Influence of fatty acid synthase inhibitor orlistat on the DNA repair enzyme O^6-methylguanine-DNA methyltransferase in human normal or malignant cells *in vitro*

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Abstract. Tetrahydrolipstatin (orlistat), an inhibitor of lipases and fatty acid synthase, is used orally for long-term treatment of obesity. Although the drug possesses striking antitumor activities *in vitro* against human cancer cells and *in vitro* and *in vivo* against animal tumors, it also induces precancerous lesions in rat colon. Therefore, we tested the *in vitro* effect of orlistat on the expression of O^6-methylguanine-DNA methyltransferase (MGMT), a DNA repair enzyme that plays an essential role in the control of mutagenesis and carcinogenesis. Western blot analysis demonstrated that 2-day continuous exposure to 40 μM orlistat did not affect MGMT levels in a human melanoma cell line, but downregulated the repair protein by 30-70% in human peripheral blood mononuclear cells, in two leukemia and two colon cancer cell lines. On the other hand, orlistat did not alter noticeably MGMT mRNA expression. Differently from lomeguatrib (a false substrate, strong inhibitor of MGMT) orlistat did not reduce substantially MGMT function after 2-h exposure of target cells to the agent, suggesting that this drug is not a competitive inhibitor of the repair protein. Combined treatment with orlistat and lomeguatrib showed additive reduction of MGMT levels. More importantly, orlistat-mediated downregulation of MGMT protein expression was markedly amplified when the drug was combined with a DNA methylating agent endowed with carcinogenic properties such as temozolomide. In conclusion, even if orlistat is scarcely absorbed by oral route, it is possible that this drug could reduce local MGMT-mediated protection against DNA damage provoked by DNA methylating compounds on gastrointestinal tract epithelial cells, thus favoring chemical carcinogenesis.

Introduction

Tetrahydrolipstatin (orlistat), a well-known irreversible inhibitor of pancreatic and gastric lipases, is extensively used as anti-obesity drug (1). This agent, that is administered by oral route, is minimally absorbed by the gastrointestinal tract and is able to prevent the absorption of a large percentage of lipids, thereby reducing lipid supply from outside sources.

Of particular interest is the finding that orlistat is also a potent inhibitor of fatty acid synthase (FASN), an enzyme that plays an important role in tumor growth and progression (2) and is considered a metabolic oncogene (3). Accordingly, the agent shows antiproliferative activity against cancer cells both *in vitro* and in animal models (2).

The antitumor activity of orlistat, however, does not seem to be entirely dependent on its activity against FASN. A large amount of experimental data indicates that the antineoplastic effects of the agent may also be due to the inhibition of fatty acid synthesis through different metabolic pathways without altering FASN activity (4) or impairing mitochondrial respiration (5). In any case, orlistat produces cytotoxic effects through activation of apoptotic pathways preceded by endoplasmic reticulum stress (6), although it is not clear whether this mechanism is preferentially involved in the suppression of neoplastic cells.

It has been previously shown that saturated fatty acids downregulate cell response to DNA damage, thus favoring malignant transformation (7). According to this mechanism, one would expect that FASN inhibition could be of value not only as a device for attaining antitumor effects, but also as an anticancer treatment modality. In addition, orlistat has been found to augment pro-apoptotic NOXA protein (8), thus

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reinforcing its possible cancer protective activity. However, preclinical studies performed on rats exposed to predisposing factors for colon cancer (i.e., receiving high fat diet alone or combined with the carcinogen compound methyl hydrazine), showed that long-term treatment with orlistat lead to severe crypt alterations of colonic mucosa, that are considered colon cancer biomarkers (9,10).

These results do not seem to be in line with the expected cancer prevention activity of orlistat. Therefore, we decided to obtain further insight into the pharmacodynamic properties of orlistat by evaluating its activity on other cell-associated biochemical functions. In view of the extensive protective role against malignant transformation played by several DNA repair enzymes, we elected to dedicate our attention in particular to O^6-methylguanine-DNA methyltransferase (MGMT). This protein plays a primary role in the defense against chemical carcinogenesis (reviewed in ref. 11) since it removes methyl adducts at O^6-guanine in DNA, which are produced by several DNA mono-methylating agents, including environmental carcinogenic compounds such as N-nitroso derivatives involved in colorectal cancer (12). Moreover, it has been found that MGMT loss could be responsible of PIK3CA mutations present in human colon cancer (13).

The results of the present study pointed out that orlistat is able to downregulate MGMT protein expression at a concentration that could be reasonably attainable at the level of intestinal epithelial cells under oral long-term treatment with the drug (i.e., 120 mg three times a day). It follows that our findings support the indication that long-term use ($\geq$1 year) of high doses of orlistat in overweight subjects would require appropriate surveillance for possible gastrointestinal carcinogenesis threats.

Materials and methods

Cell lines. The human colon cancer cell line HCT116, kindly provided by Dr G. Marra (Institute of Molecular Cancer Research, University of Zurich, Zurich, Switzerland), was maintained in McCoy's 5A medium (Sigma-Aldrich, Milan, Italy) supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS, Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), and 50 μg/ml gentamicin (Euroclone, Milan, Italy).

The human colon cancer cell line HT-29, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), was routinely grown in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich), supplemented with 2 mM L-glutamine, 60 μM gentamicin and 10% heat-inactivated FCS (Sigma-Aldrich), hereafter referred to as complete medium D (CMD). Both HCT116 and HT-29 cells growing as plastic adherent cells were removed using a solution of 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS) without calcium and magnesium.

The human Jurkat CD4+ T cell leukemia cell line and the human promyelocytic leukemia cell line HL-60 were obtained from the ATCC and were cultured at 37°C in a 5% CO_2 humidified atmosphere in RPMI-1640 (Sigma-Aldrich), supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), and 50 μg/ml gentamicin (Euroclone) hereafter referred to as complete medium (CM).

The human melanoma cell line M10, kindly donated by Dr D. Del Bufalo (Regina Elena Cancer Institute, Rome, Italy), was originally established from a cutaneous metastasis of advanced melanoma. The line, regularly cultured in CM in our laboratory, shows fairly high levels of MGMT activity (14). M10 cells were removed from continuous culture as described for the colon cancer cell lines.

Peripheral blood mononuclear cells were obtained by centrifugation on a Ficoll-Hypaque (Lymphoprep™, Axis-Shield, Oslo, Norway) gradient of buffy coats from healthy blood donors, and washed twice in RPMI-1640 medium. Non adherent mononuclear cells (NAMNC) were separated by plastic adherence at 37°C for 1 h in CM. Informed consent was obtained from blood donors according to our institutional guidelines.

Drugs. Orlistat was purchased from Sigma-Aldrich, dissolved in sterile DMSO (Sigma-Aldrich) at the concentration of 20 mM, aliquoted and stored at -70°C until use. Lomeguatrib (LM) was a generous gift from Professor G. Margison (Centre for Occupational and Environmental Health, University of Manchester, Manchester, UK). The compound was dissolved in DMSO at the concentration of 10 mM, aliquoted and stored at -70°C until use. Temozolomide (TMZ) was supplied by Schering-Plough Co. (Milan, Italy) and was always prepared freshly in culture medium and added immediately to cell suspensions, because the drug readily decomposes in aqueous solution.

MGMT activity assay. MGMT activity was determined by measuring the transfer of ^{3}H-methyl groups from a DNA substrate to the MGMT protein (15). Briefly, cell pellets (1×10^6 cells) were re-suspended in 1 ml of lysis buffer (0.5% CHAPS, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 3 mM dithiothreitol, 100 mM NaCl, 10% glycerol) supplemented with a cocktail of protease inhibitors (Roche, Mannheim, Germany) and incubated for 30 min at 4°C. Cell lysates were then centrifuged at 18,000 x g for 10 min at 4°C. Aliquots of supernatants were then diluted in 50 mM Tris-HCl buffer, pH 8.3, containing 1 mM EDTA, and 3 mM dithiothreitol, and incubated with 10 μg of ^{3}H-methylated-DNA at 37°C for 1 h. DNA was then hydrolyzed by heating samples at 75°C for 45 min, in the presence of 1 N perchloric acid, and protein precipitated using 1 mg bovine serum albumin as carrier. Pellets were washed with 1 N perchloric acid, re-suspended in 0.01 N NaOH, and radioactivity measured in a liquid scintillation counter (TRI-CARB 1900, Packard Instruments Co., Meriden, CT, USA), after addition of scintillation liquid (Ultima Gold, Packard Instruments Chemical Operation, Groningen, The Netherlands). Protein concentration in supernatants was evaluated according to the method of Bradford using the Bio-Rad Protein Assay Dye reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA) and bovine serum albumin as standard. MGMT activity was expressed in terms of fmoles of ^{3}H-methyl groups transferred per mg of protein in cell extract.

Preparation of cell extracts. Cells were washed with PBS. The cell pellet was suspended in 100 μl extraction buffer [(12.5 mM Na_2HPO_4, pH 7.2; 94 mM NaCl; 50 mM NaF;

1% di-Triton X-100; 2 mM EGTA, 1% protease inhibitor cocktail (Sigma-Aldrich), 0.5% saponin (Sigma-Aldrich) and 1 mM Na_3VO_4: (Sigma-Aldrich)] kept on ice for 30 min, and centrifuged for 30 min at 15,000 x g at 4˚C in an Eppendorf microcentrifuge. The protein concentrations were determined using Bio-Rad Protein Assay Dye. The samples were stored at -80˚C until use.

Electrophoresis and immunoblotting. The proteins were heated in a boiling water bath for 2 min and separated by running cell extracts on 10% polyacrylamide pre-cast gels (NuPAGE® Novex Bis-Tris, Invitrogen, Life Technologies, Grand Island, NY, USA), using XCell SureLock™ Mini-Cell apparatus (Invitrogen) following the instructions of the producer. At the end of the electrophoretic separation, proteins were transferred to Hybond-ECL nitrocellulose filters (GE Healthcare Life Sciences, Pittsburg, PA, USA) by electrotransfer with miniblot apparatus (Invitrogen). The transfer was carried out at 25 V overnight. Thereafter, the membranes were incubated with 3% non-fat dry milk (Bio-Rad) in Tris-buffered saline (TBS) at pH 7.5, for 60 min at room temperature and then incubated with an anti-actin rabbit polyclonal antibody (Sigma-Aldrich) diluted 1:3,000 in TBS containing 0.05% Tween-20 (TBST) and mouse monoclonal antibody against MGMT (Millipore, Billerica, MA, USA) diluted 1:500 in TBST for 60 min. The membranes were washed twice with TBST and incubated for 45 min with the secondary alkaline phosphate-conjugated antibody. Bands were developed using westernBreeze chemi-luminescent immunodetection kit (Invitrogen), according to the manufacturer's instructions. The film was scanned on a GS-710 Calibrated Imaging Densitometer and analyzed by means of Quantity One software, version 4.1.1 (Bio-Rad Laboratories). The optical density (OD) of MGMT and actin bands was expressed as arbitrary units. MGMT expression was evaluated on the basis MGMT:actin ratio (OD-R).

RNA isolation and RT-qPCR. Total RNA was extracted, from $2x10^6$ viable cells using 1 ml of TRI Reagent solution (Ambion, Life Technologies, Monza, Italy) according to the manufacturer's instructions. RNA purity and concentration were checked with a NanoQuant Infinite M200 instrument (Tecan Group Ltd., Mannedorf, Switzerland). Two microliters of RNA was purified by clearance of DNA traces using Turbo DNA-free kit (Applied Biosystems, Life Technologies, Monza, Italy). cDNA was synthesized from 2 μg of RNA by reverse transcription using TaqMan RT kit (Applied Biosystems), according to the manufacturer's instructions. Five microliters (i.e., 2 μg) of cDNA/sample was amplified, according to the manufacturer's instructions, by the Stratagene Mx3005P qPCR System (La Jolla, CA, USA) using a TaqMan gene expression assay kit (Applied Biosystems, Assay-On-Demand: code no. Hs.00172470_m1 for MGMT). Levels of MGMT mRNA were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene expression (Applied Biosystems, code no. 4326317E). All RT-qPCR reactions were performed in triplicate.

The relative quantification of MGMT was performed using the comparative threshold cycle (C_T) method (as described by Applied Biosystems protocol) that uses an arithmetic formula ($2^{-\Delta\Delta C_T}$). $\Delta\Delta C_T$ is the difference between the ΔC_T of the sample under investigation and the ΔC_T of the calibrator sample. The RNA obtained from Jurkat cells was chosen as the calibrator sample.

Statistical analysis. Statistical significance among different mean values was assessed using two-tailed Student's t-test analysis.

Results

Effect of orlistat treatment on MGMT expression in tumor cells or in normal NAMNC. In order to determine the concentration range of orlistat to be utilized in the experiments illustrated in the present report, a concentration/effect study was performed using Jurkat target cells. Leukemic cells were cultured in the presence of graded concentrations of orlistat for two days. Control cultures were exposed to DMSO alone at the concentration corresponding to that utilized for orlistat 40 μM. At the end of the *in vitro* treatment, leukemic cells were lysed and subjected to western blot (WB) analysis. The results, illustrated in Fig. 1A, indicate that orlistat, at the concentration of 40 μM, was able to reduce by >50% the MGMT level, whereas little or no effect was found when lower concentrations were used. These findings were qualitatively confirmed by four additional experiments conducted with Jurkat cells (data not shown), although downregulation of MGMT protein expression appears to be noticeably variable (i.e., inhibition among different experiments ranging from ~30 to 70%). Based on these results, we decided to utilize the concentration of 40 μM as the standard concentration of the agent in the majority of the experimental procedures herein described.

Time-course studies were performed on Jurkat cells in order to explore the effect of orlistat on MGMT expression when the leukemic blasts were exposed to the drug for a total of 72 h. Incubation of target cells with orlistat (40 μM) started on day 0, and MGMT levels were tested by WB analysis on days 1, 2 and 3. The results (Fig. 1B) show that downregulation of MGMT by orlistat is not detectable on day 1, starts on day 2 and persists up to day 3.

Similar concentration/effect and time-course studies were conducted with the epithelial colon cancer HCT116 cells. The results, illustrated in Fig. 1C, confirm that downregulation of MGMT expression is produced by orlistat in these cells at the concentration of 40 μM. This effect was even more pronounced after 4-day exposure to the agent, showing a decline of MGMT protein concentration >70%.

Analysis of the effect of continuous exposure to orlistat was extended to normal NAMNC and to other tumor cell types, such as melanoma M10, promyelocytic leukemia HL-60, and colon cancer HT-29 cell lines. After 2-day incubation with the agent, the results, illustrated in Fig. 1D, confirm that the drug provoked an ~50% reduction of MGMT level in all target cells except for melanoma M10 cells that showed no downregulation of the protein.

Comparative studies on the effect of orlistat and LM on MGMT expression. Among well established MGMT inhibitors, LM represents one of the best molecule able to rapidly and potently inhibit MGMT functional activity (16). This agent behaves as a false substrate that binds MGMT irreversibly and promotes
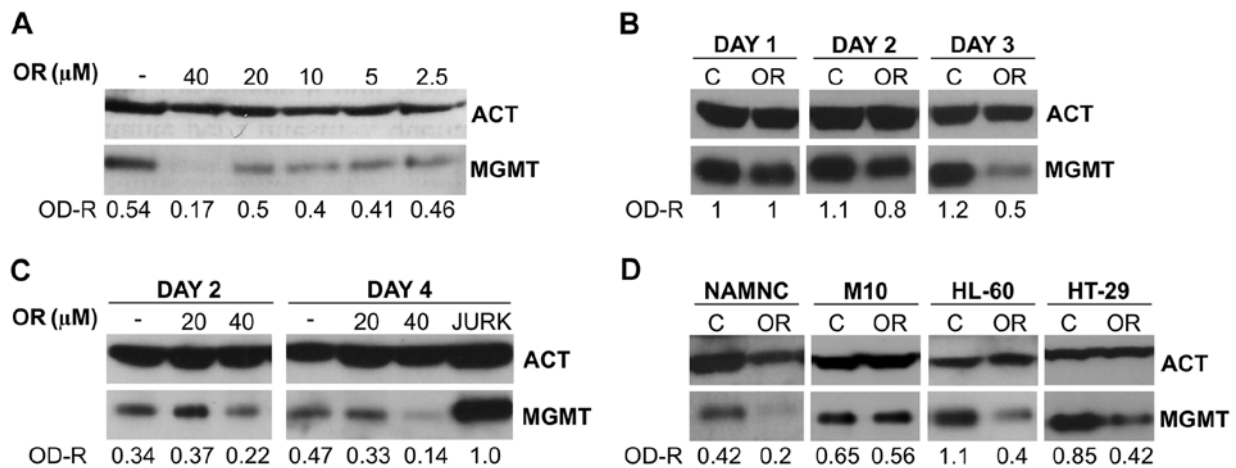
Figure 1. Effect of orlistat on MGMT protein levels in different tumor cell lines or normal NAMNC. The images represent western blot analysis of cell extracts. OR, orlistat; ACT, actin; OD-R, ratio between the optical density of MGMT and ACT band measured in terms of arbitrary units. The results were confirmed in at least two independent experiments. (A) MGMT protein levels of Jurkat cells incubated with the indicated concentrations of orlistat for 2 days. (B) Time-course analysis of the effect of continuous exposure to orlistat ($40\,\mu$M) on MGMT protein levels of Jurkat cells. (C) MGMT protein levels of HCT116 cells incubated with the indicated concentrations of orlistat for 2 or 4 days. JURK, untreated Jurkat cell control. (D) Effect of a 2-day exposure to orlistat ($40\,\mu$M) on MGMT protein levels of normal NAMNC or of other malignant cell lines (i.e., M10 melanoma, HL-60 promyelocytic leukemia, HT-29 colon cancer).

its degradation through the ubiquitin-proteasome pathway (17). This mechanism implies that MGMT function is almost immediately suppressed by LM and that the inactive protein undergoes a fairly rapid degradation within the cell. Thereafter, the cell starts to synthesize *de novo* the MGMT protein in order to restore its DNA repair functional activity. These observations prompted us to compare the effect of orlistat with that of LM on the kinetics of MGMT protein level decline in a short-term assay. Previous studies pointed out that $10\,\mu$M LM was able to downregulate markedly the MGMT function in target cells (18). Therefore, we employed this inhibitor concentration in all experiments described in the present report. Jurkat cells were incubated with orlistat ($40\,\mu$M) alone, or with LM alone. At different time intervals (i.e., 1, 3, 6 and 24 h) the cells were collected and tested for MGMT protein expression by WB analysis. The results, illustrated in Fig. 2A, confirmed that no noticeable downregulation of MGMT protein level was found at all time intervals explored when target cells were treated with orlistat. In contrast, a manifest decline of MGMT protein was detected in LM-treated cells as early as 3 h after start of treatment, followed by a further reduction of ~70% at 24 h.

Additional experiments were conducted with the intent to establish whether orlistat could be able to influence the functional activity of MGMT with a mechanism conceivably similar to that of LM. Therefore, HCT116 and Jurkat cells were treated with $40\,\mu$M orlistat or $10\,\mu$M LM, and MGMT activity was tested as early as 2 h after start of treatment. As expected, and in line with previous reports (16,18), LM provoked a drastic decrease of MGMT function in both cell lines, whereas minimal or no significant downregulation of the enzymatic activity was detected in Jurkat and HCT116 samples, respectively, exposed to orlistat (Table I).

Further studies were performed to evaluate the possible influence of orlistat and LM on MGMT gene transcription. RT-qPCR performed on Jurkat cells at different times after exposure to orlistat ($40\,\mu$M) or to LM ($10\,\mu$M) pointed out that no remarkable changes of MGMT transcription respect to DMSO-treated controls were detectable in orlistat-treated cells, for ≤24 h after start of treatment (Fig. 2B). However, a transient increase of MGMT mRNA was found at 48 and 72 h. Limited downregulation of MGMT expression was found instead, shortly after exposure to LM. In this case, mRNA values returned to normal levels at 72 h of incubation with the MGMT inhibitor.

Effects of orlistat combined with LM on MGMT protein expression. The results illustrated in the previous section favor the hypothesis that orlistat inhibits MGMT expression with a mechanism distinct from that involved in LM activity. Therefore it was of interest to explore the combined effects of the two agents on MGMT protein levels of normal or leukemic cells.

Normal NAMNC were exposed to $40\,\mu$M orlistat alone, to $10\,\mu$M LM alone, or to orlistat + LM for 6 consecutive days. WB analysis was performed on cells collected shortly (i.e., on day 1), or late (i.e., on day 6) from the onset of drug treatment. The results, shown in Fig. 3A, demonstrate that, in line with the previous results, orlistat alone had marginal effect on MGMT protein levels on day 1, whereas LM reduced MGMT expression by >50%. Combination of the two agents was slightly additive, as confirmed by other two experiments that gave similar results. When the WB assay was performed on day 6, both orlistat and LM showed remarkable inhibitory effects, that were higher when the two drugs were used in combination.

In a second set of experiments Jurkat cells were incubated with either $40\,\mu$M orlistat or $10\,\mu$M LM or with the combination of the two drugs. In this case, however, target cells were incubated with the agents for 24 h only. Thereafter, the cells were washed carefully and all cultures were reconstitute in culture medium alone. MGMT protein levels were determined on days 2, 3 and 6 of total culture time. The results,
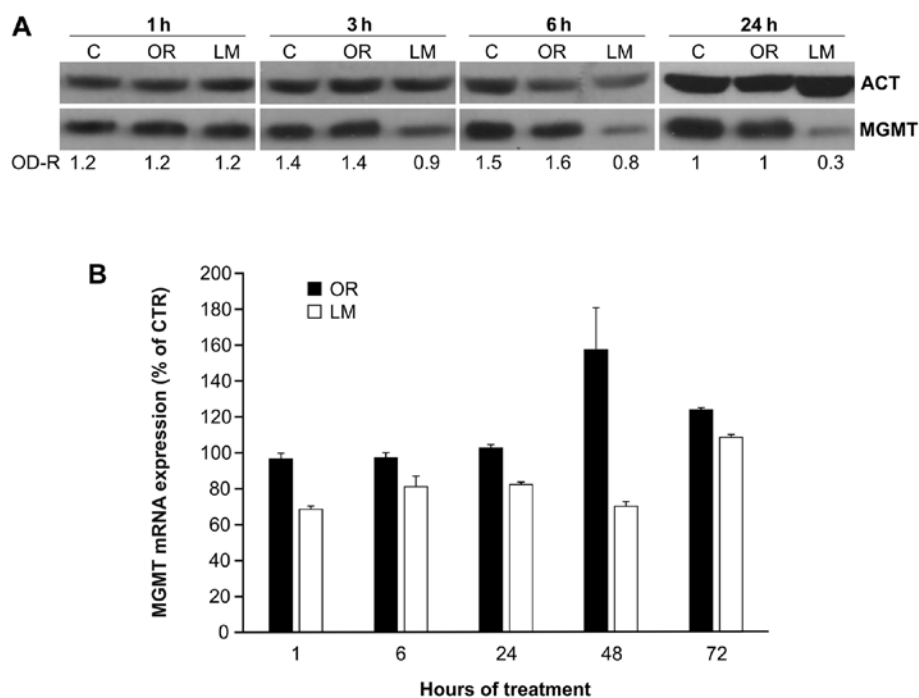
Figure 2. Time-course analysis of the effect of orlistat (OR, 40 μM) and lomeguatrib (LM, 10 μM) on MGMT protein and mRNA levels in Jurkat cells. (A) Western blot analysis. Jurkat cells were incubated with orlistat for the indicated time intervals and then analyzed for MGMT protein levels. The results were confirmed in two independent experiments. OD-R, ratio between the optical density of MGMT and actin (ACT) band measured in terms of arbitrary units. (B) Real-time RT-PCR. Jurkat cells were incubated with orlistat for the indicated time intervals and then analyzed for MGMT mRNA levels. Data are expressed in terms of percentage of MGMT mRNA expression of drug-treated cells with respect to control cells. Bars indicate standard error of the mean relative to 3 determinations per point. The amount of MGMT mRNA detected in target cells exposed to orlistat for 48 h was significantly higher than that of untreated control (P<0.01, according to Student's t-test analysis). Comparable results were obtained in one additional independent experiment.

Table I. Effect of orlistat or LM on MGMT enzymatic activity of Jurkat and HCT116 cells.

Cell line	Control (DMSO)[a]			Orlistat (40 μM)[a]				LM (10 μM)[a]	
	Exp 1	Exp 2	Mean (SE)[b]	Exp 1	Exp 2	Mean (SE)[b]	P-value[c]	Exp 1	Exp 2
Jurkat	641	592	616.5 (24.5)	527	543	535 (8)	<0.05	<1	<1
HCT116	102	108	105 (2.8)	102	105	103.5 (1.8)	NS	<1	<1

[a]Target cells were treated with DMSO (0.2% in culture medium) or the indicated concentration of orlistat or lomeguatrib (LM) for 2 h, washed and tested for MGMT enzymatic activity as described in Materials and methods. [b]MGMT activity is expressed in terms of fmoles of ^{3}H-methyl groups transferred per mg of protein in cell extract. Each value represents the mean of two independent experiments (Exp 1 and Exp 2). SE, standard error of the mean. [c]P, probability according to Student's t-test analysis performed comparing mean values relative to MGMT activity of orlistat-treated cells with that of untreated controls. NS, not significant.

illustrated in Fig. 3B, show that progressive decline of MGMT levels occurred during the time-course study in all groups treated with LM, alone or in combination with orlistat, with the combination being particularly active on day 2. However, since this type of experiment was conducted with target cells exposed to drugs for 24 h only, it was possible to detect a complete recovery of MGMT levels in the group treated with orlistat alone. On the contrary, the effect of LM, alone or in combination was fully detectable up to 6 days, as confirmed by other two experiments (data not shown).

Effects of orlistat combined with TMZ on MGMT expression. It is known that methylation of oxygen 6 of DNA guanine by endogenous or exogenous DNA methylating agents is

an important step in chemical carcinogenesis leading to mutation-mediated malignant transformation (19,20). MGMT is crucially involved in the physiological defenses against carcinogenesis consequent to methylation of O^6-guanine and undergoes inactivation and proteasome-mediated degradation after removing, in a stoichiometric reaction, methyl adducts from O^6-guanine (21). We therefore decided to investigate the effects of a combined treatment with low concentrations of a mono-methylating agent followed by orlistat on MGMT protein levels.

We directed our attention to TMZ, a triazene compound that is activated *in vitro* and is capable of inducing DNA O^6-guanine methyl adducts (20). This drug has been found recently to increase and accelerate tumorigenesis in intestinal
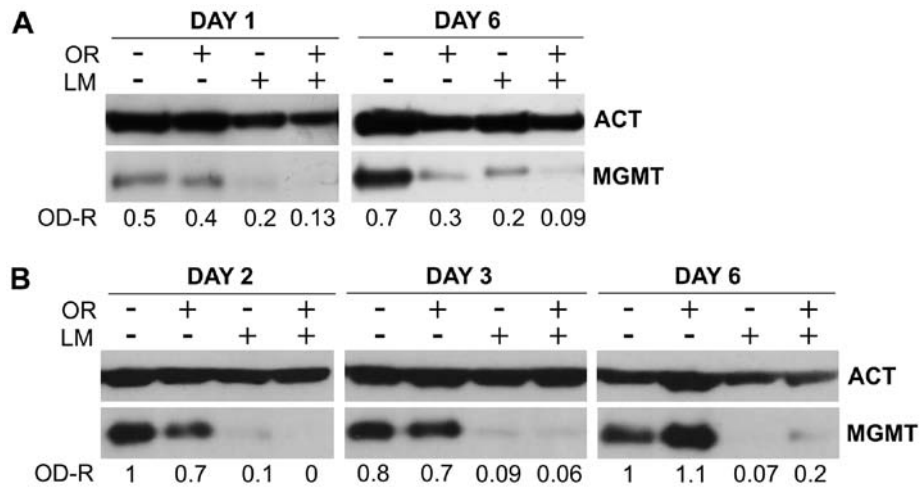
Figure 3. Effects of orlistat (OR, 40 μM) and lomeguatrib (LM, 10 μM), alone or in combination, on MGMT protein expression in normal NAMNC or Jurkat cells (for abbreviations, see legend of Fig. 1). (A) MGMT protein levels of NAMNC after 1 or 6 days of exposure to the agents alone or in combination. The results were confirmed in an additional experiment performed with a different NANMC batch. (B) Effect of treatment on MGMT protein levels of Jurkat cells. In this case target cells were exposed to the drugs for 24 h only. Thereafter leukemic cells were centrifuged, washed twice with culture medium alone, and cultured for additional 5 days in the absence of the drugs. On days 2, 3 and 6 of total culture time, aliquots of cell cultures were collected and processed for WB analysis. A similar experiment conducted with the same malignant cells provided comparable results.

cells of mice in a murine model of Lynch syndrome (22). Removal of TMZ-induced methyl adducts operated by MGMT is necessarily followed by depletion of the enzyme. Therefore, it is not surprising that TMZ downregulates the enzymatic activity (23) and the cellular levels of MGMT as a result of the DNA repair process.

In the first set of experiments, the ability of the triazene compound to downregulate the level of MGMT protein in a short-term assay was tested using HCT116 target cells that were incubated with graded concentrations of TMZ (40-320 μM) for 24 h. No influence of TMZ was detectable on MGMT levels when drug concentrations were lower than 160 μM (data not shown). At the concentration of 160 or 320 μM the drug provoked a marginal or a strong (close to 70%) downregulation of MGMT, respectively (Fig. 4A), thus confirming that TMZ was able to produce the expected enzymatic depletion in our model.

Drug combination experiments were performed on HCT116 cells that were exposed to TMZ (40 μM) for 24 h. Thereafter, the drug was removed by thorough washing and the cells were incubated for additional three days with 40 μM orlistat. The results confirm that MGMT protein levels were not affected by 40 μM TMZ, either when the protein amounts were evaluate at the end of drug treatment (Fig. 4B, day 1) or after three additional days of culture in the absence of the drug (Fig. 4B, day 4). Conversely, 3-day incubation with orlistat alone (i.e., not preceded by TMZ treatment) reduced MGMT by ~50% respect to untreated controls. Of particular interest is the finding that the group treated in sequence with TMZ and orlistat showed an overall reduction of MGMT protein level of approximately 75% with respect to untreated control. A similar set of experiments were performed with the colon cancer cell line HT-29 (Fig. 4C). In these studies, cancer cells were exposed to TMZ (40 μM) for 24 h, washed and incubated with orlistat (40 μM) for four days (i.e., from days 1-5). The results of WB analysis performed on day 1 show that TMZ, at the concentration of 40 μM, did not influence MGMT level. On day 5 of culture, HT-29 cells treated with 40 μM TMZ for 24 h, followed by
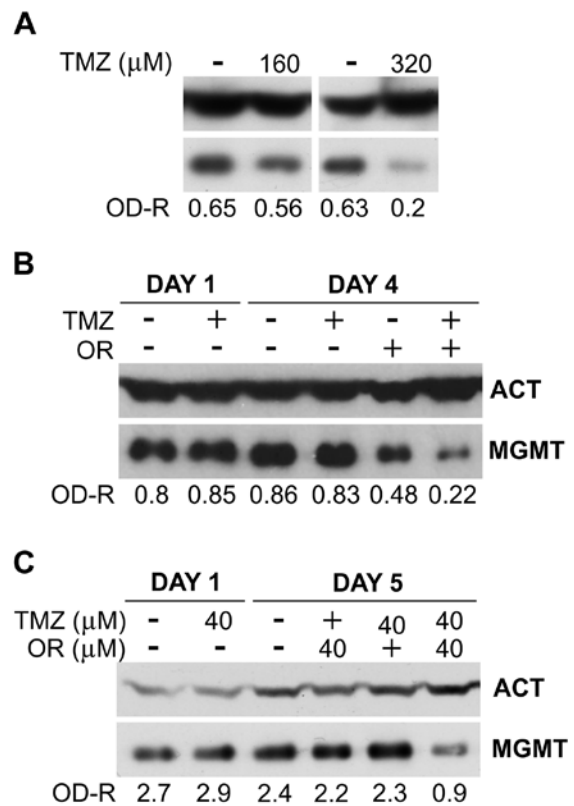

Figure 4. Effect of temozolomide (TMZ) or orlistat (OR, 40 μM), alone or in combination, on MGMT protein expression in colon cancer cells (for abbreviations, see legend of Fig. 1). (A) MGMT levels in HCT116 cells after 1 day of culture with TMZ alone. (B) Effects of TMZ and orlistat, alone or in combination, on MGMT protein levels in HCT116 cells. Target cells were cultured with medium alone or with TMZ (40 μM) for 24 h. Thereafter the cells were washed twice with medium alone, and cultured for additional 3 days with medium alone or with orlistat (40 μM). The results were confirmed in two additional independent experiments. (C) Effects of TMZ and orlistat, alone or in combination, on MGMT protein levels in HT-29 cells. Target cells were cultured in medium alone or with 40 μM TMZ for 24 h. Thereafter, the cells were washed twice with medium alone and cultured for additional 4 days with medium alone or with orlistat (40 μM). Similar results were obtained in one additional independent experiment.

4-day culture without drugs, did not display changes of MGMT protein levels. Orlistat alone, at the concentration of 40 μM produced marginal (~8%) inhibition, probably due to lower susceptibility to orlistat of HT-29 cell line with respect to that of HCT116 after 4-day incubation with the agent. However, most importantly, orlistat combined with TMZ at the same molar concentration (i.e., 40 μM), reduced MGMT levels by >60% respect to control, suggesting that the combined effects of the two agents on MGMT could be synergistic.

Discussion

The results of the present study reveal for the first time that orlistat downregulates MGMT protein expression, increases addictively the effects of a classical MGMT inhibitor such as LM and amplifies the MGMT suppression induced by a DNA monomethylating agent such as TMZ.

Alkylation and, in particular, methylation of O^6-guanine of DNA is a highly mutagenic event produced by either endogenous (24) or exogenous chemical compounds. If not repaired by MGMT, and in the absence of a functional mismatch repair system (25), the biochemical lesion is ignored and DNA synthesis proceeds leading to frequent G-C→A-T transitions, as described by Ito *et al* (26). The mismatch repair system is estimated to be of great relevance in the control of mutational events, being engaged in the rapid correction of the base substitutions that compromise fidelity of the new DNA strand generated during DNA synthesis (27). Therefore, failure of the functional activity of this enzymatic system is connected with a number of different pathologies including malignant transformation, that has been documented extensively in colon rectal cancer development in human subjects (28). It follows that both MGMT and mismatch repair play a remarkable role in the control of carcinogenesis affecting various target organs, with particular regard to the colorectal segment of the digestive tract. In any case, it is obvious that the presence of suitable levels of MGMT ensures striking protection against mutagenesis whenever O^6-methylguanine (O^6-MeG) is induced on DNA molecules (29), even in the absence of adequate levels of mismatch repair function. The MGMT protein behaves as an acceptor of the methyl adduct that is transferred in a stoichiometric reaction from DNA O^6-MeG to a cysteine residue associated with the active site of MGMT. It follows that the biochemical lesion is repaired, whereas MGMT is inactivated according to a sort of suicide process (21) leading to ubiquitination followed by proteasome-mediated degradation of the repair protein (17,30,31). Affected cells restore their MGMT levels through an entirely *de novo* synthesis of the protein. This mechanism helps in the understanding why the functional activity of the MGMT is rapidly suppressed by molecules that behave like pseudo substrates able to compete with DNA O^6-MeG for interaction with the repair protein. This is in line with the results of the present study that pointed out that LM, a potent competitor of O^6-MeG (32-34), suppressed entirely MGMT activity of Jurkat or of HCT116 cells within 2 h (Table I). On the other hand, the MGMT protein of Jurkat cells started to decline at 3 h or later, reaching low levels 24 h later, as shown by the WB assay (Fig. 2A).

In oncology the biological significance of MGMT has been considered from two opposite perspectives. The protein

has been considered responsible of malignant cell resistance against alkylating agents such as triazenes, being capable of eliminating drug-induced methyl adducts to DNA O^6-G. Consequently, agents able to suppress MGMT activity have been used in association with triazene compounds in the clinic (16,32-35); reviewed in ref. 36. On the contrary, the role of MGMT as a protective protein against not only endogenous, but also exogenous alkylating carcinogenic compounds (37) has been firmly established by a number of *in vivo* and *in vitro* investigations. Of particular interest is the observation that MGMT protects transgenic mice from N-methylnitrosourea-induced thymic lymphomas (38), or nitrosamine-induced hepatocarcinogenesis (39). This issue has been adequately discussed in a relatively recent review by Fahrer and Kaina (12) who highlighted the crucial role that could be played by MGMT in colon carcinogenesis secondary to nitrosamines. These molecules are well known carcinogenic compounds particularly present in a variety of preserved meat products (40).

The possibility that orlistat could be directly involved in colorectal carcinogenesis has been matter of debate for several years (9,10,41). However, no conclusive results have been obtained so far, although the finding that orlistat did not show carcinogenic potential (41) does not support the hypothesis that the agent is carcinogenic *per se*.

In the present study we found that *in vitro* exposure to orlistat at the concentration of 40 μM for at least two days is able to induce 30-70% reduction of MGMT protein in four different human neoplastic cell lines and NAMNC. However, particularly resistant to this biochemical effect of orlistat appears to be the human melanoma M10, as shown in Fig. 1D. No data are presently available to explain this differential behavior, that seems to deserve further investigation. In all experiments, although orlistat at the concentration of 40 μM produced a marked reduction of tumor cell growth, cell viability was never found to be <85%. Moreover, specificity of drug effect on MGMT protein expression was confirmed by the finding that the same samples utilized for WB analysis did not show any drug-induced downregulation of other cellular proteins that have been tested (e.g., calreticulin or HSP-90, data not shown).

At the present time we are not able to provide sufficient experimental data to disclose the mechanism underlying the effect of orlistat on MGMT protein expression. The finding that the compound is unable to downregulate consistently MGMT function within 2 h of treatment (Table I), does not support the hypothesis that orlistat, similarly to LM, could behave as pseudo-substrate able to bind and inactivate MGMT, followed by a proteasome-dependent degradation process (17,30,31).

Several studies have pointed out that orlistat is a potent FASN inhibitor (42). Moreover, FASN inhibition is associated with p53 upregulation (43-45), and p53 upregulation induces inhibition of MGMT expression (46). However, if one considers the defective p53 status of at least two tumor cell lines utilized in the present study (i.e., Jurkat and HT-29 cells), the hypothesis that orlistat acts on MGMT expression via p53 signaling does not appear to be sufficiently supported.

Independently from the mechanism of action of orlistat, its inhibitory effects on MGMT protein expression raise

the question of possible indirect carcinogenic effect of the compound through a reduction of surveillance against endogenous or exogenous chemical carcinogenesis. The finding that TMZ pretreatment amplifies markedly the activity of orlistat on MGMT is of particular interest. This *in vitro* active triazene compound, able to induce large amounts of DNA O^6-guanine methyl adducts, engages actively MGMT in its 'suicidal' repair function (21). However, in order to provoke a detectable reduction of the repair protein, TMZ must be used at very high concentrations (e.g., 320 μM to produce, an ~70% decline of MGMT protein level, at least in the HCT116 tumor line, as shown in Fig. 4A). Actually, at the concentration of 40 μM the triazene compound was not able to show a noticeable MGMT downregulation (Fig. 4B). Yet, when TMZ was followed by orlistat treatment, MGMT inhibition was found to be double with respect to that operated by orlistat alone suggesting a possible synergistic interaction. In addition, when MGMT was directly downregulated by a potent pseudo-substrate such as LM, addition of orlistat resulted in additively increased suppressive activity (Fig. 3). This means that orlistat could act in concert with other MGMT 'consuming' agents including those that are endowed with carcinogenic potential such as TMZ (22).

In conclusion, the preliminary data illustrated in this report appear to disclose an unexpected source of concern about the clinical use of orlistat. Actually, the administration modality of the drug in severely overweight subjects implies that daily treatment with high doses of this agent is carried out for a period of one or two years. At the present time, we are not able to evaluate the impact that a chronic downregulation of a DNA repair enzyme such as MGMT could have on host's surveillance against chemical carcinogenesis targeting digestive tract mucosa locally exposed to high concentrations of orlistat. Therefore, the studies illustrated in this report appear to provide the ground for casting a note of caution in the long-term clinical use of orlistat suggesting that appropriate control of the gastrointestinal apparatus appears to be unreservedly advisable.

Acknowledgements

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