p53 is a necessary but not sufficient factor for immortalization of these cells (Zander and Bemark, 2004). It was also shown that loss of p53 does not rescue the embryonic lethality of Rev3L-deficient embryos (Van Sloun et al., 2002). Cells were routinely grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum at 37^{o}C and 5% CO_2.

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Determination of cell survival. Cell viability experiments were performed using the WST assay according to the manufacturer's protocol (Roche Diagnostics). Cell viability in untreated controls was set to 100%. Colony assays were performed on monolayers, growing in log phase. Cells were seeded in duplicate at appropriate cell numbers in 60 mm Petri dishes to yield approximately 100 surviving colonies. After 16 h, when cells were attached, O^6BG (10 µM) was added for 1 h and then the cells were exposed to graded doses of temozolomide or fotemustine. After 10-14 days cultures were fixed (in acetic acid: methanol: H_2O 1:1:8), stained (in 0.01% amido black) and colonies were counted. Results are from three independent experiments.

Determination of apoptosis by flow cytometry. The amount of apoptosis induced by temozolomide or fotemustine was determined by quantifying the fraction of cells containing a sub-diploid amount of DNA. Both adherent and detached cells were collected and suspended in 70% ethanol for fixation. Immediately before analysis, cells were treated with RNAse (0.03mg/ml) and subsequently stained with propidium iodide (PI; 16.5mg/ml) in Phosphate Buffer Saline (PBS). PI fluorescence was measured by flow cytometry (FACScalibur, Becton Dickinson, Heidelberg, Germany). For each sample 10.000 cells were analyzed (WinMDI Software, Joseph Trotter, http://facs.scripps.edu/software.html).

Preparation of protein extracts. Cell pellets were suspended in buffer A (10 mM HEPES–KOH, pH 7.4, 0.1 mM ethylene diaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis (b-aminoethyl ether), 250 mM sucrose, 1 µM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM dithiothreitol (DTT)). The cells were lysed by freeze/thaw/vortexing. The lysate was then centrifuged at 10000rpm for 10min and the supernatant containing the cytoplasmic proteins was used for western blot analysis. Cell extracts for MGMT activity assay were prepared as follows. Cells were harvested and homogenized by sonication (Branson SONIFIER cell disruptor B15) in buffer containing 20mM Tris-HCl, pH 8.5, 1mM EDTA, 1mM β-mercaptoethanol, 5% glycerol and the protease inhibitor PMSF (0.1 mM). The

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extract was centrifuged at 10000rpm (10min) in the cold in order to remove debris and the supernatant was snap-frozen in aliquots using liquid nitrogen and stored at -80^{o}C until use. The protein concentration was determined by the method of Bradford (Bradford, 1976).

Western blot analysis. The method used here is based on the method described previously (Renart et al., 1979). Protein (30 µg) of cytoplasmic extracts of temozolomide or fotemustine treated cells was separated in a 12% SDS polyacrylamide gel. Thereafter, proteins were transfered onto a nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany). Membranes were blocked in 5% (w/v) fat-free milk powder in Tris buffered saline (TBS) containing 0.1% Tween 20, incubated with the primary antibody (1:1000 dilution), washed with 0.1% Tween 20 in TBS, and incubated with a horseradish peroxidase-coupled secondary antibody 1:3000 (Amersham Biosciences AB, Uppsala, Sweden). Antibodies used were anti-ERK2 (Santa Cruz Biotechnology Inc.), anti-caspase-7 (Cell signaling) and anti-caspase-3 (Cell Signaling). After final washing, blots were developed by using a chemiluminescence detection system (Amersham Biosciences AB).

MGMT activity assay. The MGMT activity in *Rev3Lwt*, *Rev3L+/-* and *Rev3L-/-* cells was determined using a method based on the radioactive assay where tritium-labeled methyl groups are transferred from the O^6-position of guanine to protein in the cell extract (Preuss et al., 1995). HeLa S3 cells expressing MGMT (588±86 fmol/mg protein) and HeLa MR cells deficient in MGMT served as positive and negative controls, respectively. The radioactivity of the protein was then measured. For each assay cell extracts containing 200 µg protein was incubated with [^{3}H]methyl-nitrosourea-labelled calf thymus DNA containing O^6MeG (total 80.000 c.p.m./sample) in 700 mM HEPES–KOH (pH 7.8), 10 mM DTT, 50 mM EDTA for 90 min. Data are expressed as femtomoles of radioactivity transferred from ^{3}H-labelled DNA to protein per milligram of protein within the sample. The MGMT activity data presented are results obtained from three independent experiments.

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Single-cell gel electrophoresis. DNA single-strand breaks were determined and quantified by the highly sensitive alkaline single-cell gel electrophoresis or comet assay as previously described (Olive et al., 1990). Agarose imbedded MNNG pulse treated cells were lysed (2.5 M NaCl, 100 mM EDTA, 10 mM Tris 1% Na-Laurylsarcosinate, pH 10, 1% Triton X-100 and 10% DMSO) and subjected to electrophoresis. Analysis of DNA migration was done by staining DNA with ethidium bromide and using the image analysis system of Kinetic Imaging Ltd. (komet 4.0.2; Optilas). The mean tail moment (defined as percentage of DNA in the tail x tail length) of 50 cells per sample was determined.

Serum starvation. *Rev3Lwt* and *Rev3L-/-* cells were plated in medium containing indicated concentrations of serum. Following 72h incubation cells were trypzinised and counted. Number of cells obtained in 10% serum was set to 100% and all other cell numbers obtained at lower serum concentrations were expressed as a fraction of this number.

Immunofluorescence labeling and microscopy. *Rev3Lwt* and *Rev3L-/-* cells were seeded on cover slips. Following treatment with 0.5 mM temozolomide or 5 µg/ml fotemustine and 24h incubation for temozolomide treated cells or 48h incubation for fotemustine treated cells, the cells on the cover slips were fixed with 4% formaldehyde. A second fixation step was performed using 100% methanol (−20^{o}C, 20min). Cells and cover slips were then blocked in 5% BSA PBS (0.3% Triton X-100). The antibodies used were anti-γH2AX (Upstate) and Alexa Fluor 546 (Molecular probes). Just before mounting, DNA was stained with 100nM DAPI for 15min. Between all steps cells were washed in PBS (0.3% Triton X-100) for 5min. Slides were mounted in anti-fade medium (glycerol:PBS 1:1, 2.5% DABCO, pH 8.6 with HCl). For all time points at least 40 nuclei were scored for foci. All experiments were repeated two times. Foci were not scored in apoptotic cells.

RESULTS AND DISCUSSION

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Rev3L knockout cells are hypersensitive to temozolomide and fotemustine. This study was aimed at elucidating whether Polζ is involved in the defense against DNA damage induced by temozolomide and fotemustine, which represent methylating and chloroethylating anticancer drugs, respectively. We utilized *Rev3L* knockout cells that lack the catalytic subunit of Polζ, and compared isogenic wild-type (*Rev3Lwt*), heterozygous (*Rev3L+/-*) and homozygous (*Rev3L-/-*) knockout cells. temozolomide (Fig. 1A) and fotemustine (Fig.1B) caused a dose-dependent decrease in viability of all cells. However, for both temozolomide and fotemustine, *Rev3L* deficiency caused a clear sensitization to these agents (Fig. 1A and B). Interestingly, cells heterozygous for *Rev3L* showed an intermediate sensitivity toward temozolomide and fotemustine. Similar results were obtained with the more sensitive colony survival assay (Fig. 1C and D).

Viability as measured by the WST assay and colony survival reflects both cell cycle arrest and cell death. To specifically address cell killing, the apoptotic response of these cells was determined following temozolomide and fotemustine treatment. Again, *Rev3L* knockout cells were hypersensitive to both temozolomide (Fig. 2A) and fotemustine (Fig. 2B). Similar to what was observed in the viability and colony assays, the *Rev3L* heterozygous cells showed an intermediate level of apoptosis compared to wild type and *Rev3L-/-* cells following treatment with both temozolomide and fotemustine (Fig. 2A and B). To verify that what was observed by SubG1 flow cytometry was truly apoptosis, western blot analysis of temozolomide and fotemustine treated cells were performed. Temozolomide and fotemustine caused the activation of both caspase-3 and caspase-7 in Rev3L-/- cells, as revealed by the activated fragments (Fig. 2C and D). Collectively, the data show that Rev3L protects cells against cytotoxicity induced by temozolomide and fotemustine. Importantly, the intermediate phenotype of the Rev3L+/- cells in the survival and apoptosis assays suggests that the expression level of Rev3L may be rate-limiting in the tolerance of temozolomide and fotemustine.

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Rev3L does not sensitize cells to O^6-methylguanine. In previous work it was shown that O^6-methylguanine is the dominant apoptotic lesions induced by methylating drugs, and that MGMT is therefore a key node in cell resistance to these agents [for review see (Kaina et al., 2007)]. O^6-methylguanine is repaired by MGMT in a one step damage reversal reaction that causes protection against the killing effect of this lesion. To test the hypothesis whether *Rev3L-/-* hypersensitivity is due to lack of bypass of O^6-methylguanine adducts, the influence of MGMT on the sensitivity of these cells was determined. The cell lines used display different levels of MGMT activity, from 5 up to *Rev3L-/-* cells expressing approximately 160 fmol/mg protein (Fig. 3A). The reason for the different expression levels is unknown, although it is well established that immortalized cells display variable levels of MGMT (Harris et al., 1996). To inactivate MGMT, the cell lines were treated with the specific inhibitor O^6-benzylguanine (O^{6}BG) (Moschel et al., 1992). Thereafter, *Rev3Lwt* and *Rev3L-/-* cells were treated with 0.5 mM temozolomide (in the presence or absence of 10 µM O^{6}BG) and the frequency of apoptosis was determined 72h later (Fig. 3B). *Rev3L-/-* cells were significantly more sensitive to temozolomide, and no difference in their apoptotic response was observed when they were pretreated with O^{6}BG in order to inactivate MGMT (Fig. 3B).

The cytotoxicity of O^6-methylguanine in the presence of O^{6}BG depends on its miscoding properties during replication, followed by lethal processing by DNA mismatch repair (Karran and Bignami, 1992). Therefore, we anticipated that, if O^6-methylguanine sensitivity is mediated by misincorporation opposite O^6-methylguanine by Rev3L, Msh2 deficiency would fully rescue the sensitivity of *Rev3L*-deficient MEFs to temozolomide. However, sensitivity of Rev3L knockout MEFs to the temozolomide analog MNNG was rescued only very partially by concomitant Msh2 deficiency (Fig. 3C). This result indicates that DNA mismatch repair is not involved in damage processing provoked by lack of Rev3L. Since the extent of rescue of MNNG sensitivity by Msh2 deficiency was identical for Rev3L knockout cells as for wild-type cells (Fig. 3C) we also infer that O^6-methylguanine is not subject of TLS by Polζ. The data rather suggest that Polζ is required for the tolerance of N-alkylation lesions or gaps that arise from spontaneous depurination of lesions such as N3-methyladenine or N3-methylguanine, or from BER intermediates like AP sites. In line with this is the observation that *Rev3L-/-* cells are also hypersensitive to methyl methanesulfonate (MMS) (Okada et al., 2005; Takenaka et al., 2006; Wittschieben et al., 2006), which produces very low amounts of O^6MeG (Beranek, 1990). Inhibition of BER, e.g. by methoxyamine, was shown to ameliorate the killing response of cells to temozolomide (Taverna et al., 2001), which was taken to demonstrate that non-repaired N-methylation lesions contribute to temozolomide-induced cytotoxicity and BER protects against it. Here, we extend this finding showing that a translesion DNA polymerase, Polζ, contributes to resistance against temozolomide. Therefore, Polζ appears to be a potential target for anticancer chemotherapy.

Rev3L sensitizes cells to O^6-chloroethylguanine. Whilst O^6BG did not impact on the killing response of temozolomide in *Rev3L-/-* cells, it clearly ameliorated cell death by apoptosis upon treatment with fotemustine in Rev3L lacking cells (Fig. 3B). This indicates that O^6-chloroethylguanine adducts are a substrate for Polζ mediated TLS. Recently it has been shown that Polζ is involved in TLS of many different lesions, including mitomycin C, MMS, benzo[a]pyrene adducts, AP sites, cisplatin induced guanine-guanine crosslinks, 4-hydroxyequilenin-cytosine and thymine-thymine 6-4 photoproducts (Shachar et al., 2009). Our data indicate that Polζ may also be involved in TLS across O^6-chloroethylguanine. Of note, O^6-chloroethylguanine is a rather unstable adduct, undergoing rearrangement to N1-O^6-etheno-guanine and finally N1-guanine-N3-cytosine interstrand crosslinks. Whether O^6-chloroethylguanine or the etheno adduct derived from it is subject of TLS by Polζ remains to be determined.

Rev3L does not have an influence on the formation of base excision repair intermediates. Most of the DNA lesions induced by alkylating agents are repaired by base excision repair (BER), and BER defective cells (e.g. Polβ knockout cells) are hypersensitive to methylating agents such as MNNG (Sobol et al., 1996). They also display a high level of

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DNA repair intermediates, which can be detected in the alkaline comet assay (Ochs et al., 2002). To elucidate the level of BER intermediates in *Rev3Lwt* and *Rev3L-/-* cells, cells were treated with a pulse of MNNG and single-strand breaks (ssb) were determined. As shown in Fig. 3D, the frequency of ssb increased with the dose of MNNG. There was no difference in the tail moment (TM) between *Rev3Lwt* and *Rev3L-/-* cells. Fig. 3E demonstrates the decline in the TM level with post-incubation time. Again, no difference was observed between *Rev3Lwt* and *Rev3L-/-* cells. The data suggest that BER is not different in wild-type and *Rev3L* knockout cells and Rev3L has no impact on the formation of ssb after treatment with methylating agents.

Rev3L knockout causes replication dependent toxicity and the increased formation of DNA double-strand breaks. Temozolomide and fotemustine are used as chemotherapeutics in the treatment of cancer. Cancer cells are replicating and, therefore, it is important to know whether replicating cells will be targeted. To this end, *Rev3L-/-* cells were grown under conditions of different serum concentrations and relative cell growth was determined. As the serum concentration increased, a significant increase in cell proliferation was observed following incubation for 72h (Fig. 4A). When determining the influence of proliferation on apoptosis induced by ether 0.5 mM emozolomide or 5 µg/ml fotemustine, an increase in apoptosis was observed with increasing proliferation rate (Fig. 4B). The data clearly show that the killing effect of both temozolomide and fotemustine is strongly dependent on cell proliferation in *Rev3L-/-* cells. Since *Rev3Lwt* cells were quite resistant to the doses of temozolomide and fotemustine used, we can also infer from the data that Rev3L specifically protects proliferating cells from DNA damage induced by temozolomide and fotemustine.

If Rev3L is responsible for bypass of temozolomide and fotemustine lesions during DNA synthesis in S-phase, then the lack of Rev3L may lead to more replication blocks that could collapse and form DNA double-strand breaks (DSBs) (Van Sloun et al., 2002). DSBs are

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effective triggers of apoptosis (Lips and Kaina, 2001). To determine whether *Rev3L-/-* cells show more DSBs than *Rev3Lwt* cells, both cell lines were treated with 0.5 mM temozolomide or 5 µg/ml fotemustine and the formation of γH2AX foci, a very good indicator of DSBs, was determined using fluorescence microscopy (for an example of γH2AX foci formation following chemotherapeutic treatment see Fig. 4C). These foci were scored and plotted. In both temozolomide and fotemustine treated cells, *Rev3L-/-* showed significantly higher levels of foci formation that the *Rev3Lwt* cells (Fig. 4D). Consistent with what was observed in the survival assays, loss of Msh2 was not able to correct for DSB formation in Rev3L-/- cells following MNNG treatment (Fig. 4E). This result confirms that Rev3L is involved in TLS of methylated bases other than O^6MeG.

In conclusion, the data obtained highlight a pivotal role for Polζ in the protection against two important groups of anticancer drugs, i.e. methylating and chloroethylating agents that are used as first-line therapy of brain tumors and malignant melanomas. Since the heterozygous cells displayed a phenotype of intermediate sensitivity, the levels of Rev3L may determine drug tolerance. In this context it is important to note that Rev3L is over-expressed in gliomas (Wang et al., 2009) and can presumably be up-regulated upon drug exposure (Wakana et al., 2000). Combined with our finding that Rev3L levels may be rate-limiting in the development of drug tolerance, Rev3L may be a primary determinant in the development of tolerance to alkylating chemotherapy. Therefore, determination of the expression levels of Rev3L in tumors, prior and post treatment, is desirable.

Polζ was shown to cooperate with other DNA polymerases, notably Polη and Polκ, that insert nucleotides opposite the lesion. Polζ in turn performs the extension step until DNA replication can proceed in a normal manner (Shachar et al., 2009). The extension mediated by Polζ is required for TLS across many lesions, including AP sites, which explains why Polζ is an exceptionally important player in tolerating critical DNA damage. It has been shown that it is involved in the tolerance of cisplatin-guanine-guanine intrastrand adducts (Shachar et al.,

2009) and interstrand crosslinks formed by MMC (Wittschieben et al., 2006). As expected, Polζ knockdown sensitized cells to cisplatin while stable over-expression caused resistance (Wang et al., 2009). Here, we extend this finding to O^6-alkylating chemotherapeutics and show that for these agents Polζ is a marker of resistance, acting in concert with MGMT, BER, MMR and DSB repair in protecting against toxic alkylation damage. Similar to strategies of down-regulating or inhibiting MGMT and BER in tumor cells, it is pertinent to consider Polζ as a novel target in cancer therapy.

Acknowledgement

We acknowledge Georg Nagel for technical assistance.

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Footnotes

Work was supported by Deutsche Forschungsgemeinschaft [DFG-Ka 724], the German Cancer Foundation [Ka-106748], the Dutch Cancer Foundation [UL 2001-2517] and the European Union [EU-IP FP6-512113].

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Figure legends

Fig. 1: Contribution of Rev3L to the protection of cells against temozolomide and fotemustine. Viability of temozolomide **(A)** and fotemustine **(B)** treated Rev3Lwt, Rev3L+/- and Rev3L-/- cells. Cells were treated with indicated doses and viability was determined 72h later using the WST assay. Colony survival following temozolomide **(C)** and fotemustine **(D)** in Rev3Lwt, Rev3L-/+ and Rev3L-/- cells. Cells were treated with the indicated concentrations and colonies were scored after appearance 10-12 days later. For all experiments, in order to deplete MGMT, 10 µM O^6BG was added to the medium 1 h before temozolomide of fotemustine treatment.

Fig. 2: Activation of apoptosis and the executing caspases following temozolomide and fotemustine. Apoptosis induced by temozolomide **(A)** and fotemustine **(B)** in Rev3Lwt, Rev3L-/+ and Rev3L-/- cells. Cells were treated with the indicated concentrations and apoptosis was determined 72h later by the SubG1 method using flow cytometry. The activated fragments of caspase-3 **(C)** and caspase-7 **(D)** were assayed in Rev3Lwt and Rev3L-/- cells using immunoblotting at indicated times following 0.5mM temozolomide or 5µg/ml fotemustine treatment. For all experiments 10 µM O^6BG was added to the medium 1 h before temozolomide of fotemustine treatment.

Fig. 3: Influence of Rev3L on O^6-alkylguanine triggered apoptosis and BER. **(A)** MGMT repair activity in Rev3Lwt, Rev3L+/- and Rev3L-/- cells. **(B)** Apoptosis induced by temozolomide (0.5 mM) or fotemustine (10 µg/ml) in the presence or absence of the MGMT inhibitor O^6BG (10 µM). Results obtained by SubG1 determination using flow cytometry for Rev3Lwt and Rev3L-/- are shown. Apoptosis frequency was determined 72h after treatment. **(C)** Survival of Rev3Lwt, Msh2-/-, Rev3L-/- and Rev3L;Msh2-/- double knockout cells following 250 µM MNNG treatment. **(D)** Single-cell gel

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electrophoresis results obtained in Rev3Lwt and Rev3L-/- cells treated for 1h with indicated doses of MNNG. **(E)** Time kinetics for Rev3Lwt and Rev3L-/- cells pulse treated for 1h with 25 µM MNNG, samples were stopped at indicated time points.

Fig. 4: Replication dependence of apoptosis induction in Rev3L-/- cells. **(A)** Relative growth in relation to serum concentration in Rev3L-/- cells, cells were grown in medium containing indicated concentrations of serum and growth was determined by counting cell numbers after 72 h and plotting it as a relative number compared to cell number obtained in 10 % serum. **(B)** Relative apoptotic response of Rev3L-/- cells. Results were obtained by treating cells grown in indicated concentrations of serum with 0.5mM temozolomide or 5µg/ml fotemustine and determining the apoptotic response 72h later using SubG1. Amount of apoptosis observed in 10% serum was set to 100%. **(C)** γH2AX foci formation in Rev3Lwt cells treated with 0.5 mM temozolomide. **(D)** Quantification of γH2AX foci formation in Rev3Lwt and Rev3L-/- cells following 0.5 mM temozolomide or 5 µg/ml fotemustine treatment, for temozolomide treated cells foci were scored 24h after treatment and for fotemustine 48h after treatment. Foci were not scored in apoptotic cells. **(E)** γH2AX formation in Rev3Lwt, Msh2-/-, Rev3L-/- and Rev3L;Msh2-/- double knockout cells following 100 µM MNNG treatment at indicated time points.

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Figure 1

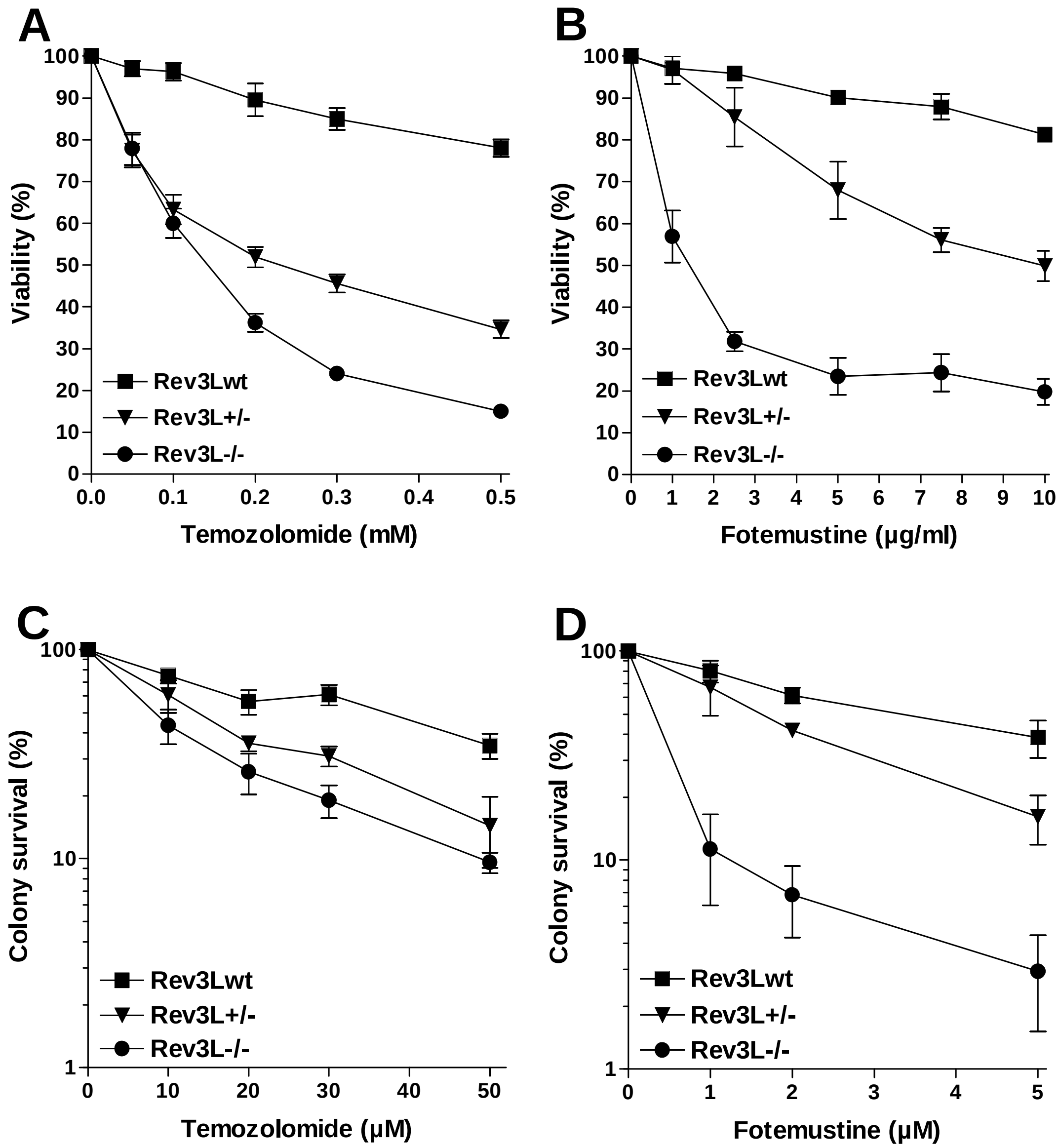

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Figure 2

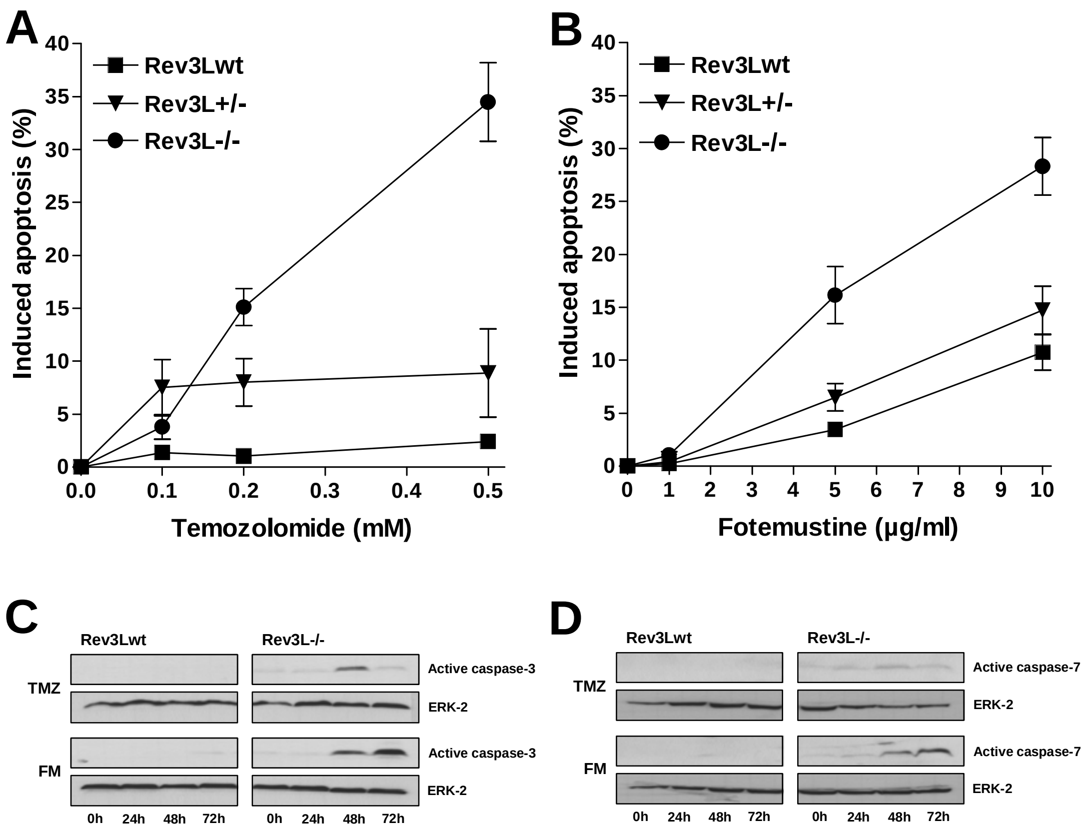

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Figure 3

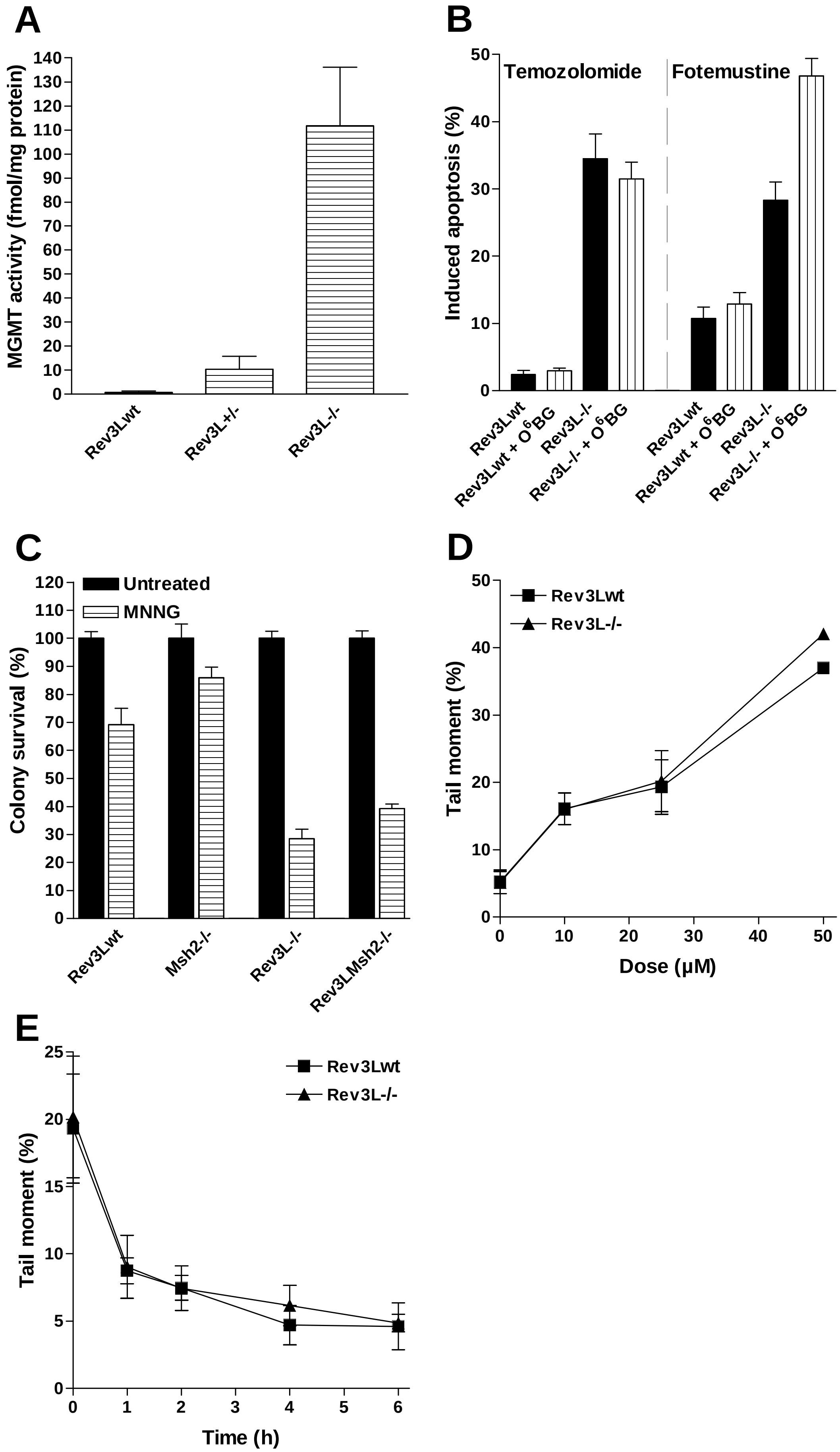

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Figure 4