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ORIGINAL ARTICLE

Tyrosine kinase inhibitors and multidrug resistance proteins: interactions and biological consequences

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Abstract Although multidrug resistance (MDR) proteins are known to play a role in drug resistance and modification pharmacodynamic characteristics of certain conventional chemotherapeutics, information about their interactions with tyrosine kinase inhibitors (TKIs) remains fragmentary and somewhat controversial. The chronic administration of TKIs in many clinical situations strongly suggests that any possible interactions with MDR transporters should be studied as a function of time. For example, short periods of exposure to TKIs could provide insights into the nature of the binding to MDR-related proteins, either as substrates or as inhibitors, whereas prolonged exposure to TKIs could provide insights into cellular responses to binding/inhibition of MDR-related proteins. In this report, we provide evidence that suggests that both Gefitinib and Vandetanib may act as transported substrates for Breast Cancer Resistance Protein (BCRP, ABCG2). Conversely, the interaction of Gefitinib and Vandetanib with P-glycoprotein (PgP, MDR1) appeared to be as inhibitors alone. Consistent with this, short periods of exposure ($\leq$24 h) to either Gefitinib or Vandetanib increased the effectiveness of SN-38, the active metabolite of CPT-11. Conversely, prolonged exposure (5 days) decreased SN-38 effectiveness, and was associated with BCRP up-regulation and reduced cell accumulation in S-phase, possibly though reduced intracellular accumulation of SN-38. This report underlines the needs for more detailed characterisation new biologically targeted anticancer drugs, in particular analysing periods of both short and prolonged drug exposure reflecting potentially distinct situations in the clinic in order to optimise future development in combination with established chemotherapeutic approaches.

Keywords Gefitinib · Vandetanib · Resistance · BCRP · P-glycoprotein

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Introduction

In recent years pharmaceutical research in oncology has produced multiple agents targeting important mitogenic pathways in multiple tumor types. Nonetheless, although these targeted agents have produced impressive preclinical results, the clinical benefits in certain disease settings have not been less than hoped for. One of the possible explanations could be that while a great deal of effort has gone into understanding specific targets and/or cellular pathways relatively little effort has gone into evaluating the dynamic complexity of drug effects over time.

For example, several reports have described the efficacy of TKIs as antitumor agents when given alone or in

combination with conventional chemotherapeutics [1, 2] and the molecular mechanisms involved [3–7], but only more recently has attention been focused properties of these agents such as antiangiogenic effects or MDR modulation [8–10].

Previous reports on the interaction of TKIs and MDR related proteins are somewhat controversial and fragmentary [11–16]. Nonetheless, this remains an important and relevant aspect of investigation since TKIs could potentially modify the pharmacodynamic characteristics of certain conventional chemotherapeutics and therefore positively or negatively impact on the overall response to anticancer therapy.

In the present study, two TKIs, Gefitinib and Vandetanib, were investigated. Each of these novel agents is based on a quinazoline chemical backbone, but each has a different selectivity and potency in terms of biological targets. Gefitinib is an orally available EGFR TKI, already in clinical use as a monotherapy in non-small cell lung cancer patients [17], whereas Vandetanib is an oral VEGFR2 TKI, with additional activity against EGFR and RET tyrosine kinases, currently in phase III clinical trials in several solid tumor types either alone or in combination with docetaxel or pemetrexed (http://www.clinicaltrials.gov).

It has been reported previously that Gefitinib could reduce drug resistance by acting as an inhibitor or a substrate of BCRP which is, together with PgP and MRP1, one of the main MDR transporters responsible of the establishment of resistance to certain conventional chemotherapeutics in cancer [10, 13, 18–20]. Previous reports on the interaction of Gefitinib or Vandetanib with the other transporters are limited but suggest both drugs may act as inhibitors of PgP [21, 22]. However, these reports seemed to be in disagreement with our previous results, where pre-exposure to Gefitinib reduced the effectiveness of camptothecin (CPT-11), where we hypothesised that this was due increased expression of BCRP which we observed after prolonged exposure to either Gefitinib or Vandetanib [11, 23]. We reasoned that the underlying reasons for these discrepancies could be due to the different exposure times to Gefitinib that were used.

In this paper, a series of direct and indirect experimental evidences is presented on the interactions of Gefitinib and Vandetanib with BCRP and PgP that may, in part, rationalise the previous apparently contradictory data. Our results suggest that TKIs may modulate MDR by two distinct mechanisms (1) a direct interaction with MDR transporters and (2) changing the expression levels of MDR proteins, possibly as a result of key cell functions such as cell cycle progression or protein synthesis.

Methods

Drugs and chemicals

Gefitinib (ZD1839/Iressa) and Vandetanib (ZD6474/Zactima) were provided by AstraZeneca Pharmaceuticals (Macclesfield, UK.). Stock solutions were prepared at 20 mM in DMSO and stored in aliquots at −20°C. SN-38 and mitoxantrone were provided by Aventis and Sigma Aldrich (USA), respectively and dissolved in DMSO at 20 mM; aliquots were kept at −20°C. Doxorubicin was provided by Sigma Aldrich (USA), dissolved in sterile water at 10 mM and aliquots were kept at −80°C. Further dilutions were made in medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 50,000 UL^{-1} penicillin and 80 μM streptomycin. Ko143 was a gift of Dr. A.H. Schinkel (The Netherlands Cancer Institute, Amsterdam).

Cell lines

Colon Cancer cell lines, HT-29 and CaCo-2, were routinely cultured in McCoy's and DMEM, respectively, supplemented with 10% foetal bovine serum, 50,000 UL^{-1} penicillin and 80 μM streptomycin in a humidified incubator at 37°C with an atmosphere containing 5% CO_2. Cells were trypsinized once a week with trypsin/EDTA (0.25%/0.04%) and medium was changed twice a week. Madin-Darby canine kidney cells wild type and transfected with either human MDR1 (MDR1-MDCK) and BCRP (BCRP-MDCK) were obtained from The Netherlands Cancer Institute (Amsterdam, The Netherlands) and routinely cultured in DMEM.

Permeability experiments

Preparation of Caco-2 monolayer

This procedure has been previously reported by Colabufo [24]. Briefly, Caco-2 cells were harvested with trypsin-EDTA and seeded onto MultiScreen Caco-2 assay system at a density of 10,000 cells/well. The culture medium was replaced every 48 h for the first 6 days and every 24 h thereafter, and after 21 days in culture, the Caco-2 monolayer was utilised for the permeability experiments. The Trans-Epitelial Electrical Resistance (TEER) of the monolayers was measured daily before and after the experiment using a epithelial voltohmmeter (Millicell-ERS; Millipore, Billerica, MA, USA). Generally, TEER values obtained are greater than 1,000 Ω for a 21 day culture.

Drug transport experiment

Apical to basolateral (P_{app} A–B) and basolateral to apical (P_{app} B–A) permeability of drugs were measured at 120 min and at various drugs concentrations (1–100 μM). Drugs were dissolved in Hank's balanced salt solution (HBSS, pH 7.4) and sterile filtered. After 21 days of cell growth, the medium was removed from filter wells and from the receiver plate. The filter wells were filled with 75 μL of fresh HBSS buffer and the receiver plate with 250 μL per well of the same buffer. This procedure was repeated twice, and the plates were incubated at 37°C for 30 min. After incubation time, the HBSS buffer was removed and drug solutions added to the filter well (75 μL). HBSS without the drug was added to the receiver plate (250 μL). The plates were incubated at 37°C for 120 min. After incubation time, samples were removed from the apical (filter well) and basolateral (receiver plate) side of the monolayer and then were stored in a freezer (−20°C) pending analysis. The concentrations of compounds were analysed using UV–vis spectroscopy. The apparent permeability (P_{app}), in units of nm/s, was calculated using the following equation [25]:

$$P_{app} = \left(\frac{V_A}{\text{Area} \times \text{time}}\right) \times \left(\frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{initial}}}\right)$$

where V_A is the volume (in mL) in the acceptor well; area is the surface area of the membrane (0.11 cm^2 of the well); time is the total transport time in seconds (7,200 s); (drug) acceptor is the concentration of the drug measured by UV spectroscopy; (drug) initial is the initial drug concentration (1×10^{-4} M) in the apical or basolateral wells. In the same experimental conditions the Apparent Permeability in the presence of 100 μM verapamil were determined.

Cell ATP availability assay

This experiment was performed as reported in technical sheet of ATPlite 1step Kit for luminescence ATP detection using Victor3, from PerkinElmer Life Sciences [26]. Caco-2 cells were seeded into 96 well microplate in 100 μL of complete medium at a density 2×10^4 cells/well. The plate was incubated overnight in a humidified atmosphere 5% CO2 at 37°C. The medium was removed and 100 μL of complete medium in the presence or absence of different concentrations of test compounds was added. The plate was incubated for 2 h in a humidified atmosphere 5% CO2 at 37°C. Then, 50 μL of mammalian cell lysis solution was added to all wells and the plate stirred for 5 min in an orbital shaker. In all wells 50 μL of substrate solution was added, the plate stirred for 5 min as above reported. The plate was dark adapted for 10 min and the luminescence was measured in Victor3.

[^{3}H]-mitoxantrone transport inhibition

Caco-2 cells were seeded onto MultiScreen Plates 10,000 cells/well for 21 days measuring the integrity of the cell monolayers by Trans-Epithelial Electrical Resistance (TEER, Ω × cm^2) with an epithelial voltohmmeter. Mature Caco-2 cell monolayer exhibited a TEER >800 Ω × cm^2 prior to use in transport experiments. Transport experiments for tested compounds were carried out as described by Taub [27]. In each well to basolateral (BL) compartment in the absence and in the presence of Gefitinib and Vandetanib (from 0.20 to 400 μM) 20 nM [^{3}H]mitoxantrone for 120 min at 37°C was added and its appearance in the apical (AP) compartment was monitored. At 120 min a 20 μL sample was taken from donor compartment to determine the concentration of radioligand remaining in the donor chamber at the end of the experiment. Samples were analysed using LS6500 Beckman Counter. For each compound, [^{3}H]mitoxantrone transport inhibition was calculated as radioactivity difference between radioligand in the presence and absence of compounds. These differences were expressed as percentage of inhibition effect for each single drug concentration. In the same experimental conditions the apparent permeability in the presence of 100 μM verapamil were determined.

Evaluation of cytotoxicity

Determination of the IC_{50} was performed using the 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay. On day 1, 10,000 cells/well in a volume of 200 μL were plated in 96-wells plates. In each plate, one column contained cells not exposed to drugs (control), and five columns contained cells exposed to increasing concentrations of drugs. Each drug or drug combination was repeated in six identical wells. On day 2, Vandetanib (0.01, 0.1, 1, 10 and 100 μM) was added for 5 days. For each drug, results were expressed as dose-effect curves with a plot of the fraction of unaffected (surviving) cells versus drug concentration. The IC_{50} was defined as the drug concentration yielding a fraction of affected (no surviving) cells = 0.5, compared with untreated controls and was calculated utilising CalcuSyn ver.1.1.4 software (Biosoft, UK). Each experiment was done in triplicate.

Drug combination studies

To define the best schedule for the combination of Vandetanib and SN-38, either simultaneous or sequential exposures were tested [23]. SN-38 was utilised with a short time-exposure (1 day) and Vandetanib with a long time-exposure (5 days), in three different combination. Schedules:

1. *Simultaneous* drugs exposure followed by Vandetanib alone: Vandetanib and SN-38 were given simultaneously for 1 day, then, after two wash-steps performed with the medium, Vandetanib was given alone for 4 days.
2. *Vandetanib after*: SN-38 was given for 1 day, then, after two wash-steps performed with the medium, Vandetanib was given for 5 days.
3. *Vandetanib before*: Vandetanib was given for 5 days, then, after two wash-steps performed with the medium, SN-38 was given for 1 day.

Each drug combination was tested in seven different concentrations, using a constant ratio of 1:1 with respect to their equiactive concentrations (IC_{50}). Growth inhibition by MTT assay was analysed with the Median Drug Effect Analysis software to determine the interaction between Vandetanib and SN-38, expressed as a non-exclusive case combination index (CI) using CalcuSyn software (Biosoft).

To test the ability of TK inhibitors to interfere with chemotherapeutics effectiveness, firstly the subtoxic MDCK wt, MDR1-MDCK and BCRP-MDCK were tested for TK inhibitors effectiveness, exposing cells to each of drug (0.01–100 μM) until 3 days. To test drugs combination, cells were exposed to Gefitinib or Vandetanib for 1 h, then, after two wash-steps performed with the medium, mitoxantrone or doxorubicin was given for 1 day.

HPLC drug analysis

The stock solutions of SN-38 and drug solved in McCoy's medium were used as controls. The HPLC consisted of a LC9010 system coupled with a UV detector (Varian Inc, Palo Alto, CA, USA). The mobile phase comprised 10 mM ammonium acetate and methanol (80:20 [v/v]). Cells, after drug(s) exposure, were harvested, washed in ice-cold PBS (pH 7.4), lysed by the addition of ice-cold water and then mechanically detached from the plastic by scraping. SN-38 was purified by eluting samples through Aspec Bond Elut-C2 column (Varian Inc) using a solution of ammonium acetate and methanol (90:10 [v/v]).

Cell cycle perturbation

Cells were harvested, washed twice in ice-cold PBS (pH 7.4), fixed in 4.5 mL of 70% ethanol at −20°C, and washed once in ice-cold PBS. The pellet was resuspended in PBS containing 1 mg/mL RNase, 0.01% NP40 and the cellular DNA was stained with 50 μg/mL propidium iodide (Sigma). Cells were stored in ice for 1 h prior to analysis. Cell cycle analysis performed using a FACScan flow cytometer (Becton Dickinson), and data were interpreted using CellQuest software, provided by the manufacturer.

BCRP and PgP expression analysis

BCRP and PgP expression were characterised in HT-29 and Caco-2 cells by western blotting and flow cytometry. In all experiments, DMSO was added in each control to evaluate the possible cytotoxicity of the solvent and antibodies utilised were the monoclonal antibody to BCRP (BXP-21) was from Alexis (Lausen, Switzerland), monoclonal anti-P-glycoprotein (MDR) clone F4 (Sigma, Missouri, USA) and β-actin monoclonal antibody was from Sigma-Aldrich (USA).

Western blot Analysis

Total proteins were extracted from cell culture by homogenisation in radioimmunoprecipitation assay (RIPA) buffer (0.5 M NaCl, 1% Triton X-100, 0.5% NP40, 1% deoxycholic acid, 3.5 mM sodium dodecyl sulfate), with PMSF (Sigma, Missouri, USA) and measured by Bradford method. Total cellular proteins were separated by electrophoresis on an 8% SDS-polyacrylamide gel and electro transferred onto PVDF membranes. Membranes were incubated with the primary antibody, probed with horseradish peroxidase-labelled secondary antibody and signal was detected by ECL chemoluminescence assay from Amersham Pharmacia Biotech (Upsala Sweden). β-actin expression level was used to normalize the sample values.

Flow cytometry (FCM)

Cells were harvested, washed twice in ice-cold PBS pH 7.4, fixed in 4.5 mL of 90% methanol and stored at −20°C. Fixed cells were washed in ice-cold PBS for one time and, to analyse PgP or BCRP expression, cells were incubated overnight at 4°C with the monoclonal antibody. To determine the unspecific fluorescence due to the fluorescein-conjugated secondary antibody, untreated cells were incubated with an appropriate isotype control in the same experimental conditions (isotype control). After 15 min incubation with 0.5 ml 0.5% FBS in PBS, cells were centrifuged and washed once in 0.5 ml 0.5% FBS in PBS. The pellet was resuspended in 0.5% FBS in PBS in the presence of the goat anti-mouse IgG (H&L) fluorescein-conjugated affinity purified secondary antibody (Chemicon international USA) (1:50) and incubated for 1 h at 4°C. After a wash step with 0.5 mL 0.5% FBS in PBS, PgP protein determination was performed using a FACScan flow cytometer (Becton Dickinson). Fluorescence analysis was gated to include single cells on the basis of forward and side light scatter and was based on the acquisition of data from 5,000 cells. Log fluorescence was collected and displayed as single parameter histograms.

Statistical analysis

All the in vitro experiments were performed in triplicate, and results have been expressed as the mean ± standard deviation (SD) unless otherwise indicated.

Results

In order to evaluate whether Gefitinib or Vandetanib were transported by, or were substrates/inhibitors of BCRP and/or PgP [24, 28, 29] we use three biological assays, (1) monolayer efflux assay, (2) [^{3}H]-mitoxantrone transport inhibition assay and (3) cell ATP availability assay. These, together with the utilisation of verapamil, a selective PgP inhibitor, allowed us to define the interaction of TKIs and MDR related proteins. To confirm the specificity of TK inhibitors interaction with BCRP or PgP, excluding the involvement of other MDR transporters, a canine kidney in vitro model, stably transfected with either human MDR1 or BCRP, was utilised [30, 31]. Moreover, since TKIs are generally used chronically in the clinic we also investigated the effects of prolonged exposure to either Gefitinib or Vandetanib on BCRP

Firstly, expression levels of both MDR-1 and BCRP were evaluated in all cell lines and in Fig. 1, FCM analysis is reported. The initial biological characterisation was carried out using Caco-2, a human colon cancer cell line expressing both PgP and BCRP (Table 1). The contribution of each transporter was estimated by performing the same assay in the presence or absence of 100 μM verapamil to inhibit PgP activity; experiments carried out without verapamil measured the contribution of both BCRP and PgP conversely, in the presence of the PgP inhibitor only BCRP involvement is determined. Caco-2 cells form a stable monolayer, allowing determination of Apparent Permeability (P_{app}) and BCRP and PgP interacting mechanism.

Apparent permeability (P_{app}) evaluation was determined by equilibrating each TKI in apical position and measuring it in the corresponding basolateral compartment (A–B flux). This evaluation represented both BCRP and PgP active transport of TKI. In the meantime, the opposite flux from basolateral to apical compartment (B–A flux) was measured, at the same concentration. This evaluation represented passive transport of TKI. BA/AB ratio is a parameter to establish if the analysed compound was transported by BCRP and PgP transporters. For BA/AB ratio ≤2, a TKI is considered not to be transported by pumps while a TKI displaying BA/AB ratio >2 is considered a transported substrate. In our experiments, Gefitinib and Vandetanib displayed similar BA/AB ratios (12.3 and 12.4, respectively). The same measurements, carried out in the presence of verapamil, displayed similar results (BA/AB ratios of 11.4 and 13.9 for Gefitinib and Vandetanib, respectively), demonstrating that both compounds appeared to act as BCRP substrates and that PgP efflux transport was negligible for each TK inhibitor.

In order to confirm BCRP involvement, [^{3}H]mitoxantrone transport inhibition was evaluated in Caco-2 cells in the presence and absence of verapamil. Results were reported as a dose-response curve plot and the EC_{50} values.

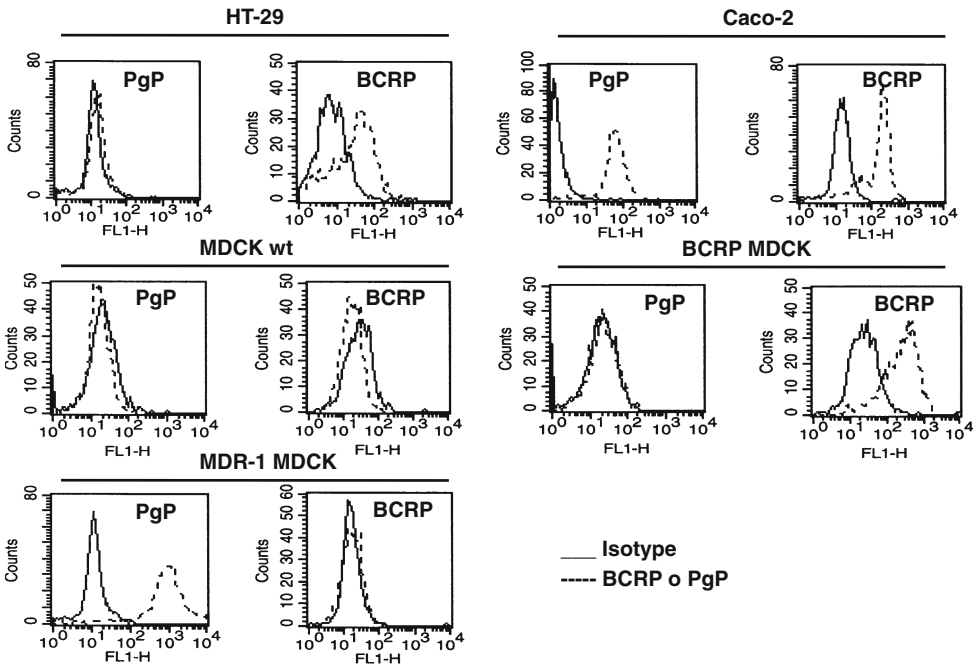


Fig. 1 PgP and BCRP expression. In HT-29, Caco-2, MDCK wt, BCRP-MDCK, MRD1-MDCK cells, BCRP and PgP expression levels were determined by flow cytometry as described in "Methods"

Table 1 Biological characterisation of TK Inhibitors interaction with ABCG2 and/or PgP

	[^{3}H]mitoxantrone transport inhibition EC_{50}, μM ± SEM[a]	ATP-ase activation (%)[b]	P_{app} B–A nm/s	P_{app} A–B nm/s	BA/AB
Gefitinib	3.0 μM	Yes	1,906	155	12.3
Gefitinib plus verapamil[c]	0.5 μM	Yes	1,761	155	11.4
Vandetanib	1.2 μM	Yes	1,848	149	12.4
Vandetanib plus verapamil[c]	0.3 μM	Yes	1,870	134	13.9
verapamil[c]	27%				

[a] The results are the mean of two independent experiments, samples in triplicate

[b] This effect has been determined for each compound at 1 μM. It is considered a positive value for >20%

[c] At 100 μM

The EC_{50} values for 50% inhibition of [^{3}H]mitoxantrone transport were 3.0 and 1.2 μM for Gefitinib and Vandetanib, respectively. In the presence of verapamil, [^{3}H]mithoxantrone transport was more effectively inhibited by both compounds (EC_{50} = 0.5 and 0.3 μM for Gefitinib and Vandetanib, respectively). This latter result could be considered as an apparent discrepancy with respect to the unchanged permeability values determined in the absence and in the presence of verapamil. A possible explanation could be that verapamil was able to modulate BCRP pump; in fact, a partial inhibition (about 27%) of BCRP-mediated [^{3}H]mithoxantrone efflux was observed at 100 μM verapamil.

Cellular ATP depletion in the same biological system was also measured. MDR substrates deplete cellular ATP concentrations whilst inhibitors do not change ATP levels, although this is not a specific test. Gefitinib and Vandetanib determined depleted cellular ATP concentrations (by 20–50%) in Caco-2 cells in both the absence and the presence of verapamil.

In summary, these assays produced similar results for Gefitinib and Vandetanib. Both agents appeared to be transported by BCRP, both agents competed for BCRP-mediated [^{3}H]mithoxantrone transport and depleted cellular ATP levels. Therefore, these compounds could be considered substrates for BCRP. Moreover, our experimental data allowed us to exclude both Gefitinib and Vandetanib as transported substrates of PgP, but did not exclude other types of interaction. Several aspects remain to be clarified such as the effect of verapamil on the BCRP pump in these assays, and the possible involvement of other ATP-binding cassette (ABC) transporters such as multi-drug related protein (MRP1) in Gefitinib and Vandetanib efflux.

To validate our results, the ability of both TK inhibitors to interfere with chemotherapeutics effectiveness was evaluated in a canine kidney in vitro model (MCDK), in which MDR-1 or BCRP were stably transfected. This model was firstly characterised for the efficacy of drug(s) alone and then, in combination. MDCK wt were utilised as control in each experiment. Gefitinib and Vandetanib subtoxic concentrations were measured by exposing MDCK wt, MDR1-MDCK and BCRP-MDCK cells to each TK inhibitors at various concentration (0.1–100 μM) until 3 days. Our data evidenced that 1 μM Gefitinib and 1 μM Vandetanib did not induce any cell growth inhibition (data not shown). Then, MDR1-MDCK and BCRP-MDCK cells were exposed to "classical substrates" doxorubicin and mitoxantrone, respectively. Wild type cells showed an higher sensitivity to chemotherapeutics as respect to transfected cells; in fact, 1 μM of mitoxantrone or doxorubicin induced a cell growth reduction of about 40 and 25% in MDCK wt. Conversely, it was necessary 10 μM of each drug to induced a comparable cell growth reduction (about 30%) in BCRP-MDCK and MDR1-MDCK, respectively. This different sensitivity could be justifiable by the presence of MDR-1 or BCRP in MDR1-MDCK and BCRP-MDCK, respectively; these transporters, extruding drug(s), induced a reduction of their intracellular concentration. Finally, the combined administration of each TKIs with doxorubicin or mitoxantrone, in a sequential schedule with Gefitinib or Vandetanib given before at subtoxic concentration and for short time, increased cell growth inhibition only in the transfected cells as reported in Fig. 2, suggesting an increase in chemotherapeutics intracellular concentration for MDR transporters blocking.

We next characterised the effects of Gefitinib and Vandetanib on the effectiveness of a known BCRP substrate following brief (1 h) or prolonged (5 days) exposure to the TKIs.

The scientific basis of these experiments was that in the clinic TKIs are characteristically dosed chronically [17 and clinicaltrials.gov]. In addition, published data demonstrated synergism of Gefitinib with BCRP substrates [10, 12–15]. However, our previous data showed that 5 days Gefitinib treatment, given 1 day before SN-38, reduced the camptothecin's effectiveness in HT-29, a colon cancer cell line; in this paper, the analysis of the dose-response interactions,

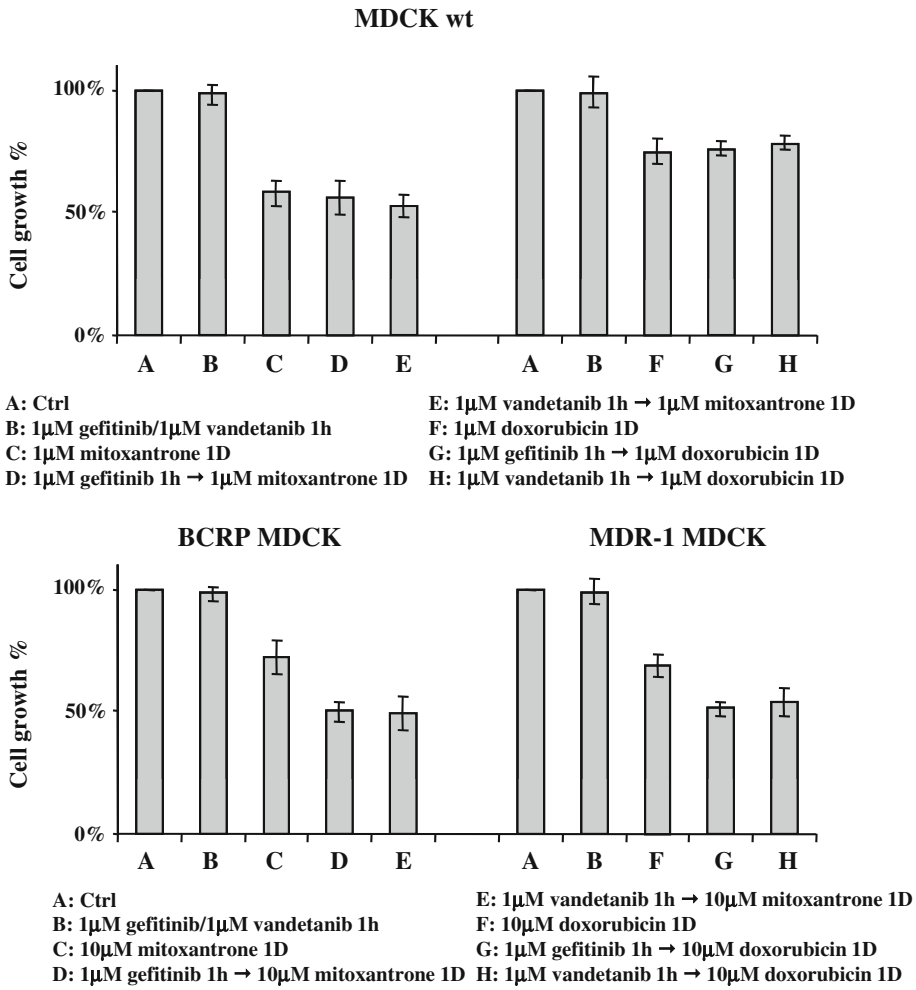
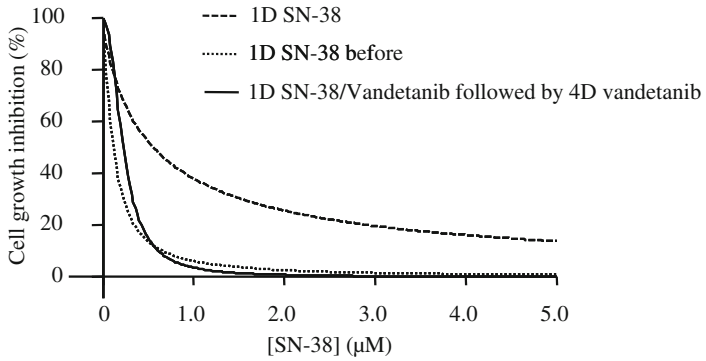


Fig. 2 Cell growth modulation in animal in vitro model. Cells, pre-incubated to Gefitinib or Vandetanib for 1 h, were exposed to doxorubicin or mitoxantrone for 1 day and the cell growth was analysed by MTT assay. Experiments were performed in triplicate and results are expressed as mean ± SD from three experiments

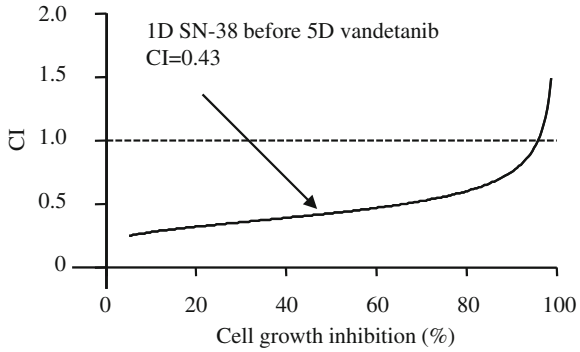
expressed as a non-exclusive case combination index (CI), demonstrated a strong antagonism between Gefitinib and SN-38 (CI = 4.624 ± 0.23) [23]. Then, we also showed that prolonged exposure to Gefitinib or Vandetanib induced an upregulation of BCRP in HT-29 cells, which constitutively expressed BCRP but not PgP [11].

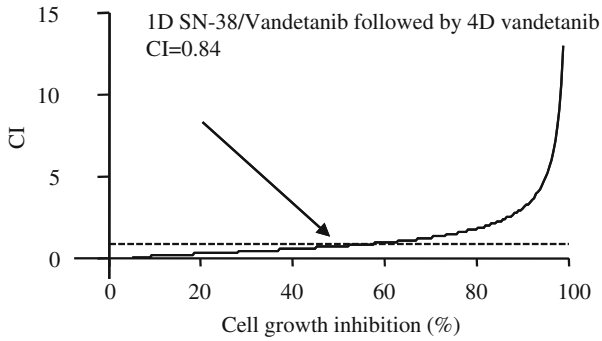
Here, we determined whether Vandetanib was similar to Gefitinib in modulating SN-38 activity. To determine IC50s of Vandetanib and SN-38, HT-29 cells were incubated with each drug at various concentrations and exposure-times; the ability to reduce cell growth, expressed as IC50, has been summarised in Fig. 3. Common criteria of clinical utilisation of these drugs suggested to utilise SN-38 for short time-exposure (1 day) and Vandetanib for longer time-exposure (5 days). To determine whether the two drugs in combination had any synergic, additive or antagonistic effect and so the optimal schedule, HT-29 cells were exposed to drugs, by using a constant ratio (1:1) of their IC50, in three different sequences: SN-38 1 day followed by Vandetanib 5 days; simultaneous exposure to the two drugs for 1 day followed by Vandetanib 4 days and Vandetanib 5 days before SN-38 1 day. The growth inhibition measured with MTT assay and the results analysed with the Median Drug Effect Analysis software are showed in Fig. 3 as dose/growth inhibition and growth inhibition/CI plots. Our data evidenced a synergic interaction when SN-38 was given at the beginning of treatment conversely, a strong antagonism was evident when the TK inhibitor was given before, with a combination index of 3.846 ± 1.15. These results, very similar to data reported for Gefitinib, are consistent with an increase in SN-38 efflux as a consequence of the TKI-induced BCRP overexpression [11]. In Fig. 4, we characterised the ability of both Gefitinib and Vandetanib to induce an increased expression of BCRP in HT-29 in function of time exposure.

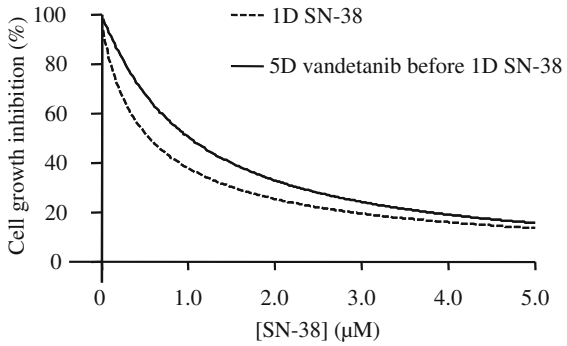
We next measured the intracellular accumulation of SN-38 following prolonged (5 day) exposure to TKIs by HPLC analysis. HT-29 cells were pre-incubated (5 days) in the presence or absence of Gefitinib or Vandetanib. Cells were then incubated for 1 day with SN-38, and the intracellular SN-38 levels were measured immediately following drug wash out from the culture medium and over the next 24 h. In HT-29 cells not pre-treated with a TKI, HPLC analysis showed a reduction of intracellular SN-38 from 80 to



	IC50 μM	
exposure time	vandetanib	SN-38
18 hours	80±4.8	0.77±0.20
1 day	59±3.6	0.50±0.12
2 days	19±1.8	0.26±0.04
3 days	10±0.4	0.13±0.01
5 days	3±0.4	N.D.







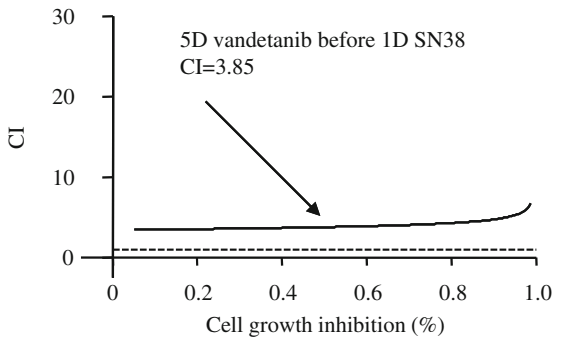


Fig. 3 Cytotoxicity of SN-38 plus Vandetanib. HT-29 cells were incubated with Vandetanib and SN-38 alone or in combination utilising three different. Each experiment was carried out as reported in Materials and Methods and the results are showed as [SN-38] (μM)/cell growth inhibition (%) and cell growth inhibition/CI plots of the mean of three different experiments. In the table, IC50 of Vandetanib and SN-38 in HT-29 cells at various times of incubation are reported

40 ng/10^6cells over 24 h. In contrast, pre-incubation of HT-29 cells with a TKI for 5 days markedly (about 4-fold) reduced the SN-38 levels in cells measured immediately after 1 day of SN-38 treatment, and within 1 h of washout from the culture medium, intracellular SN-38 was not detectable. Gefitinib and Vandetanib showed similar results. The data for Gefitinib are shown in Fig. 5.

To assess effects on cell cycle, HT-29 cells, pre-exposed or not to (5 days) Gefitinib or Vandetanib, were incubated for 1 day with SN-38. Cells were then evaluated by flow cytometry to assess accumulation in S-phase, a characteristic of exposure to camptothecins. A strong reduction of cells accumulation in S phase, SN-38 dependent, was evident after pre-treated with Gefitinib. Conversely, Vandetanib pre-exposion induced a slight phenomenon (Fig. 6). In further experiments, the effect of brief pre-exposures (1 h) to TKIs on growth and S-phase fraction of SN-38 treated HT-29 cells was investigated in the presence or absence of BCRP inhibition. Combination with Ko143, an irreversible BCRP inhibitor, with subtoxic concentrations (10% IC_{50}) of Gefitinib or Vandetanib, reduced HT-29 cell proliferation (Fig. 7a). This effect was similar to that observed for known BCRP substrates (SN-38 and mitoxantrone) in the same assay system. Moreover, the effect of brief exposure to TKIs on cell cycle was carried out as previously described. Analysis of cell cycle in

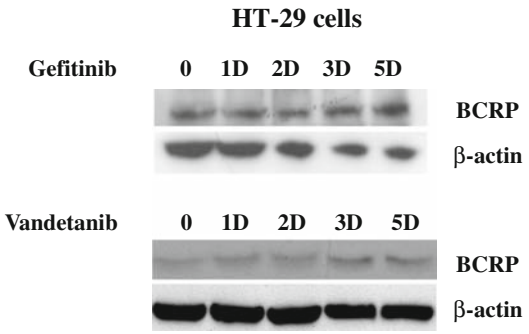


Fig. 4 Increased ABCG2 expression after exposure to Gefitinib or Vandetanib. HT-29 cells were incubated with Gefitinib or Vandetanib for 1–5 days and drug-dependent increases in ABCG2 expression were determined by Western blotting

HT-29 cells by flow cytometry showed that 1 h pre-exposure to either Gefitinib or Vandetanib increased the proportion of tumor cells in S-phase following SN-38 treatment (Fig. 7b).

The experiments with brief (1 h) pre-exposures to TKIs provide additional evidence that both Gefitinib and Vandetanib are transported substrates of the BCRP multi-drug transporter, possibly competing with camptothecins for binding and inhibiting drug efflux, thereby increasing the antitumor effects of SN-38 treatment. However, after prolonged (5 days) exposure to Gefitinib or Vandetanib, the impact on the biological effects of SN-38 is different. In this scenario, we propose that TKI pre-treatment leads to increased BCRP multi-drug transporter protein expression, increasing SN-38 transport, thereby reducing the antitumor effects of SN-38. We further hypothesise that in this model, the inhibitory effects of Gefitinib or Vandetanib on SN-38 transport by BCRP are not offset the increased levels of BCRP produced following prolonged exposure to these TKIs, with the net result that the anti-tumour effects of SN-38 are reduced.

Discussion and conclusions

In this report, we have investigated the effects of the TKIs Gefitinib and Vandetanib with the MDR related proteins, BCRP and PgP after both brief (1 h) and prolonged (5 day) exposure periods. Characterisation of TKI transporter binding, as a substrate or an inhibitor, requires short exposure times, whereas we suggest that the cellular effects should be examined over a longer time period, consistent with the anticipated chronic exposure to these agents in the clinic.

We have demonstrated that Gefitinib and Vandetanib may be substrates for BCRP transport. In contrast, there is no evidence that Gefitinib or Vandetanib are substrates for PgP, although our work and the work of others suggests that they may act as inhibitors for known PgP substrates [22]. Consistent with this, brief exposure to either Gefitinib or Vandetanib, or the known BCRP substrates, SN-38 and

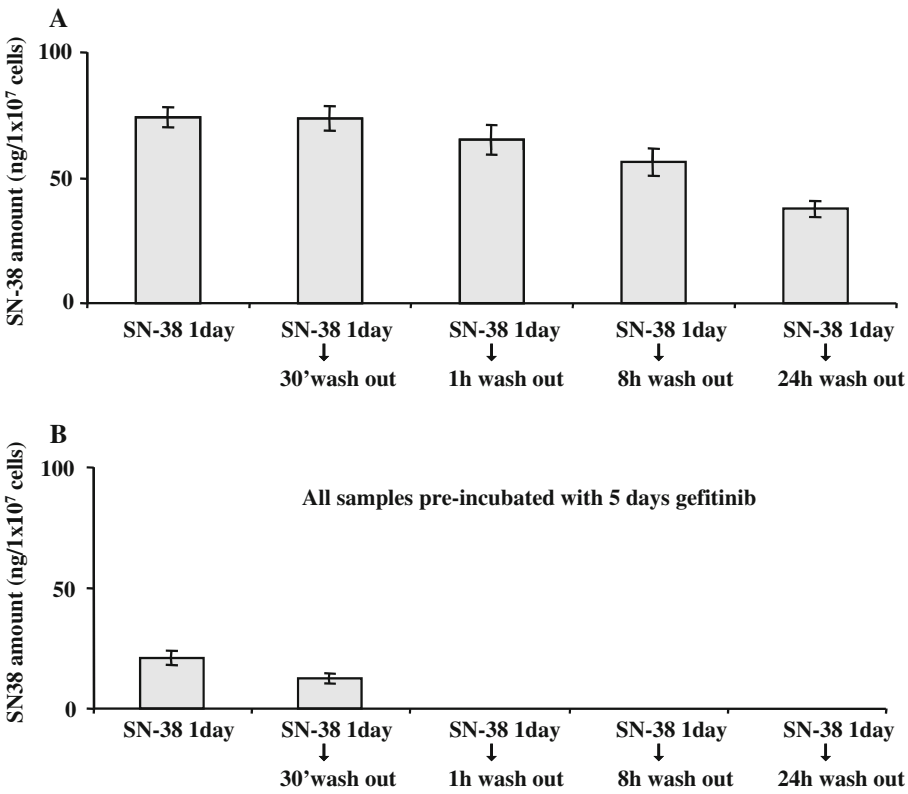


Fig. 5 SN-38 intracellular accumulation. Analysis of Gefitinib ability to reduce SN38 accumulation. **a** HT-29 cells were treated with 0.56 μM SN-38 for 1 day and, after 0, 30 min, 1, 8 and 24 h drug wash out, cells were detached and stored at −20°C. The amount of SN-38 in each sample was determined by HPLC. **b** HT-29 cells, pre-exposed to 3.8 μM Gefitinib for 5 days, were treated as described in A. Experiments were performed in triplicate and results are expressed as mean ± SD from three experiments

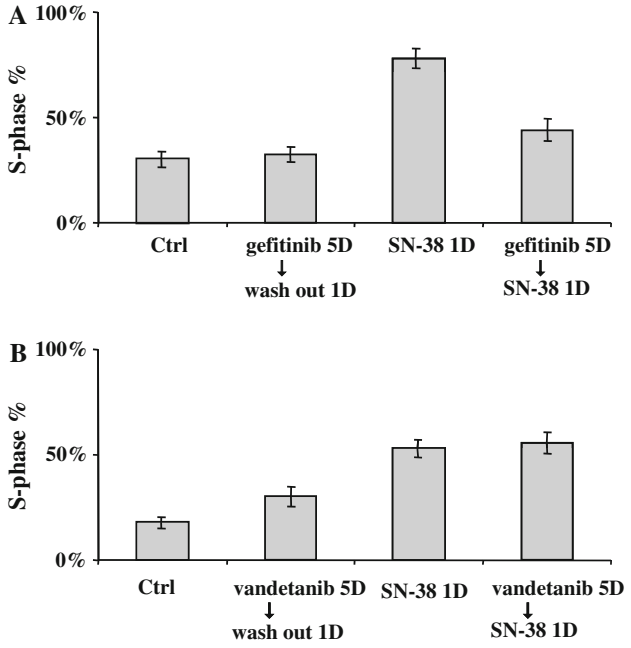


Fig. 6 Cell cycle modulation after TK inhibitor prolonged exposure. HT-29 cells, pre-incubated to 3.8 μM Gefitinib or 3 μM Vandetanib for five days, were exposed to 0.56 μM SN-38 for 1 day and the cell cycle was analysed by flow cytometry analysis. Experiments were performed in triplicate and results are expressed as mean ± SD from three experiments

mitoxantrone, produced greater anti-tumour effects in vitro when the BCRP transporter was blocked. Furthermore, brief exposure to Gefitinib or Vandetanib increased the effectiveness of SN-38.

To confirm the specificity of Gefitinib and Vandetanib interaction with BCRP or PgP, cell growth inhibition by doxorubicin or mitoxantrone, with or without a preexposure to TK inhibitors, was evaluated in a "clean" in vitro model, in which was possible to selectively analyse drug interaction with human PgP or BCRP. Our results confirmed the already well known capability of BCRP and PgP to extrude mitoxantrone and doxorubicin, respectively and the effectiveness of TK inhibitors, after short exposure, to limit chemotherapeutics extrution, acting as competitive substrates of both MDR transporters.

Prolonged exposure to Vandetanib, as already reported for Gefitinib [23], reduced the effectiveness of the SN-38 with a clear antagonism between the two agents. This antagonism may be explained, at least in part, by increased expression of BCRP [11] reducing SN-38 levels in tumour cells, and consequently reducing the antitumor effects of SN-38 such as S-phase accumulation.

Previous reports have shown that Gefitinib is able to revert MDR related resistance in various in vitro preclinical cancer models [13–15, 32–34]. Our data, after brief

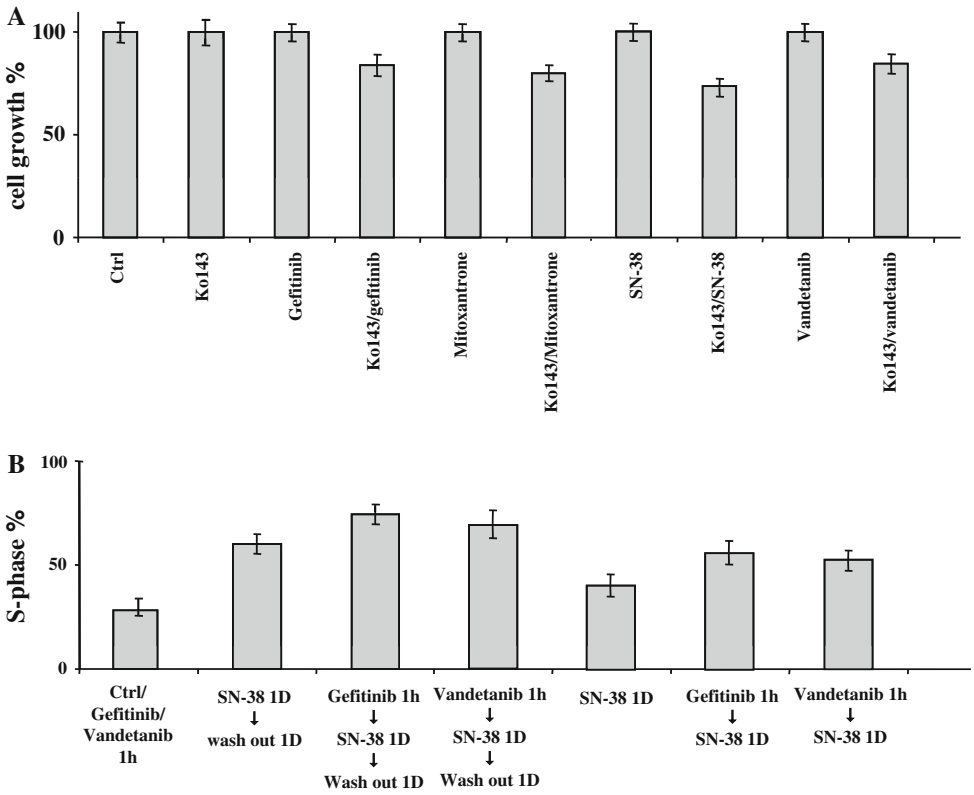


Fig. 7 Cell cycle modulation and cytotoxicity after TK inhibitor short exposure. TK Inhibitors short exposure (1 h) induced cell growth inhibition and enhanced SN-38 activity. **a** HT-29 cells were incubated with 0.38 μM Gefitinib, 0.3 μM Vandetanib, 0.056 μM SN-38, 0.2 μM mitoxantrone in the presence or absence of 20 μM Ko143 and the survival of cells was determined using MTT assay. **b** HT-29 cells were incubated, for 1 h with 3.8 μM Gefitinib or 3 μM Vandetanib followed by 0.56 μM SN-38 for 1 day and the cell cycle was analysed by flow cytometry analysis

exposure to Gefitinib, are in agreement with these previous reports and suggest that Gefitinib (and Vandetanib) could enhance the anti-tumour activity of agents that are substrates for the BCRP transporter. Although both Vandetanib and Gefitinib show similar effects on BCRP, the selectivity and potency of these compounds as TKIs are quite distinct, although both agents inhibit EGFR tyrosine kinase activity [35, 36]. Since the structures of both of these compounds are based around an anilinoquinazoline core, we suggest that further studies using chemically unrelated EGFR TKIs or anilinoquinazoline-based TKIs which do not inhibit EGFR may be required to better understand the basis of the interactions of Gefitinib and Vandetanib with the BCRP transporter.

Moreover, our results, after prolonged exposure to either Gefitinib or Vandetanib could be provide a possible explanation results reported by Usuda [37], who described a case of non-small cell lung cancer (NSCLC) in which expression of BCRP was associated with acquired resistance to Gefitinib, independent of EGFR mutations.

Gefitinib and Vandetanib are currently in clinical usage or in phase III clinical trials, respectively, for NSCLC patients. Our results suggest that the interaction of these agents with multi-drug resistance proteins may be an important determinant of drug resistance, particularly when these agents are combined with certain chemotherapeutic agents, and we further suggest that this should be considered in clinical trial designs. Moreover, our data demonstrate that even where the molecular mechanisms of drug activity are understood through detailed preclinical pharmacology, these data need also to take more account of the effects of chronic exposure that will be used in the clinical situation. Our data clearly show different in vitro antitumour effects of Vandetanib or Gefitinib on the activity of SN-38, depending on the time of exposure to the TKIs. Only with this approach can the optimal doses and schedules of novel therapeutics be identified, either as monotherapy, or in combination with chemotherapies. Further studies evaluating additional cellular endpoints followed by in vivo validation in preclinical models are warranted to facilitate rationale clinical trial designs that could test these hypotheses for the benefit of patients.

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