O6-methylguanine DNA methyltransferase (MGMT) is a molecular determinant for potency of the DNA-epidermal growth factor receptor (EGFR) targeting combi-molecule ZRS1

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Abstract

To enhance the potency of current epidermal growth factor receptor (EGFR) inhibitors, we developed a novel strategy that seeks to confer them an additional DNA damaging function, leading to the design of drugs termed “combi-molecules”. ZRS1 is a novel combi-molecule that contains an EGFR tyrosine kinase targeting quinazoline arm and a methyltriazene-based DNA damaging one. We examined its effect on human tumour cell lines with varied levels of EGFR and O6-methylguanine DNA methyltransferase (MGMT). ZRS1 was more potent than the clinical methylating agent temozolomide (TEM) in all cell lines, regardless of their MGMT status. However, its potency was in the same range as or less than that of Iressa, an EGFR inhibitor, against MGMT-proficient cells. In the MGMT-deficient or in MGMT-proficient cells exposed to the MGMT inhibitor O6-benzylguanine, its potency was superior to that of Iressa and TEM or a TEM+Iressa combination. Cell signaling analysis in A549 (MGMT+) and A427 (MGMT-) showed that ZRS1 strongly inhibited EGFR phosphorylation and related signaling pathways. In addition, the p53 pathway was activated by DNA damage in both cell lines but apoptosis was significantly more pronounced in A427 cells. Using MGMT shRNA to block endogenous MGMT protein expression in A549 resulted in significant sensitization to ZRS1. Furthermore, transfection of MGMT into A427 greatly decreased the potency of ZRS1. These results conclusively demonstrate that MGMT is a critical molecular determinant for the full-blown potency of the dual EGFR-DNA targeting combi-molecule.
Introduction

The epidermal growth factor receptor (EGFR) is overexpressed in a large number of cancers including breast, colon and lung cancers, leading to aggressive tumor growth and poor patient prognosis (1, 2, 3). Another member of the EGF family of receptors, ErbB2 or Her2, is also frequently overexpressed in various cancer types (4). Ligand stimulation of EGFR results in activation of several downstream signalling pathways, including the RAS/RAF/MAPK and PI3K/Akt pathways. These pathways in turn regulate a variety of other proteins that are involved in cell differentiation, proliferation, motility and metastasis. Small molecule inhibitors have been designed to inhibit the tyrosine kinase activity of EGFR (5). Two such agents Iressa and Tarceva are now in use in the clinical management of lung cancer (6). However, resistance and low response rates to these inhibitors have been observed (5), suggesting that improvement of the potency of the current drugs or the design of new approaches constitutes an urgent need.

Over the past years, we developed a novel tumour targeting approach termed “combi-targeting” that sought to design molecules designated as “combi-molecules” to act as an EGFR tyrosine kinase inhibitor and a DNA-damaging agent (7, 8). This has inspired the design of other type of EGFR-DNA targeting molecules (9, 10). One such molecule, ZRS1 contains an EGFR tyrosine kinase targeting quinazoline arm and a methyltriazene-based DNA damaging one. Like its predecessors, under physiological conditions, ZRS1 is designed to undergo hydrolysis to generate an aminoquinazoline FD105 (an EGFR inhibitor) and a methyl diazonium that damages DNA (11). The DNA methylating pharmacophore (3-methyl-1,2,3-triazene) of ZRS1 is similar to that of clinical
chemotherapeutic methylating agents dacarbazine and TEM. As depicted in figure 1A TEM is hydrolyzed to 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC) which in turn is further converted to methyl diazonium and 4-amino-5-imidazole-carboxamide (AIC). Likewise, ZRS1 is hydrolyzed to release the methyl diazonium and an aromatic amine (FD105) (Fig. 1A). This common methylidiazonium species released by the two agents attacks DNA at the O6- or N7 positions of guanine and at the N3 position of adenine (12). N7 methylguanine and N3-methyladenine account for 80% of all damage whereas O6-methylguanine accounts for only 6 % (12). Despite the greater abundance of N7-methylguanine adducts, the cytotoxic effects of methylating agents are imputed to the O6-methylguanine adducts that generate mismatches with cytosine and thymine. It is believed that the futile attempt by the mismatch repair system to correct these lesions ultimately triggers apoptotic cell death (13). It should be noted that common assays designed to quantitate DNA damage induced by methylating agents require alkaline conditions to generate breaks at N7-methylguanine. The traditional alkaline elution assay (14) that consisted of comparing the rate of elution of DNA from control and treated cells on a polycarbonate filter is now widely replaced by the comet assay. This assay originally described by Olive et al. (15) consists of electrophoresing whole cell nuclei generated in situ on an agarose gel. O6- and N3-methyl adducts are primarily analyzed by liquid chromatography of DNA from cells treated with the radiolabeled alkylating agent.

The O6-methylguanine lesion, which is now widely accepted as the primary cytotoxic lesions induced by methylating agent, is efficiently repaired by the DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT) that removes the methyl adducts
from the O6 positions of guanine by transferring it to its internal cysteine residues, resulting in its own inactivation \(^\text{(16, 17)}\). Tumour expression of MGMT varies and correlates with therapeutic response to methyltriazene-based chemotherapy in the treatment of glioma \(^\text{(18)}\). It has been shown in many models that tumours expressing MGMT are remarkably resistant to methylating agents. To circumvent this problem, an inhibitor of MGMT termed O6-benzylguanine (BG) has been designed that significantly potentiates the action of temozolomide in xenograft models and is currently under clinical investigation \(^\text{(17, 19)}\).

Recently, in an attempt to increase the potency of methylating agents, we studied a novel strategy that seeks to confer them an EGFR inhibitory arm that can alleviate EGFR-mediated growth and anti-apoptotic signaling, thereby sensitizing the cells to the DNA methylating lesions regardless of their MGMT content. Previous studies by our group demonstrated the feasibility of the latter type of EGFR/DNA-directed agents termed “combi-molecules” and showed that their antiproliferative effect was superior to that of combinations of EGFR- and DNA targeting molecules \(^\text{(7, 8, 9)}\). However, little is known about the molecular mechanisms regulating the optimal potency of the combi-molecules. In the present study, we first examined the molecular events underlying the response to the dual actions of the novel stabilized combi-molecule ZRS1 and subsequently investigated the importance of the cytotoxic O6-methylguanine lesion in its mechanism of action by modulating its direct repair enzyme MGMT. We have chosen to perform the study in lung cancer cells, a type of tumour in which Iressa and other EGFR inhibitors are most commonly indicated \(^\text{(6)}\). The study focused on the lung cancer cell lines A549 and
A427, which were genetically engineered to provide two more variants: A427M, its MGMT transfectant and A549sh in which endogenous expression of MGMT was knocked down. Our hypotheses were strategically tested under isogenic conditions to prevent biases introduced by differences between cell lines. Importantly, the cells were selected on the basis of their p53 wild type status, which is required for activation of the DNA damage response pathway. It is now well known that in wild type p53 cells, activation of p53 in response to DNA damage is associated with a rapid increase in its levels, binding to DNA and transcriptional activation of a number of genes, the products of which trigger cell cycle arrest and apoptosis. One such product is Bax that migrates to the surface of the mitochondria to form pores that allow the release of cytochrome c and subsequent triggering of apoptosis. Thus the ideal mechanism by which, enhanced potency can be triggered by combi-molecules could be one in which it blocks both growth and antiapoptotic signaling mediated by EGFR while activating apoptosis through the p53-activated pathway. Under conditions where the action of the DNA damaging arm would be mitigated by DNA repair, one would expect the drug to exert potency only through its EGFR blocking arm. To test this hypothesis we used the MGMT expressing A549 lung cancer cells and abrogated MGMT in the latter both pharmacologically by exposing the cells to O6-benzylguanine or biologically by shRNA technology. Conversely, we used the MGMT-deficient A427 cells in which we challenge our hypothesis by restoring MGMT using gene transfection technology. Our results demonstrated that in EGFR and MGMT-proficient cells, despite being able to block EGFR signaling (MAPK and PI3K-AKT pathways), the potency of ZRS1 was comparable or equal to that of the EGFR-inhibitor Iressa. This was associated with its
remarkably delayed activation of the p53 pathway that resulted in low levels of apoptotic cell death. By contrast, in MGMT-deficient cells A427 or in MGMT-proficient cells A549 in which MGMT was pharmacologically or biologically suppressed (see A549sh), rapid activation of the DNA damage response and down-regulation of the PI3K pathway led to significantly high levels of apoptotic cell death. This is the first report on the optimal conditions for synergistic action of the two mechanistic arms of a methylating combi-triazene.

Materials and Methods

Cell Culture

The human tumor cell lines A549, A427 (lung carcinoma) and HT29 (colon carcinoma), MDA-MB-468 and MDA-MB-231 (breast carcinoma) were obtained from the American Type Culture Collection. The human tumor cell lines HCT116 and HCT116 (p53-/-) (colon carcinoma) were generous gifts from Dr. Janusz Rak from the Montreal Children Hospital (Montreal, Canada). All cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine and antibiotics (Wisent, St. Bruno, Canada). All cells were maintained in logarithmic growth and in monolayer culture at 37°C in a humidified atmosphere of 5% CO₂ in air.

Drug Treatment

Iressa was extracted from a Tablet provided by AstraZeneca. Temozolomide was extracted from a Temodal tablet from Schering Plough. ZRS1 was synthesized in our
laboratory according to known procedures described elsewhere. In all assays, drugs were dissolved in DMSO (dimethylsulfoxide) and further diluted in DMEM media before added to cells. The concentration of DMSO never exceeded 0.2% (v/v) during treatment.

**Antibodies and Reagents**

Anti-phosphotyrosine, anti-EGFR, anti-p53, anti-GADD45α, anti-MGMT and donkey anti-goat IgG-HRP antibodies were from Santa Cruz Biotechnology. Anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-AKT, anti-AKT, anti-phospho-Bad (Ser112), anti-phospho-Bad (Ser136), anti-Bad, anti-Bax antibodies were from Cell Signaling Technology. Anti-tubulin α antibody was from Neomakers and anti-actinin antibody was from Sigma Aldrich. Secondary goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were obtained from Jackson ImmunoResearch Laboratories.

**Growth Inhibition Assay**

Cells were plated in 96-well microtiter plates and the next day cells e exposed to different concentrations of a drug continuously for four days and stained with sulforhodamine B as previously described (8, 20). The resulting colored residue was dissolved in 200 μl Tris base (10 mM, pH 10.0) and optical density was recorded at 492 nm using a Bio-Rad microplate reader. The results were analyzed by GraphPad Prism (GraphPad Software, Inc., San Diego, CA) and the sigmoidal dose response curve was used to determine 50% cell growth inhibitory concentration (IC50). Each point represents the average of at least two independent experiments run in triplicate.
Western Blot Analysis

To evaluate the effect of ZRS1 on EGFR signalling pathway, cells were grown to 80% confluence in 6-well plates, serum starved for 24hrs (DMEM phenol red-free medium without FBS), followed by 2hr incubation with ZRS1 at the indicated concentrations. Cells were then stimulated with EGF (50 ng/ml) for 20 min and collected by scraping in PBS on ice, centrifuged and lysed in ice-cold lysis buffer for 30 min (20 mM Tris-HCl pH 7.5, 1% NP-40, 10 mM EDTA, 150 mM NaCl, 20 mM NaF, 1mM Na3VO4, protease inhibitor cocktail (Roche Molecular Diagnostics, Laval, Qc)). Lysates were analyzed by western blotting as previously described (8, 9). Membranes were incubated with antibodies coupled with horseradish peroxidase at room temperature for 1 h. Proteins were visualized by enhanced chemiluminescence (Super West Pico Chemiluminescent Substrate, Pierce). When the same blot was probed for another protein, the membrane was stripped in Restore Western Blot Stripping Buffer (Pierce) and probed with a secondary primary antibody.

Alkaline comet assay for quantitation of DNA damage

The alkaline comet assay was performed as previously described (7, 8). Comets were visualized at 10X magnification using Leica microscope after staining with SYBR Gold (1:10,000, Molecular Probes, Eugene, OR) for 45 min. DNA damage was quantified using Comet Assay IV software (Perceptive Instruments, UK) and the degree of DNA damage was expressed as a tail moment parameter (i.e., the distance between the barycenter of the head and the tail of the comet multiplied by the percentage of DNA within the tail of the comet (40). A minimum of 50 comets was analyzed for each drug.
treatment and the mean tail moments were calculated from at least two independent experiments.

**Generation of Stable Cell Lines**

MGMT plasmid (ORF clone that contains full-length of Homo Sapiens MGMT cDNA) was purchased from OriGene Technologies and transfected into A427, an MGMT-deficient cell line, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Transfected cells were then split at a 1:10 dilution into fresh growth medium 24 h after transfection and maintained in selective medium containing 750μg/ml G418 for stable clone selection. The resultant A427 subclone expressing MGMT was named “A427M”. MGMT-targeted short hairpin RNA (shRNA) vector was purchased from Santa Cruz Biotechnology and transfected into A549, an MGMT-proficient cell line, using Lipofectamine 2000 as described. Transfected cells were maintained similarly as described in selective medium containing 5μg/ml puromycin for stable clone selection. The resultant A549 subclone was named “A549sh”. Western blot analysis was used to evaluate MGMT expression.

**Flow Cytometry Analysis**

To determine cell cycle distribution, cells were grown in 6-well plates and then incubated with drugs for 24 h. Cells were then harvested, washed in PBS, fixed in cold 70% ethanol, and then stained with 50μg/ml propidium iodide. Samples were analyzed on a Becton Dickinson Flow Cytometer and cell cycle analysis was done with the ModFit software. To evaluate apoptosis, cells were grown in 6-well plates and then incubated with drugs
for 48 h. Cells were then collected, washed with PBS, centrifuged and subsequently incubated with Annexin V-FITC and propidium iodide using the apoptosis detection kit (BD Bioscience PharMingen). Data were collected using logarithmic amplification of both FL1 (FITC) and FL2 (propidium iodide) channels and quantitated by quadrant analysis of coordinate dot plots.

Results

ZRS1 exhibits the highest potency in an MGMT-deficient cell line

To examine the growth inhibitory potency of the novel combi-molecule ZRS1, its IC50 values were determined by the sulforhodamine B assay (20) as described in the Method section in a panel of human cancer cell lines, including the lung cancer cells (A549, A549sh, A427 and A427M), colon cancer [HT29, HCT116 and HCT116 (p53-/-)], and breast cancer (MDA-MB-468 and MDA-MB-231) cell lines. ZRS1 exhibited the highest potency in A427 cells (IC50= 0.5 µM), an MGMT-deficient cell line (Table 1A). In addition, ZRS1 was more potent than the clinical methylating agent temozolomide in all the cell lines tested regardless of their MGMT status (Table 1A). However, its potency was in the same range as or less than Iressa, a single-targeted EGFR inhibitor, against MGMT-proficient cells (Table 1A). Taking into account that ZRS1 is a combi-molecule with two divergent targets, its efficacy was compared with that of a combination of either equi-molar concentration (i.e. combination of the two drugs at equal concentration) or equi-effective dose of Iressa and temozolomide in A427 cell line. Our results demonstrated that the IC50 value of ZRS1 was 15-fold lower than that of equi-molar and equi-effective combinations of Iressa + temozolomide (Table 1B), indicating that in the
absence of MGMT, ZRS1 can induce stronger potency than Iressa (a monotargeted EGFR inhibitor), temozolomide (a monotargeted methylating agent) and their corresponding 2-drug combinations.

**ZRS1 inhibits EGFR-mediated signalling pathways**

To elucidate the molecular mechanism underlying the anti-proliferative potency of ZRS1, its effect on EGFR-mediated signalling pathways was examined at equi-dose in two lung cancer cell lines A549 (MGMT+) and A427 (MGMT-) expressing wild type p53. The examination was performed at equi-dose to clearly define at equivalent EGFR inhibitory strength the extent of inhibition of signal transduction in both cells. ZRS1 inhibited the EGF-stimulated EGFR phosphorylation in a dose-dependent manner at equal strength in both cell lines (Figure 1B and C). Likewise, this translated into the inhibition of the phosphorylation of two major EGFR downstream proteins, ERK1/2 and Akt, in a dose-dependent manner and at almost equal strength in both cells (Figure 1B and C). Bad is a proapoptotic member of the Bcl-2 family and its phosphorylation leads to the release of the antiapoptotic protein Bcl2 (21, 22). Since activation of ERK1/2 and Akt leads to phosphorylation of Bad at serine residues 112 and 136 respectively (21, 22), inhibition of phosphorylation of the latter residues was investigated. As shown in figure 1B and C, ZRS1 inhibited the phosphorylation of Bad (S112) and Bad (S136) in a pattern corresponding to the inhibition of EGFR, ERK1/2 and Akt. Inhibition of p-Bad-112 was slightly stronger in the A549 and in A427 cells. Taken together, our results strongly suggest that the quinazoline-based EGFR targeting arm of ZRS1 functions as an EGFR inhibitor and suppresses both the growth signalling and anti-apoptotic pathways.
stimulated by EGFR activation in these cells. Overall, little difference was observed in the strength of inhibition of EGFR-mediated signal transduction by ZRS1 in the two cell types, indicating that as expected, MGMT expression has no significant influence on the action of the EGFR inhibitory arm of the combi-molecule.

**ZRS1 induces DNA damage but differentially affects proteins involved in the DNA damage response in A549 and A427**

To examine whether or not the methyltriazene-based DNA damaging arm of ZRS1 induces DNA damage, A549 and A427 cells were treated with ZRS1 at various concentrations for 2hrs and then alkaline comet assay was used to evaluate the DNA damage. It should be noted that the purpose of this experiment was to detect the levels of DNA damage induced by ZRS1 in both cell lines. Breaks detected by the comet assay are primarily due to N7-methylguanine under the alkaline condition of the assays as previously reported by Lacoste et al. (23). The analysis was performed 2 h after drug treatment to minimize the impact of any DNA repair mechanism. In both cell lines, ZRS1 induced significant DNA damage at concentrations as low as 6 µM, and the extent of DNA damage was dose-dependent (Figure 2A and 2B). These results indicated that ZRS1 was capable of inducing significant levels of DNA damage in the two cell types. While the latter analysis was performed at equi-doses to determine the extent of DNA lesions after a 2-h drug exposure, due to the marked difference in sensitivity between the two cell types to ZRS1 (ZRS1 IC50 growth inhibition, A549-16.6 µM, A427-0.5 µM), the analysis of p53 activation and transactivated proteins was performed at equi-effective doses at 4, 24, 48 hr. In both cell lines, ZRS1 induced accumulation of p53 in a dose-
dependent manner (Figure 2C and 2D). However, much lower concentrations of ZRS1 were able to induce more noticeable changes in p53 in A427 (0.5 µM) than in A549 (15 µM). Interestingly, accumulation of p53 did not lead to accumulation of p21 in both cell lines (unpublished data).

Furthermore, we focused our analysis on two important proteins that characterize the DNA damage response: as Bax and GADD45 (growth arrest and DNA damage-inducible gene) (24, 25, 26). Like Bad, Bax is a member of the Bcl-2 family and it promotes apoptosis in response to DNA damage (24, 25). GADD45 is a p53-regulated stress protein that plays an important role in cell cycle checkpoint following exposure to certain types of DNA damaging agents such as UV irradiation and alkylating agents (26). Within 4hrs, ZRS1 induced accumulation of both Bax and GADD45 in A427 in a dose-dependent manner (Figure 2D), while Bax and GADD45 were barely detectable in A549 cells (Figure 2C). Therefore, the effect of ZRS1 on these proteins was further examined after 24hrs or 48hrs treatment. As shown in Figure 2, the protein level of Bax became evident only after 48hrs and GADD45 became noticeable after 24hrs in A549. In contrast, the levels of both proteins were induced as early as 4 h and increased in a dose- and time-dependent manner in A427 cells, indicating that the DNA damage response pathway was more rapidly and more strongly activated in the sensitive MGMT-deficient A427 cells than in MGMT-proficient A549 and leads to transactivation of growth arrest GADD45 and pro-apoptotic Bax protein.

**ZRS1 induces significantly higher levels of apoptosis in the MGMT-deficient cells**
Given that multiple proteins that were affected in response to ZRS1-induced DNA damage play critical roles in cell cycle checkpoint and apoptosis, the effect of ZRS1 on cell cycle distribution and apoptosis was examined in A549 and A427 at equi-doses to assess the strength of the arrest in both cell types. In both cell lines, ZRS1 caused G2/M arrest after 24hr-treatment (Figure 3A-3B). However, this effect could only be seen at high doses for the MGMT-proficient A549 cells (15-30 μM). In contrast, for the MGMT deficient A427 cells, cell cycle perturbation was observed at concentrations as low as 0.5 μM, with an S-phase arrest shifting to G2M arrest in a dose-dependent manner. Importantly, ZRS1 induced significant cell death by apoptosis in A427 cells after 48hr-treatment in a dose-dependent manner (Figure 3C, filled columns). In contrast, within the same period (48 h), it did not trigger cell death by apoptosis in the MGMT+ A549 cells (Figure 3C, empty columns), indicating that MGMT may play a significant role in blocking the onset of events that lead to apoptosis.

**MGMT modulates the efficacy of DNA damaging arm of ZRS1**

To investigate the molecular mechanisms underlying the difference between the efficacy of ZRS1 in A549 and A427, we hypothesize that it may strictly depend on the DNA repair protein MGMT. The growth inhibitory effects of ZRS1 in the absence and presence of O6-benzylguanine (O6-BG) were compared in the MGMT-proficient cell lines A549 using the SRB assay. The IC50 value of ZRS1 in A549 was dramatically decreased to 0.7 μM (from 15.6 μM) and similarly, exposure to O6-BG considerably decreased the IC50 values of temozolomide in A549 (Figure 4A). However, exposure to O6-BG did not affect the efficacy of Iressa (Figure 4A). Next, we investigated the
molecular events associated with the potentiation of ZRS1 by O6-BG in A549 by probing the DNA damage response proteins induced following its administration to tumour cells. A549 cells were pretreated with O6-BG for 12hrs and then exposed to various concentrations of ZRS1 for only 4hrs. As expected, not only the protein level of p53 but also those of Bax and GADD45 increased in a dose dependent manner after exposure to ZRS1 (Figure 4A), suggesting that the DNA repair activity of MGMT is involved in the delayed activation of the DNA damage response pathway and subsequent triggering of apoptosis.

To further confirm our hypothesis that MGMT antagonized the cytotoxicity of the DNA damaging arm of the combi-molecule ZRS1, we stably transfected the MGMT-proficient cell line A549 with MGMT shRNA to knock down endogenous expression of MGMT, leading to the resultant cell line A549sh. Western blot analysis showed that the expression level of MGMT was significantly reduced in A549sh as compared with wild type cell line (Figure 4B). The anti-proliferative ability of ZRS1 in A549sh was tested using SRB assay and our results showed that the IC50 values of both ZRS1 and temozolomide decreased by 2-fold in A549sh when compared with A549 (Figure 4B). However, the IC50 of Iressa remained in the same range (Figure 4B).

Our results showed that knockdown of MGMT expression enhanced the anti-proliferative effect of the two methylating agents ZRS1 and temozolomide. Conversely, we stably transfected the MGMT-deficient cell line A427 with MGMT cDNA to express MGMT, leading to the resultant cell line A427M. Western blot analysis confirmed that MGMT
was detected in A427M (Figure 4C). The results showed that the IC50 values of both ZRS1 and temozolomide dramatically increased (Figure 4C), suggesting that MGMT expression dampened the ability of the two methylating agents to inhibit cell growth.

Finally, we examined the cell cycle distribution and apoptosis induced by ZRS1 in these two stable cell lines. ZRS1 caused G2/M arrest in both cell lines (Figure 5A-5B). However the arrest was significantly less in the MGMT transfectant. In addition, ZRS1 caused stronger G2/M arrest in A549sh than its parental A549 cell line (Figure 3A and 5A). Likewise, ZRS1 induced stronger G2/M arrest in the MGMT-deficient A427 cell line than its A427M transfectant (Figure 3B and 5B). Importantly, MGMT transfection suppresses the ability of ZRS1 to induce apoptosis in A427 cells (Figure 5C, empty columns versus Figure 3C filled columns). Conversely, depletion of MGMT levels by shRNA transfection confers the ability to induce significant levels of apoptosis in A549 cells after 48hr treatment (Figure 5C, filled columns versus Figure 3C, empty columns).

Experiments in the wild type, transfected and shRNA knocked down cells, allowed us to define a growth inhibitory profile for ZRS1, TEM and Iressa in the presence or depletion of MGMT in cells with p53 wild type. As outlined in figure 4D-E, ZRS1 was consistently more potent than TEM (20 ~ 26 fold) in all the cells independently of their MGMT status. However, in the presence of MGMT, the growth inhibitory potency of ZRS1 against these cells was comparable or equal to that of Iressa. More importantly, as depicted in 4D-E, even under conditions where MGMT is depleted or absent, ZRS1 was consistently more potent than TEM or Iressa, an indirect indication that when MGMT is absent both
the EGFR inhibitory and DNA damaging arm add to the cytotoxic or growth inhibitory potency of ZRS1.

Discussion

Over the past few years, we demonstrated the feasibility of a novel tumour targeting strategy termed “combi-targeting” that seeks to develop novel drugs termed “combi-molecule” capable of blocking growth factor-mediated signaling while inducing cytotoxic DNA damage. Combi-molecules such as SMA41 and RB24 have been shown to block EGFR and to methylate DNA (7, 27). Although numerous studies from our group have confirmed the dual actions of combi-molecules (7, 8, 9), little is known about the optimal condition under which they exhibit their optimal potency. With the purpose of increasing their stability, we recently reported in a stability optimization study, the synthesis of ZRS1 carrying an acetoxymethyl carbamate moiety that stabilizes the triazene moiety (11). Thus, for the current study, we chose to test our hypothesis with ZRS1, a stable and water soluble combi-molecule. We thoroughly investigated herein the molecular mechanisms underlying the dual actions of ZRS1 and attempted to determine conditions under which it can exert its optimal potency.

The ability of ZRS1 to induce DNA damage and to block EGFR phosphorylation in the cells under study was a *sine qua non* of further mechanistic study. Thus, we first demonstrated that it could induce DNA damage using the comet assay and block EGFR-mediated signalling. Also, we demonstrated that the potency of ZRS1 was in the same range or less than that of Iressa, a clinical EGFR-targeting kinase inhibitor, in all cell
lines, except in the A427 and the A549sh cells in which MGMT was depleted (Fig. 4D-E). The A427 cell line does not express the DNA repair enzyme MGMT and harbours EGFR wild type (28, 29). More importantly, its p53 status is wild type (30), suggesting that its DNA damage response pathway may be intact. Thus we investigated the mechanism underlying its activity in the latter cell line and compared the observed signalling events with those triggered in A549, a methylating-agent-resistant lung cancer cell line with a similar characteristics (e.g. EGFR proficient and p53 wild type status), but expressing MGMT (30, 31, 32). Genetic engineering was used to decrease MGMT activity in A549 and increase it in A427. The activity of MGMT in A549 could also be depleted with O6-BG. Cellular engineering and pharmacological inhibition have served herein as a powerful platform to resolve a long standing challenge posed by the dissection of the contribution of individual arms to the overall cytotoxicity of methylating combimolecules. While EGF-induced activation of EGFR has now been shown to induce DNA repair enzymes ERCC1, XRCC1 and DNA PK (32, 33), from the current study, it can be inferred that there is no such signalling link between EGFR activation and MGMT activity or expression. ZRS1 inhibited EGFR and Erk1/2 phosphorylation in both cell lines but its activity was significantly reduced in the MGMT-proficient one, indicating that EGFR inhibition was not associated with depletion of MGMT activity or simply that EGFR blockade and DNA damage in the latter cells are not synergistic events for cell-killing. Interestingly, the IC50 values for ZRS1 against A549 cells (15.6 μM) provided a measure of the antiproliferative impact of EGFR blockade: it was in the same range (18 μM) as that of the single-targeted clinical inhibitor Iressa in these cells. More importantly, when A427 cells were transfected with MGMT, the IC50 was brought to the same range
as that of Iressa, and conversely when MGMT was pharmacologically abrogated in A549, IC50 values reached the same levels as that against A427 cells [0.5 μM (A427 wild type, 0.7 μM (A549+O6-BG), Table 1]. This convincingly suggests that MGMT is the main abrogator of the potency of the DNA damaging arm or indirectly that O6-methylguanine is the critical cytotoxic adduct that mediates the cytotoxicity of the DNA targeting arm of the combi-molecule. Importantly, one would be tempted to consider the significant potency of ZRS1 under these conditions to be solely due to depletion of MGMT. However, the fact that both pharmacological and biological abrogation of MGMT in A549 cells did not bring the activity of methylating agent TEM to the same levels as ZRS1 in these cells is a strong counterargument (See figure 4E). It is also to be noted that the potency of ZRS1 in the absence of MGMT was even stronger than equimolar and equieffective combinations of temozolomide and Iressa (Table 1B), suggesting that the subcellular distribution of the combi-molecule could also play a role in its increased potency. Previous studies suggest that the preferential perinuclear distribution of combi-molecules enhanced the levels of DNA damage that they induce (34). The relationship between biodistribution and potency of combi-molecules is discussed elsewhere and is beyond the scope of the current study.

The suppression of EGFR, ERK1/2 and Akt phosphorylation by ZRS1 led to further inhibition of the phosphorylation of downstream protein Bad, indicating that it alleviates the notorious anti-apoptotic effect activated through the PI3K pathway (35). Bad is a pro-apoptotic member of Bcl-2 family. However, survival factors inhibit the apoptotic activity of Bad by phosphorylation it at serine 112 and serine 136 (21, 22, 35). Inhibition
of phosphorylation at the latter two residues by ZRS1 strongly suggests that it can alleviate anti-apoptotic signalling through its EGFR inhibitory arm. More importantly, through the p53-mediated DNA response pathway, ZRS1 induced the pro-apoptotic protein Bax. By contrast, in A549, where the biological effects of DNA methylation were mitigated by MGMT, Bax expression was dramatically delayed. The mechanism of DNA repair by MGMT is based on its ability to remove the methyl group of O6-methylguanine by transferring onto its own cysteine residue. In the absence of MGMT, the O6-methylguanine lesion persists and attempt to repair it by the mismatch repair (MMR) is known to be responsible for triggering apoptosis (13, 36). The A549 and A427 are MMR proficient cells (37, 38). By significantly alleviating the EGFR-mediated antiapoptotic signaling, activation of the DNA damage response pathway as indicated by p53, Bax, GADD45 induction and cell cycle arrest, ZRS1 was able to trigger full-blown apoptosis, leading to submicromolar potency in these cells.

In summary, our study demonstrates that in the presence of MGMT, the growth inhibitory activity of ZRS1 is in the same range as Iressa, with no apparent contribution of the DNA damaging arm. In contrast, as outlined in figure 5D, in the absence of MGMT, its ability to not only block the EGFR-MAPK and EGFR-PI3K pathways (Target 1) but also activate p53 pathway and Bax expression (Target 2), translates into significant cell killing by apoptosis. Our current finding provides insight into the optimal conditions under which our methylating combi-molecules exert their potency: intact EGFR pathway, intact DNA damage response and absence of EGFR-independent DNA repair protein such as MGMT that directly repairs the cytotoxic lesions. This analysis will
lead to a more rational development and choice of tumours for which this novel class of 
drugs can be indicated.

**Abbreviations**

EGFR: epidermal growth factor receptor; MGMT: O6-methylguanine DNA 
 methyltransferase; TEM: temozolomide; shRNA: short-hairpin RNA; O6-BG: O6- 
benzylguanine

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fellowship to Ying Huang.
References


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Figure legends

Figure 1. Decomposition of ZRS1 and effect of ZRS1 on EGFR-mediated signaling pathways. A. Under physiological condition, the combi-molecule ZRS1 undergoes hydrolysis, releasing a methylidiazonium ion (a DNA methylating species) and FD105 (an inhibitor of EGFR) B. A549 cells were serum starved for 24hrs and subsequently incubated with the indicated concentrations of ZRS1 for 2hrs before stimulation with 50ng/ml EGF for 15min. C. A427 cells treated as in B.

Figure 2. Comparison of the effect of ZRS1 on DNA damage response in A549 and A427. A. A549 cells were treated with the indicated concentrations of ZRS1 for 2hrs followed by assessment of drug-induced DNA damage using alkaline comet assay. Tail moments were calculated by Comet IV software (Perceptive Instruments). Each point represents the average of at least 50 comet tail moments for each concentration from two independent experiments and normalized to base comet tail moment at 0µM. B. A427 cells were treated with the indicated concentrations of ZRS1 for 2hrs DNA damage measured as described in A. C. A549 cells were treated with indicated concentrations of ZRS1 for 4, 24 or 48hrs and lysates analysed by western blotting. D. A427 cells were treated with indicated concentrations of ZRS1 for indicated time points and cell lysates were analyzed by western blot as described in C.

Figure 3. Comparison of the effect of ZRS1 on cell cycle distribution and apoptosis in A549 and A427 cells. A. A549 cells incubated with the indicated concentrations of ZRS1 for 24hrs and processed for flow cytometry as described in Materials and Methods.
Proportions of cells in each cell cycle phase were mean ± s.e.m. of at least two independent experiments run in duplicate. One representative image of cell cycle profile at ZRS1-0 µM and ZRS1-15 µM is shown respectively. Arrows indicate G2/M. **B.** A427 cells were treated with the indicated concentrations of ZRS1 for 24hrs and analyzed as in A. One representative image of cell cycle profile at ZRS1-0 µM and ZRS1-15 µM is shown respectively. Arrows indicate G2/M. **C.** A549 cells (empty columns) and A427 cells (filled columns) were incubated with ZRS1 at the indicated concentrations for 48hrs and subsequently analyzed by flow cytometry with Annexin V-FITC/propidium iodide staining to assess cell death. Results were mean ± s.e.m. of at least two independent experiments. *, p < 0.05; **, p < 0.01.

Figure 4. Modulation of the efficacy of ZRS1 by MGMT. **A.** (left panel) A549 cells were treated with 20µM O6-BG continuously for four days. The IC50 values were determined by SRB assay and represented as mean ± s.e.m. of at least two independent experiments run in triplicate. (right panel) A549 cells were pretreated with 20µM O6-BG for 12hrs and exposed to ZRS1 at the indicated concentrations for 4hrs. Cell lysates were subjected to western blot analysis using specific antibodies. **B.** (upper panel) A549 cells were stably transfected with MGMT-shRNA, resulting in A549sh cell line, and western blot analysis was used to evaluate MGMT expression. (lower panel) The anti-proliferative effects of ZRS1, Iressa and Temozolomide on A549sh cells were evaluated by SRB assay and the IC50 values were mean ± s.e.m. of at least two independent experiments run in triplicate. **C.** (upper panel) A427 cells were stably transfected with MGMT-DNA, resulting in A427M cell line, and western blot analysis was used to
evaluate MGMT expression. (lower panel) The anti-proliferative effects of ZRS1, Iressa and Temozolomide on A427M cells were evaluated by SRB assay and the IC50 values were mean ± s.e.m. of at least two independent experiments run in triplicate. D. IC50 values of ZRS1 were summarized and compared to IC50 values of Iressa and Temozolomide in wild type cell line A549, pharmacologically treated A549(O6-BG) and biologically engineered A549sh. Arrows highlights the IC50 values of ZRS1. E. IC50 values of ZRS1 were summarized and compared to IC50 values of Iressa and Temozolomide in wild type cell line A427 and biologically engineered A427M. Arrows highlights the IC50 values of ZRS1.

**Figure 5. Comparison of the effect of ZRS1 on cell cycle distribution and apoptosis on A549sh and A427M cells.** A. A549sh cells incubated in the absence (control) or presence of 15µM or 30µM ZRS1, were processed for flow cytometry as described in Materials and Methods. Proportions of cells residing in each cell cycle phase were mean ± s.e.m. of at least two independent experiments run in duplicate. B. A427M cells incubated in the absence (control) or presence of 15µM or 30µM ZRS1 and cell cycle analyzed as in A. Data represent mean ± s.e.m. of at least two independent experiments run in duplicate. C. A549sh cells (filled columns) and A427M cells (empty columns) were incubated with ZRS1 for 48hrs and then analyzed by flow cytometry with Annexin V-FITC/propidium iodide staining to assess cell death. Results were mean ± s.e.m. of at least two independent experiments. *, p < 0.05; **, p < 0.01. D. Schematic representation of the pathways underlying the optimal potency of ZRS1. O6-MeG represents the O6-methylguanine DNA lesion.
**Table 1A.** Anti-proliferative effect of ZRS1 on isogenic and established cancer cell lines.

<table>
<thead>
<tr>
<th>Cancer Cell Lines</th>
<th>IC50(μM) of ZRS1</th>
<th>IC50(μM) of Iressa</th>
<th>IC50(μM) of Temozolomide</th>
<th>MGMT Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549 (lung)</td>
<td>15.6 ± 0.1</td>
<td>18.3 ± 1.6</td>
<td>321.0 ± 10.3</td>
<td>+</td>
</tr>
<tr>
<td>A549sh (lung)</td>
<td>6.8 ± 1.7</td>
<td>17.1 ± 2.1</td>
<td>139.3 ± 23.3</td>
<td>-</td>
</tr>
<tr>
<td>A427 (lung)</td>
<td>0.5 ± 0.0</td>
<td>5.9 ± 0.2</td>
<td>12.4 ± 3.0</td>
<td>- -</td>
</tr>
<tr>
<td>A427M (lung)</td>
<td>10.0 ± 1.6</td>
<td>8.2 ± 0.9</td>
<td>348.3 ± 31.5</td>
<td>+</td>
</tr>
<tr>
<td>HT29 (colon)</td>
<td>28.4 ± 2.7</td>
<td>8.6 ± 0.3</td>
<td>630.9 ± 15.3</td>
<td>+</td>
</tr>
<tr>
<td>HCT116 (colon)</td>
<td>24.4 ± 0.9</td>
<td>8.8 ± 0.1</td>
<td>406.9 ± 31.0</td>
<td>+</td>
</tr>
<tr>
<td>HCT116 (p53-/-) (colon)</td>
<td>22.0 ± 1.0</td>
<td>17.0 ± 0.3</td>
<td>422.1 ± 30.2</td>
<td>+</td>
</tr>
<tr>
<td>MDA-MB-468 (breast)</td>
<td>5.2 ± 0.2</td>
<td>4.9 ± 1.0</td>
<td>110.3 ± 25.2</td>
<td>+</td>
</tr>
<tr>
<td>MDA-MB-231 (breast)</td>
<td>39.3 ± 1.8</td>
<td>29.9 ± 0.1</td>
<td>174.9 ± 24.2</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) MGMT-proficient  
(-) depleted by MGMT-targeted shRNA  
(-->) MGMT null

**Table 1B.** Comparison of the efficacy of ZRS1 with the efficacy of Iressa and Temozolomide combination in A427 lung cancer cell line.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC50(μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZRS1</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>Iressa + Temozolomide</td>
<td>7.4 ± 0.4</td>
</tr>
<tr>
<td>(equal concentration)</td>
<td></td>
</tr>
<tr>
<td>Iressa + Temozolomide</td>
<td>8.1 ± 0.1</td>
</tr>
<tr>
<td>(equal efficacy)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.

A. 

TEM \xrightarrow{+H_2O \text{-CO}_2} MTIC \xrightarrow{} AIC

ZRS1 \xrightarrow{\text{H}_2\text{O}} \text{H}_3\text{C}^+\text{N}==\text{N}\xrightarrow{\text{-AcO}^-, \text{-H}_2\text{CO} \text{-CO}_2} \text{DNA}\xrightarrow{} \text{EGFR} \text{FD105}

B. A549

<table>
<thead>
<tr>
<th></th>
<th>-EGF</th>
<th>+EGF</th>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.25</td>
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<tr>
<td>ZRS1(\mu M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-EGFR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total EGFR</td>
<td></td>
<td></td>
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<tr>
<td>p-ERK1/2</td>
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<tr>
<td>Total ERK1/2</td>
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<tr>
<td>p-Akt</td>
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<tr>
<td>Total Akt</td>
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<td></td>
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<tr>
<td>p-Bad (S112)</td>
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<tr>
<td>Total Bad</td>
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<td></td>
</tr>
<tr>
<td>Tubulin</td>
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</tr>
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</table>

C. A427

<table>
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<th>-EGF</th>
<th>+EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.25</td>
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<tr>
<td>ZRS1(\mu M)</td>
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<td>p-EGFR</td>
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<td>Total EGFR</td>
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<td>Total ERK1/2</td>
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<td>p-Akt</td>
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<tr>
<td>Total Akt</td>
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<tr>
<td>p-Bad (S112)</td>
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<tr>
<td>Total Bad</td>
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<td></td>
</tr>
<tr>
<td>Tubulin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.

A. **A549**

![Graph showing tail moment % of control for varying ZRS1 concentrations (µM).](image)

B. **A427**

![Graph showing tail moment % of control for varying ZRS1 concentrations (µM).](image)

C. **A549**

![Western blot images for 4 hrs, 24 hrs, and 48 hrs showing p53, Bax, GADD45, and Actinin levels with varying ZRS1 concentrations (µM).](image)

D. **A427**

![Western blot images for 4 hrs, 24 hrs, and 48 hrs showing p53, Bax, GADD45, and Actinin levels with varying ZRS1 concentrations (µM).](image)
Figure 3.

A. A549

B. A427

C. A549 and A427 death by apoptosis

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Figure 4.

A. Table showing IC50 values for ZRS1, Iressa, and Temozolomide in A549 and A549 treated with O6-BG.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC50 (μM) in A549</th>
<th>IC50 (μM) in A549 with O6-BG</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZRS1</td>
<td>15.6 ± 0.1</td>
<td>7 ± 0.1</td>
</tr>
<tr>
<td>Iressa</td>
<td>18.3 ± 1.6</td>
<td>17.5 ± 0.4</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>321.0 ± 10.3</td>
<td>18.4 ± 1.5</td>
</tr>
</tbody>
</table>

B. Immunoblot showing expression of MGMT and Tubulin in A549 and A549sh.

C. Table showing IC50 values for ZRS1, Iressa, and Temozolomide in A427 and A427M.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 (μM) in A427</th>
<th>IC50 (μM) in A427M</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZRS1</td>
<td>0.5 ± 0.0</td>
<td>10.0 ± 1.6</td>
</tr>
<tr>
<td>Iressa</td>
<td>5.9 ± 0.2</td>
<td>82.0 ± 0.9</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>12.4 ± 3.0</td>
<td>348.3 ± 31.5</td>
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</tbody>
</table>

D. Graph showing IC50 values for ZRS1, Iressa, and Temozolomide in A549, A549(O6-BG), and A549sh.

E. Graph showing IC50 values for ZRS1, Iressa, and Temozolomide in A427 and A427M.
Figure 5.

A. **A549sh**

<table>
<thead>
<tr>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
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<tbody>
<tr>
<td>% of total cell cycle phase</td>
<td>% of total cell cycle phase</td>
<td>% of total cell cycle phase</td>
</tr>
<tr>
<td>concentration (μM)</td>
<td>concentration (μM)</td>
<td>concentration (μM)</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
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<tr>
<td>15</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>30</td>
<td>50</td>
<td>50</td>
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</table>

B. **A427M**

<table>
<thead>
<tr>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of total cell cycle phase</td>
<td>% of total cell cycle phase</td>
<td>% of total cell cycle phase</td>
</tr>
<tr>
<td>concentration (μM)</td>
<td>concentration (μM)</td>
<td>concentration (μM)</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>30</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

C. **C.20**

<table>
<thead>
<tr>
<th>A427M</th>
<th>A549sh</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of cell death by apoptosis</td>
<td>% of cell death by apoptosis</td>
</tr>
<tr>
<td>concentration (μM)</td>
<td>concentration (μM)</td>
</tr>
<tr>
<td>0.0</td>
<td>0.5</td>
</tr>
<tr>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td>3.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

D. **D.0.0**

Ligand (EGF, TGF-α)

Cell membrane

PI3K

AKT

ANTIOPTOSIS

BAD

Bcl-XL

BAX

APOTOPSIS

GADD45

CELL CYCLE ARREST

P53

Phosphorylation and Activation of Transcription Factors

06 mg

06 mg

Raf1

MEK

MAPK

ZRS1

Target 1

Target 2

O6 MeG

O6 MeG

CELL PROLIFERATION

Phosphorylation and Activation of Transcription Factors

06 mg

06 mg

Raf1

MEK

MAPK

ZRS1

Target 1

Target 2

O6 MeG

O6 MeG

CELL PROLIFERATION

Phosphorylation and Activation of Transcription Factors

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06 mg

Raf1

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MAPK

ZRS1

Target 1

Target 2

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O6 MeG

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06 mg

Raf1

MEK

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Target 1

Target 2

O6 MeG

O6 MeG

CELL PROLIFERATION

Phosphorylation and Activation of Transcription Factors

06 mg

06 mg

Raf1

MEK

MAPK

ZRS1

Target 1

Target 2

O6 MeG

O6 MeG

CELL PROLIFERATION
Molecular Cancer Research

O6-methylguanine DNA methyltransferase (MGMT) is a molecular determinant for potency of the DNA-epidermal growth factor receptor (EGFR) targeting combi-molecule ZRS1

Ying Huang, Zakaria Rachid and Bertrand J Jean-Claude

Mol Cancer Res  Published OnlineFirst January 24, 2011.

Updated version  Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-10-0407

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