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A bioanalytical investigation on the exquisitely strong *in vitro* potency of the EGFR–DNA targeting type II combi-molecule ZR2003 and its mitigated *in vivo* antitumour activity

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ABSTRACT

ZR2003 is a type II of combi-molecule designed to target DNA and the epidermal growth factor receptor (EGFR) without requirement for hydrolysis. In human tumour cell lines cultured as monolayers, it showed 6.5–35 fold greater activity than Iressa. Further evaluation in 3D organ-like multilayer aggregates showed that it could block proliferation at submicromolar level. However, despite the superior potency of ZR2003 over Iressa *in vitro,* its activity xenograft models was not significantly different from that of Iressa. To rationalize these results, we determined the tumour concentration of both ZR2003 and Iressa *in vivo* and more importantly *in vitro* in multicellular aggregates. The results showed that in A431 and 4T1 xenografts, the level of ZR2003 absorbed in the tumours were consistently 2-fold less than those generated by Iressa. Likewise, in the multicellular aggregates model, the penetration of ZR2003 was consistently lower than Iressa. In serum containing media, the level of extractable or free ZR2003 was also inferior to those of Iressa. The results from this bioanalytical study, suggest that the discrepancy between the *in vitro* and *in vivo* potency of ZR2003 when compared with Iressa, may be imputed to its significantly lower tumour concentration.

1. Introduction

At advance stages, solid tumours are characterized by a complex network of signaling pathways that protect tumour cells against the action of antitumour drugs. Over the past ten years, the implication of several of these pathways in tumour cell response to chemotherapeutic drugs, has been elucidated. It is now commonly accepted that alternative reactivation pathways play a significant role in the ability of cancer cells to evade the cytotoxic lesions. Thus, the development of drug combinations that target multiple pathways has long been an actively pursued research goal. Within this context, over the past several years, we developed a novel concept termed "the combi-targeting approach" that seeks to design molecules referred to as "combi-molecules" capable of inducing a tandem targeting of signaling mediated by the epidermal growth factor receptor (EGFR), a receptor that is overexpressed in several solid tumours including lung, brain, breast, ovarian and prostate tumours [1–3]. Several prototypes of combi-molecules have been synthesized to verify the combi-targeting postulates. It has now been shown that: (a) they block EGF-induced phosphorylation and downstream signaling, (b) they induce significant levels of DNA damage and (c) they trigger significant levels of DNA damage [4]. More importantly, they were shown to down-regulate DNA repair enzymes, indicating that this tandem blockade of EGFR signaling is an event that deprives the cells from mechanisms required to rescue them from the cytotoxic DNA lesions. Studies on mechanisms of decomposition and targeting of combi-molecules led to their classifications in two major types: those that require decomposition to generate DNA damage and an additional EGFR targeting agent (type I) and those that do not require decomposition to induce the binary EGFR-targeting event (type II) [5]. As shown in Scheme 1, drugs like BJ2000 that require degradation of the combi-molecules to generate the DNA damaging species without requirement for hydrolysis are termed type II combi-molecules. While the mechanism of action and potency of type II combi-molecules have been well demonstrated *in vitro*, their behavior *in vivo* remained to be elucidated. Here we study the inverse potency of a type II combi-molecule ZR2003 *in vivo* when compared with its *in vitro* growth inhibitory potency. ZR2003 is the chloro analogue of ZR2002 that

Abbreviations: EGFR, epidermal growth factor receptor; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; SRB, sulforhodamine B.

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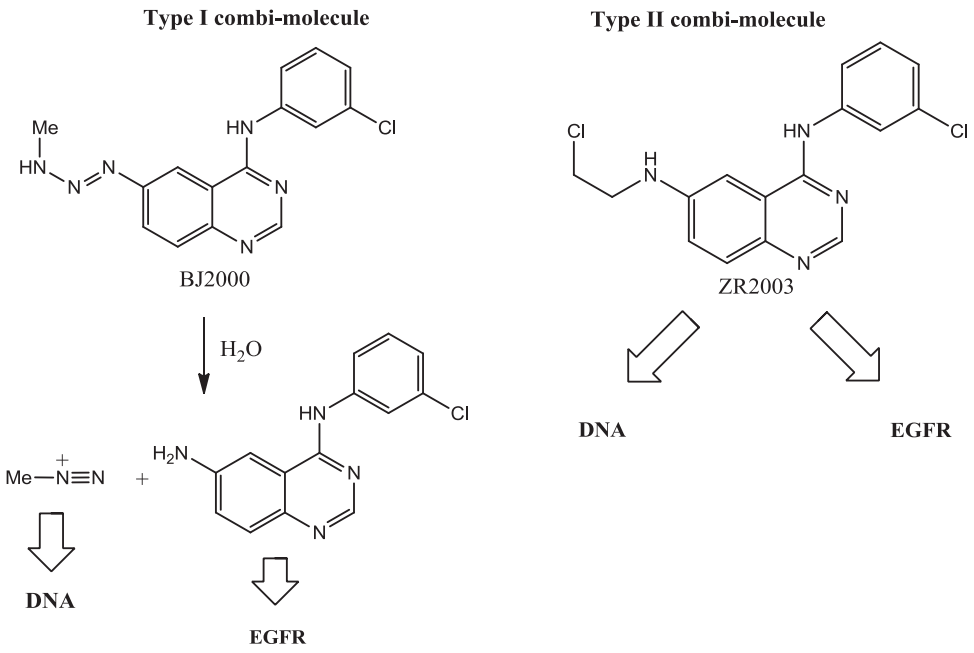

Scheme 1.

has been shown to significantly damage DNA and block EGFR [6,7]. We showed herein that *in vitro*, ZR2003 is an extremely potent EGFR–DNA targeting agent when compared with the clinical drug Iressa. However, this did not translate into greater antitumour activity than Iressa *in vivo*. A human EGFR-overexpressing A431 carcinoma of the vulva xenografts model showed no significant difference between the efficacy of Iressa and that of ZR2003. In order to rationalize these unpredicted results, we undertook an *in vivo* and *in vitro* bioanalytical study on the tumour penetration of these drugs. *In vitro*, we used 3D aggregates of the human ovarian cancer cells OV90 that form organ like compact multicellular aggregates *in vitro* [8–10]. This bioanalytical study also sought to verify whether an organ-like 3D model could mimic *in vivo* tumour absorption of ZR2003.

2. Materials and methods

2.1. Drug treatment

ZR2003 was synthesized in our laboratory [6]. Iressa was purchased from the Royal Victoria Hospital (Montreal) pharmacy and extracted from pills in our laboratory. In all assays, the drug was dissolved in DMSO and subsequently diluted in sterile RPMI-1640 or OSE media containing 10% FBS (Wisent, St. Bruno, Canada) immediately prior to the treatment of cell cultures [concentration of DMSO never exceeded 0.2% (v/v)].

2.2. Cell culture

2.2.1. Monolayer

The cell lines used in this study were the human epidermoid carcinoma of the vulva A431 purchased from the American Type Culture Collection (Manassas, VA). The mouse breast cancer 4T1 cell line was a generous gift from Dr. Thierry Muanza (Montreal Jewish General Hospital). The human ovarian cancer cells OV90 were obtained from Dr. Anne-Marie Mes-Masson (Université de Montréal). All cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS, and antibiotics as described previously [1]. Cells were incubated in a monolayer at 37 °C in a humidified environment of 5% CO_2, 95% air. The cultures were maintained in logarithmic growth by harvesting with a trypsin–EDTA solution containing 0.5 mg/ml trypsin and 0.2 mg/ml EDTA and replating before confluence. In all assays, the cells were plated for 24–48 h prior to drug administration.

2.2.2. Multicellular aggregates

OV90 cells were passaged in regular tissue culture flask. For the preparation of multilayer aggregates, they were detached and resuspended in ultra low attachment 6 or 24 well-plates. Within the first 24 h, they started form multicellular aggregates that within 7 days usually assembled into very compact organ-like structures. Of all the cells tried, including A431 and 4T1 cells, OV90 was the one forming multilayer aggregates sufficiently large for *in vitro* pharmacokinetics studies.

2.3. Growth inhibition assay

2.3.1. Sulforhodamine B assay (SRB)

Approximately 10,000 cells per well were plated in 96-well plates. After 24 h incubation at 37 °C, cell monolayers were exposed to different concentrations of each drug continuously for 6 days. All growth inhibitory activities were evaluated using the sulforhodamine B (SRB) assay [11]. Briefly, following drug treatment, cells were fixed using 50 μl of cold trichloroacetic acid (50%) for 60 min at 4 °C, washed four times with tap water, and stained for 30 min at room temperature with SRB (0.4%) and dissolved in acetic acid (0.5%). The plates were rinsed five times with 1% acetic acid and allowed to air dry. The resulting colored residue was dissolved in 200 μl of Tris base (10 mM) and optical density was read for each well at 540 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in triplicate.

2.3.2. Alamar blue viability assay

Cells were plated at a density of 10,000 per well in low attachment plate and allowed to form multicellular aggregate for 24 h. Drugs were added at specified doses and kept in the culture for 5 days after which aggregates were dissociated by pipetting and cell viability determined by adding 20 μl of alamar blue (pro-omega).

Fluorescence intensities were determined using a Spectramax microplate fluorometer [12].

2.4. EGFR phosphorylation assay

MDA-MB-468 cells were preincubated in a 6-well plate with serum-free media for 18 h, after which they were exposed to the indicated concentrations of ZR2003 for 2 h and subsequently treated with 100 ng/ml EGF for 10 min. Equal amounts of cell lysates were analyzed by Western blotting using anti-phosphotyrosine antibodies (NeoMarkers, Fremont, CA). The membrane was stripped, and reprobed with anti-EGFR antibodies (NeoMarkers).

2.5. Fluorescence microscopy analysis

MDA-MB-468 cells were grown on 2-well chamber coverglass (Nalge Nunc, Naperville, IL) for 24 h and then incubated with 10 μM ZR2003 for 30 min. Thereafter, the cells were washed with PBS, incubated in drug-free media and observed directly without fixation using LSM510 META confocal microscopy (ZEISS). The fluorescence was observed using the DAPI filter (excitation at 359 nm) and cells were observed with an oil immersion 63 × 1.4 differential interference contrast (DIC) objective.

2.6. Alkaline comet assay for quantitation of DNA damage

The modified alkaline comet assay was performed as described previously [13]. MDA-MB-468 cells were treated with the indicated concentration of ZR2003 for 2 h, harvested and re-suspended in PBS. Cell suspensions were diluted to approximately 10^6 cells/ml, and mixed with agarose (1%) in PBS at 37 °C in a 1:10 dilution. The gel was cast on Gelbond strips (Mandel Scientific, Canada) using gel casting chambers and then immediately placed into a lysis buffer [2.5 M NaCl, 0.1 M tetra-sodium EDTA, 10 mM Tris-base and 1% (v/v) Triton X-100, pH 10.0]. After being kept on ice for 30 min, the gel was gently rinsed with distilled water and immersed in a second lysis buffer (2.5 M NaCl, 0.1 M tetrasodium EDTA, 10 mM Tris-base) containing 1 mg/ml proteinase K for 60 min at 37 °C. Thereafter, the gel was rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37 °C, and electrophoresed at 19 V for 20 min. It was subsequently rinsed with distilled water and placed in 1 M ammonium acetate for 30 min. Thereafter, it was soaked in 100% ethanol for 2 h, dried overnight, and stained with SYBR Gold (1/10,000 dilution of stock supplied from Molecular Probes, Eugene, OR) for 20 min. Comets were visualized under fluorescence microscopy at 330× magnification [3,13].

2.7. Annexin V binding assay

MDA-MB-468 cells were pre-incubated in 6-well plates until confluence and then exposed to a dose range of each drug for 48 h. Thereafter, they were then harvested and incubated with annexin V-FITC and propidium iodide (PI) using the apoptosis Detection Kit (BD Bioscience Pharmingen, USA) and the supplier's protocol. Annexin V-FITC and PI binding were analyzed with a Becton-Dickinson FACScan. Data were collected using logarithmic amplification of both the FL1 (FITC) and FL2 (PI) channels. Quadrant analysis of coordinate dot plots was performed with the CellQuest software package.

2.8. In vivo studies

2.8.1. Mouse xenografts

CD-1 mice (Charles River) were maintained as per McGill animal safety protocols. Dose finding was performed with two CD-1 mice per group, and the maximum tolerated dose defined as the dose that does not induce >15% weight loss over a period of at least 14 days. For xenografts studies, each mouse were injected subcutaneously into the flank with 1×10^6 cells (human A431 cells) suspended in 0.1 ml 10% FBS RPMI medium. Treatments began when tumours became palpable. The animals were placed into two treatment groups of six mice each and one control group that received the vehicle [Tween 80 (6%)/ethanol (6%) saline solution]. The treatment groups were given drugs at 100 mg/kg in 0.4 ml of vehicle every other day. Tumour burdens were measured before each injection, and tumour volume was calculated using the formula TV = [(tumour width + tumour length)/4]$^3 \times 4/3 \times \pi$.

2.8.2. Syngeneic 4T1 model

Balb-c female mice were implanted sc with 4T1 cell (1×10^6). Tumours were allowed to grow for one week and drug administration was started at the time they were palpable. Tumour burdens were measured before each injection, and tumour volume was calculated using the formula TV = [(tumour width + tumour length)/4]$^3 \times 4/3 \times \pi$. The tumours grew extremely rapidly reaching sizes over 1.5 cm^3 after 11 days in the control group.

2.9. Biodistribution

The animals (male CD-1 nude mice, three per group) bearing A431 xenografts or Balb/c mice bearing 4T1 mouse breast cancer cells were given 100 mg/kg doses by i.p. injection. Three hours after injection, the plasma, the tumours and organs (e.g. brain, liver, and kidney) were removed, weighed, and homogenized. The homogenates were re-suspended in acetonitrile and centrifuged at 10,000 rpm for 8 min at 4 °C, and the supernatants collected. Following evaporation to dryness, the samples were reconstituted in acetonitrile and analyzed by liquid chromatography. For HPLC, samples were injected on a Ascentiss C18 (150 mm × 4.6 mm, 5 μm) column. All sample quantifications were relative to standard curves.

3. Results

3.1. Monolayer and multilayer aggregates

The ability of ZR2003 to block cell growth in comparison with the clinical drug Iressa was evaluated using SRB assay. The results showed that ZR2003 was 6-fold more potent than Iressa against the A431 cell line (Fig. 1A). Likewise ZR2003 was *ca.* 35-fold more potent than Iressa in the human ovarian cancer cell line OV90 (Fig. 1B). More importantly, further analysis of ZR2003 in multilayer aggregates showed that it was 16-fold more potent than the latter, suggesting that it is an extremely potent cytotoxic agent (Fig. 1C). As shown in Fig. 1, it is important to note that while no difference in potency was observed with Iressa under monolayer or multilayer aggregate conditions, cells in monolayer cultures were 2-fold more sensitive to ZR2003 than those in multilayer aggregates.

3.2. Reversibility of growth inhibitory activity

As shown in Fig. 2A, the only structural difference between ZR2003 and FD105 is the 2-chloroethyl group. However, under 120 h continuous exposure, the results obtained from the SRB, showed that ZR2003 (IC_{50} = 0.51 μM) was 46-fold more potent than FD105 (IC_{50} = 23.59 μM, Fig. 2C) in MDA-MB-468 cells. Importantly, reversibility study using a short exposure assay (2 h) followed by 120 h recovery and comparison with continuous exposure for 120 h, showed an almost complete loss of activity for FD105 in these cells (IC_{50} = 191.9 μM, Fig. 2C) indicating that it induced significantly reversible growth inhibitory activity. However after the 120 h recovery, ZR2003 retained significant growth inhibitory

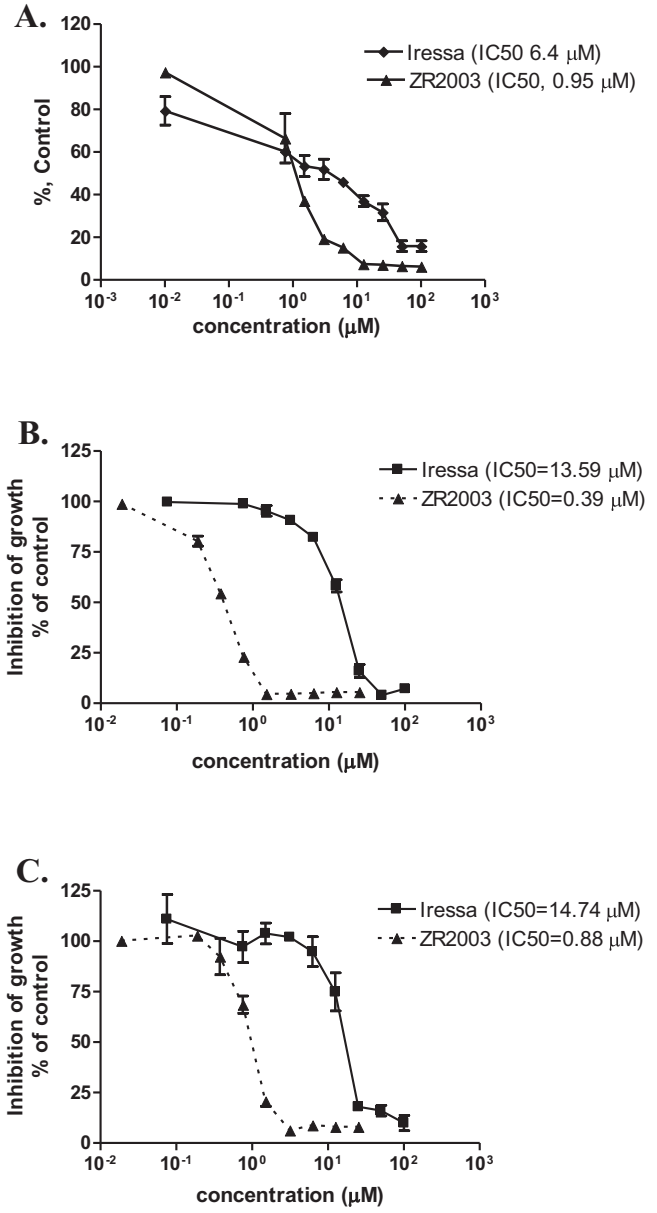

Fig. 1. Growth inhibition of cells grown as monolayers: (A) human A431 carcinoma of the vulva, (B) human ovarian OV90 or as multilayer aggregates and (C) OV90 cells.

activity (IC_{50} = 4.81 μM, Fig. 2B), indicating that it induced a more sustained growth inhibition than FD105.

3.3. Selective growth inhibition

The selective potency of the type II combi-molecule ZR2003 was evaluated in a panel of isogenic cell lines NIH3T3, NIH3T3HER14 (transfected with EGFR gene) and NIH3T3neu (transfected with ErbB2 gene). Interestingly, ZR2003 selectively blocked the growth of the transfectants (Fig. 3).

3.4. Binary EGFR–DNA targeting potential of ZR2003

Prior to embarking into more advanced studies with ZR2003, it was critical to demonstrate its dual EGFR–DNA targeting potential. This was performed by first demonstrating the ability of ZR2003 to penetrate the cells and to damage DNA. We subsequently determine whether it could block EGFR phosphorylation in whole cells.

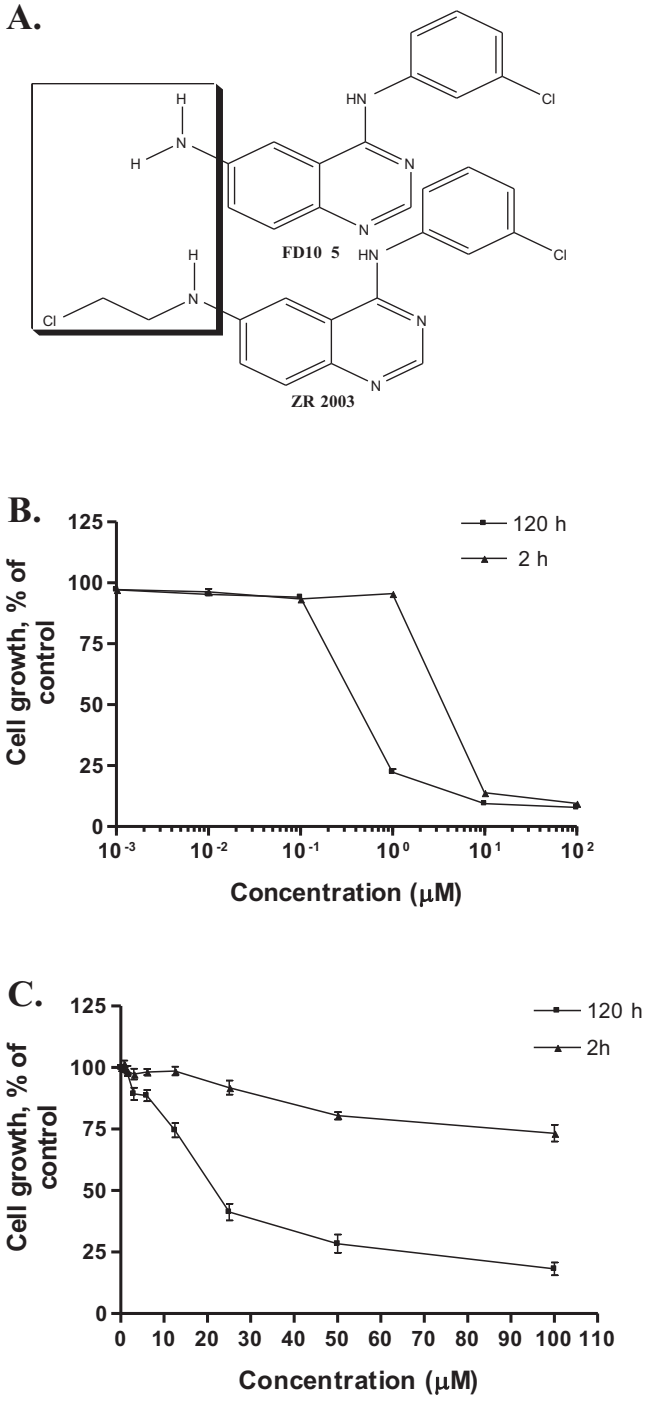

Fig. 2. (A) Difference between ZR2003 structure and FD105. Reversal of the growth inhibitory potency of ZR2003 (B) and FD105 (C), following a 2 h short exposure period.

This study was performed in the MDA-MB-468 cells in which the binary-targeting potential of several combi-molecules have been previously demonstrated [13].

3.4.1. Cell penetration and induction of DNA damaging potential

ZR2003 being a molecule that fluoresces in the blue, we analyzed its cell penetration by fluorescence microscopy. The results showed that it was readily absorbed in the cells with an apparent perinuclear distribution (Fig. 3A). More importantly it was not concentrated in the nucleus. Nevertheless, we examined its ability to damage nuclear DNA since a fraction of ZR2003 may have diffused

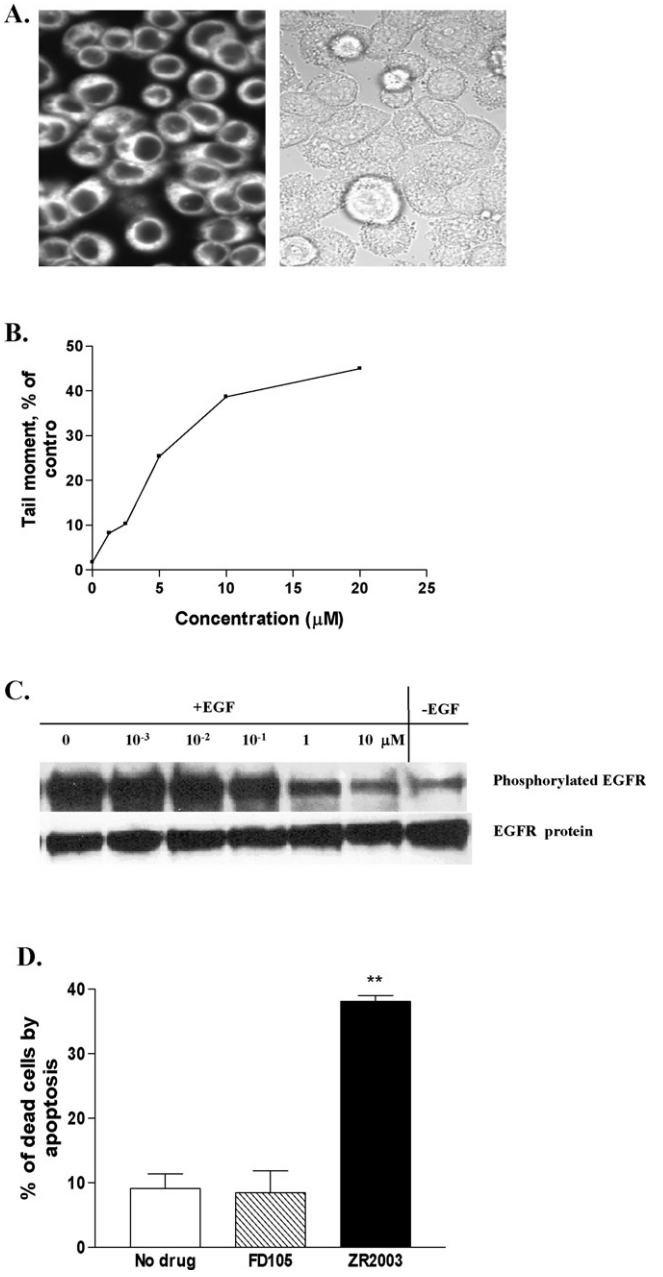

Fig. 3. (A) Subcellular distribution of ZR2003 and the corresponding phase contrast image. MDA-MB-468 cells were incubated with 10 μM ZR2003 for 30 min and examined under LSM510 META confocal microscopy. The fluorescence was observed using the DAPI filter (excitation at 359 nm) and cells were observed with a DIC 63 × 1.4 oil objective. (B) Quantitation of DNA damage using the alkaline comet assay. Tail moment was used as a parameter for the detection of DNA damage in MDA-MB-468 cells exposed to the indicated concentrations of ZR2003. (C) Inhibition of EGFR autophosphorylation by ZR2003. Serum starved MDA-MB-468 cells were preincubated for 2 h with the indicated concentrations of ZR2003 prior to stimulation with 100 ng/ml EGF for 10 min. Equal amount of cell lysates was analyzed by western blot using anti-phosphotyrosine antibody. The membrane was stripped, and re-probed with anti-EGFR antibody. (D) Induction of cell death by apoptosis in MDA-MB-468 cell line following drug treatment. Cells were untreated or treated with 12 μM ZR2003 or 12 μM FD105 for 48 h. Each point represents at least two independent experiments. Results are shown as mean ± SE. Statistical analysis was carried using Student's *t* test with two tailed, $^{**}p < 0.01$ ZR2003 vs. FD105.

to the nucleus. Using the alkaline comet assay, we indeed demonstrated that ZR2003 was capable of inducing significant levels of DNA damage in a dose dependent manner in MDA-MB-468 cells at extremely low doses (e.g. 0.5 μM) (Fig. 3B).

3.4.2. Inhibition of EGFR TK activity

Western blot analysis demonstrated that ZR2003 blocked EGF-induced EGFR phosphorylation in MDA-MB-468 cells in a dose-dependent manner, with significantly strong inhibition at concentration as low as 1 μM without affecting the levels of EGFR (Fig. 3C).

3.5. Apoptosis induced by ZR2003

Annexin V-FITC and PI staining (detected by flow cytometry) were used to distinguish viable (PI−/FITC−), early apoptotic (PI−/FITC+), dead cells by apoptosis (PI+/FITC+) and necrotic (PI+/FITC−). Apoptosis was studied after a 48 h drug exposure at 12 μM. At this concentration ZR2003 showed more cell death by apoptosis ($p < 0.01$) in MDA-MB-468 cells than its free inhibitor counterpart FD105 (Fig. 3D), indicating that the chloroethyl group has conferred significant cytotoxic properties to ZR2003.

3.6. In vivo efficacy

While the MDA-MB-468 cells lent themselves well to the analysis of reversibility and binary EGFR–DNA targeting potential of ZR2003, they express a mutant form of PTEN that confers moderate sensitivity to EGFR inhibition *in vitro* and *in vivo*. Therefore we chose to perform our *in vivo* efficacy studies in mouse bearing A431 cells, a human epidermal carcinoma of the vulva cell line that is widely used in the evaluation of the antitumour potency of EGFR inhibitors. In the latter model, drugs are often administered on a once daily schedule. However, ZR2003 being a DNA alkylating agent, in this study a once every other day schedule was used to attenuate toxicity. As shown in Fig. 4A, the results showed that 16 days after treatment, both ZR2003 and Iressa induced significant growth inhibitory activity. However, despite being a monotargeted and specific EGFR inhibitor, it appeared slightly more potent than the dual targeted ZR2003. Similarly, in a more aggressive and less EGFR-dependent mouse model, while ZR2003 growth curve overlapped with that of control, Iressa appeared to induce a slight stronger growth inhibition than ZR2003 (Fig. 4B).

3.7. In vivo tumour penetration

Having observed that the superior potency of ZR2003 over Iressa did not translate *in vivo*, we surmised that this may have been due to differential biodistribution in the tumours. To test this hypothesis, we analyzed the tumour content of the two drugs *in vivo*. Animals were given equidoses of Iressa or ZR2003 (100 mg/kg) and sacrificed 3 h after injection and the A431 tumours surgically removed. ZR2003 and Iressa tumour concentrations were determined by HPLC and expressed as nmol/g tumour. The tumour concentration of Iressa (10.4 nmol/g) was significantly higher than that of ZR2003 (4.4 nmol/g) ($p < 0.05$) (Fig. 4C). In order to ascertain this result, we repeated the analysis in a different tumour model using a mixture of the two drugs at equidoses (50 mg/kg). The results consistently showed a superior absorption of Iressa in both plasma and tumours (Fig. 5A and B).

3.8. Multilayer aggregate modeling

We surmised that the decreased tumour absorption of ZR2003 could have been imputed to metabolism and elimination. In order to determine whether this was due to intrinsic properties of the

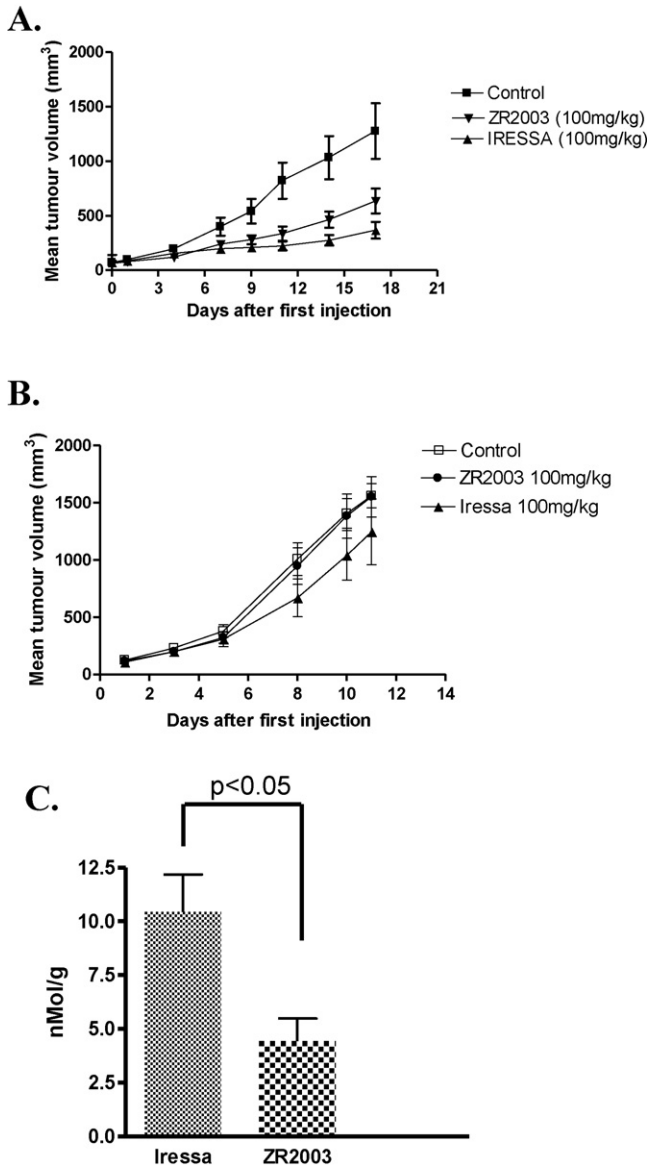

Fig. 4. *In vivo* growth inhibition of (A) human A431 xenografts and (B) mouse 4T1 tumours by ZR2003 and Iressa. The drug was administered on an every other day schedule at a dose of 100 mg/kg. (C) Levels of Iressa and ZR2003 in A431 tumours, 3 h after administration IP at a 100 mg/kg dose.

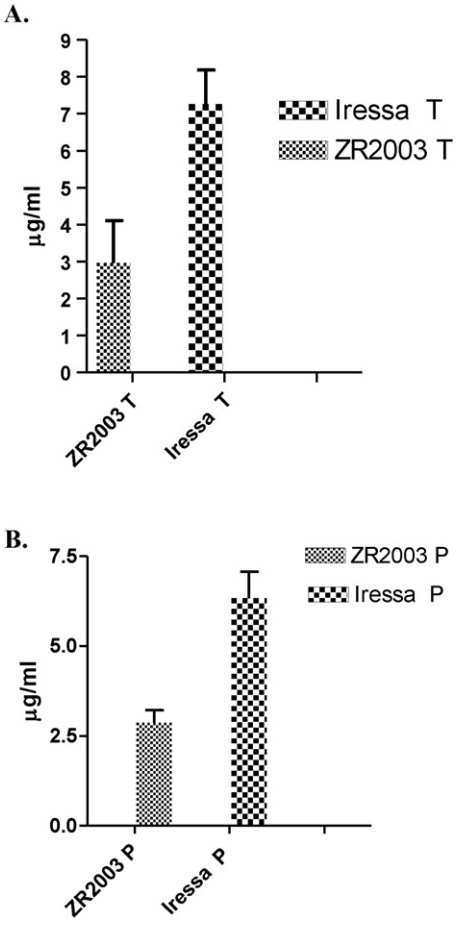

Fig. 5. Tumour concentrations of ZR2003 and Iressa in 4T1 breast tumours at equidoses (50 mg/kg) (A) and in mouse plasma (B). The T symbol represents tumours and P plasma.

molecule (e.g. reactivity of the alkylating group), we studied its absorption *in vitro* in multilayer aggregates of the OV90 cells in a context where levels of metabolic enzymes would be the minimum. The OV90 cells form compact aggregates (Fig. 6A) that lend themselves well to absorption analysis. Thus, we measured the absorption of both drugs in the aggregates and in the cell culture medium. The results showed that both Iressa and ZR2003 were absorbed in OV90 aggregates in a dose-dependent manner (Fig. 6B and C). However, the trend was toward a greater penetration of Iressa compared with ZR2003. This was confirmed by calculation of ZR2003/Iressa ratio in the multilayer aggregates that remained consistently below 1, over the entire dose range (Fig. 6D).

4. Discussion

It has become increasingly clear over the past ten years that due to the complexity of advanced tumours, drugs that have multiple tumour targets are more effective than single ones. As an example lapatinib [14,15] that targets both EGFR and Her2 is clinically more potent than Iressa, a monotargeted and specific EGFR-targeting drug. Sorafenib (Nexavar) [16–18] that targets VEGFR, CRAF, wild type BRAF, mutant BRAF, Kit, Flt-3, VEGFR-2, VEGFR-3 and PDGFR-β was approved in 2005 for the treatment of renal cell carcinoma [19]. While the multitargeting aspect of these molecules is confined to receptors of the tyrosine kinase family, our combi-targeting principles seek to "cross-target" kinase inhibitor with different macromolecules (e.g. DNA) and recently we described these principles as divergent targeting [20]. A significant body of work has accumulated to show that lead combi-molecules can not only damage DNA but also down-regulate antiapoptotic signaling pathways. ZR2002 is one such combi-molecule, which has been shown to be extremely potent *in vitro* but with mitigated activity *in vivo*. This was believed to be due to the poor water solubility of the compound [7]. Chemical alteration of the structure to introduce a chloro group instead of a bromo, gave rise to ZR2003, a more water soluble molecule [6]. We therefore expected its activity to improve *in vivo*. We demonstrated herein that it blocked EGFR in a dose-dependent manner and induced significant levels of DNA damage in human tumour cells. In the current study, we examined its potency *in vitro* in comparison with Iressa and showed that it was a 6.5-fold stronger growth inhibitory agent than Iressa and induced more sustained growth inhibitory activity than its closest analogue FD105. However, this did not translate into the same levels of potency *in vivo* in an A431 xenograft model known to be sensitive to EGFR inhibitors. In order to rationalize these results, we undertook a tumour penetration study to verify whether tumour

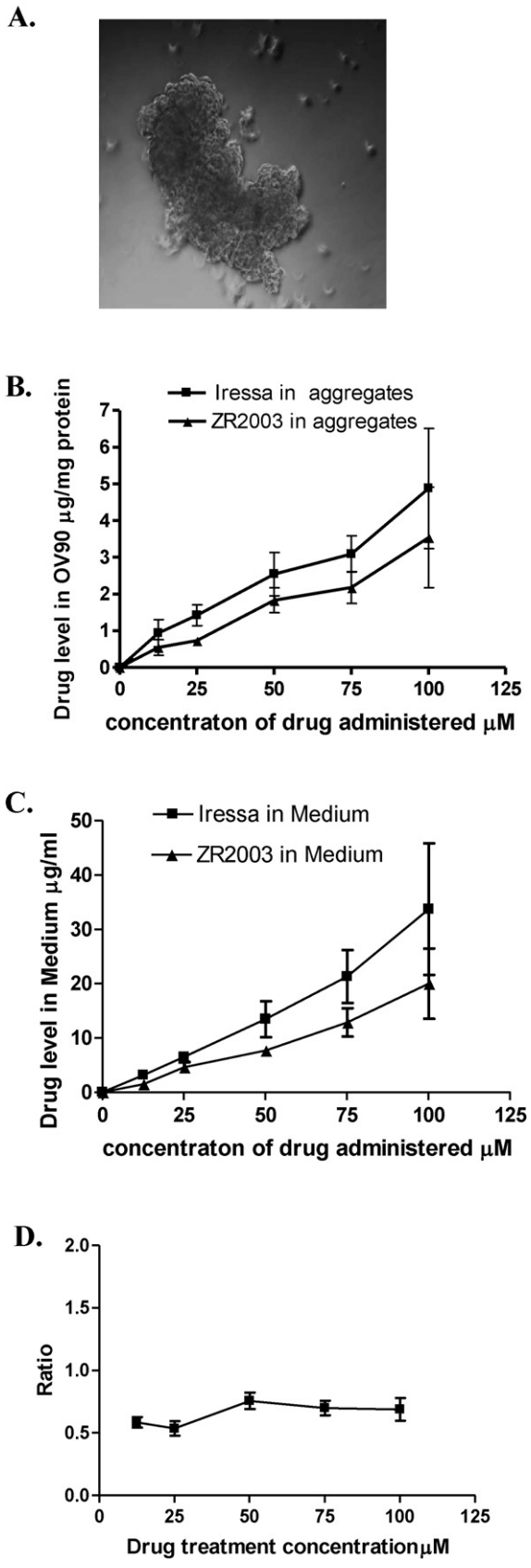

Fig. 6. Distribution of Iressa and ZR2003 in OV90 aggregates. (A) Representative picture of multilayer aggregate grown on ultra low attachment plates after 4-days post-incubation of OV90 cells; (B) dose-dependent increase in levels of each drug expressed as µg/g protein content of lysates form the aggregates; (C) concentrations of each drug in the culture medium; (D) ZR2003/Iressa distribution ratio in OV90 spheroids.

penetration could account for the observed difference in potency *in vivo*. More importantly, in order to test whether the differences in biodistribution was due to diffusion into the tumours, we studied their penetration into multilayer aggregates *in vitro*, a condition that allows to avoid the influence of metabolism, absorption and elimination on the amount of drug available for tumour penetration. Also, it is important to note that because of cell-to-cell interactions, spheroid and multilayered cultures are good mimics of tumour environments. Their compact nature (Fig. 6A) mimics the barriers imposed by tumour tissue, thereby allowing tumour pharmacokinetic measurements.

The observed results both in A431 and 4T1 models that were used primarily for tumour distribution analysis, showed a rather superior potency of Iressa over the combi-molecule ZR2003. More importantly the tumour content of ZR2003 both in A431 and 4T1 was consistently 2-fold lower than that of Iressa. Interestingly, similar results were obtained in multilayer aggregate models, indicating that the lower tumour concentration of ZR2003 compared with Iressa was not due to liver metabolism or rapid clearance *in vivo* but rather to interactions that prevent its internalization into tumours. Importantly the differential levels of ZR2003 were equally observed in serum containing medium. This suggests that perhaps ZR2003 that contains a reactive chloroethylating species may in contrast to Iressa, react with and bind to plasma proteins.

The type II combi-molecules ZR2002 and ZR2003 are the most cytotoxic combi-molecules ever synthesized and also the most potent EGFR inhibitors of the combi-molecule class with IC50 in submicromolar range [6]. They show equivalent EGFR inhibitory potency as Iressa, but 6-fold greater potency than the latter. However, their development is hampered by slightly less *in vivo* efficacy than Iressa. Here we showed that decreased biodistribution could be the main cause for such debility. Having demonstrated that the difference in biodistribution could be mimicked by 3D organ-like model, the latter could well represent a new platform for predicting the tumour penetration of these types II combi-molecules *in vivo*. It presents the advantage of allowing to measure biodistribution over a broad range of concentration, thereby allowing establishing consistent ZR2003 or analogous compounds/Iressa ratios. Here we observed that the aggregate penetration of Iressa was significantly superior to that of ZR2003.

Further studies are now ongoing in our laboratory to explore different routes of administration of ZR2003 and the development of analogues as prodrugs with lower alkylating activity in order to reduce macromolecular binding and promote tumour penetration.

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