Src Family Kinase Inhibitor Saracatinib (AZD0530) Impairs Oxaliplatin Uptake in Colorectal Cancer Cells and Blocks Organic Cation Transporters

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Abstract

Elevated Src family kinase (SFK) activity is associated with tumor invasion and metastasis. The SFK inhibitor saracatinib (AZD0530) is currently in phase II trials in patients including those with colorectal cancer (CRC), where links between SFK activity and poor prognosis are particularly striking. Saracatinib is likely to be used clinically in combination regimens, specifically with 5-fluorouracil (5-FU) and oxaliplatin, in CRC. The aim of this study was to determine the effect of saracatinib on oxaliplatin and 5-FU efficacy in CRC cells. Saracatinib did not modulate 5-FU efficacy but antagonized oxaliplatin in a schedule-specific manner through reduced oxaliplatin uptake via an SFK-independent mechanism. Saracatinib resembles the pharmacophore of known organic cation transporter (OCT) inhibitors and reduced oxaliplatin efficacy maximally in cells overexpressing OCT2. These data suggest that oxaliplatin uptake in CRC is attenuated by saracatinib via inhibition of OCT2, a potential consideration for the clinical development of this SFK inhibitor.

Introduction

The gene encoding the nonreceptor protein tyrosine kinase c-Src was the first proto-oncogene to be described when identified as the cellular homologue of v-Src, the transforming factor of the Rous sarcoma retrovirus (1). c-Src is one of the nine members of the Src family kinases (SFK) and, along with two other members, Fyn and Yes, is ubiquitously expressed (2). Of the nine SFKs, c-Src is the most comprehensively studied on its role in cancer. Increased levels of c-Src activity occur in several solid tumor types, including colorectal, breast, and lung cancers (3). In colorectal cancer (CRC), c-Src activity is elevated in more than 70% of tumors and increases with tumor progression (4–7) where high c-Src activity correlated with poor clinical prognosis (8).

A major role of c-Src activity in cancer is promotion of migration and invasion leading to a more aggressive and metastatic cancer phenotype (9). Currently, there are four SFK inhibitors in oncology clinical trials, including saracatinib (AZD0530), a potent, orally available SFK inhibitor (10). Consistent with the studies outlined above, SFK inhibition by saracatinib has varying and cell context–dependent effects on cell proliferation in vitro and in vivo but consistently inhibits migration, invasion, and metastasis across a range of cancer types (11). In common with other targeted agents, saracatinib is likely to be used clinically in combination with other chemotherapeutic agents; indeed, preclinical studies have shown that saracatinib enhances the antimigratory effects of the epidermal growth factor receptor (EGFR) inhibitor gefitinib in endocrine-resistant breast cancer cell lines (12) and restores tamoxifen sensitivity to resistant breast cancer cell lines (12, 13).

In a phase II trial in patients with previously treated metastatic CRC, saracatinib alone had negligible effect on overall survival (14). Thus, combining saracatinib with standard-of-care agents may present an alternative strategy to target SFK in CRC. Therefore, the aim of this study was to investigate the effect of saracatinib on oxaliplatin and 5-fluorouracil (5-FU) responses in CRC cell lines.

Materials and Methods

Cell culture

HCT116 cells (American Type Culture Collection) were cultured in McCoy’s 5A (Life Technologies, Inc.), WiDr (ATCC) in RPMI (Life Technologies), and parental HEK293...
(ATCC) and HEK293-overexpressing organic cation transporter (OCT) isoforms (15) in DMEM/F-12 (Life Technologies), all supplemented with 10% fetal bovine serum (Biowest) in a humidified atmosphere at 37°C and 5% CO₂. OCT-overexpressing cells were also supplemented with 800 μg/mL G418 (Life Technologies). All cell lines were authenticated using the AmpFISTR system (Applied Biosystems) during the study. Oxaliplatin (Alexis Biochemicals), cisplatin (Sigma), 5-FU (Sigma), saracatinib, and PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazol[3,4-d]pyrimidine; Calbiochem) were diluted to the indicated concentrations in media from stock solutions.

**Western blotting**

Cell lysis and Western blotting were carried out as previously described (16). The following primary antibodies were used: anti-pY576FAK, anti-pY861FAK (Biosource), anti-FAK (Santa Cruz), anti-c-Src (Upstate), anti-Yes, anti-Fyn (Wako), and anti-actin (Sigma).

**Sulforodamine B assay**

Cells were plated in 96-well plates and treated as described, fixed for 1 hour with ice-cold 10% trichloroacetic acid, and left to dry. Cells were then stained with 0.4% Sulforodamine B (SRB) for 15 minutes and washed with 1% acetic acid, the dye was solubilized with 1.5 mol/L Tris-HCl (pH 8.8), and the absorbance at 540 nm was determined.

**Comet-X assay**

After treatment, cells were irradiated with 45-Gy from an X-ray source and processed for comet-X assay as previously described (17), where 100 nuclei on two slides were measured for each treatment.

**Platinum concentration determination**

After treatment, cells were either processed with a blood and cell culture DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions to generate samples to determine DNA-associated platinum concentration, or lysed in cell lysis buffer [Cell Signaling; supplemented with protease inhibitor cocktail (Sigma)] to determine soluble and protein-associated platinum levels. DNA concentration was determined on a Nanodrop-2000 (Thermo Scientific) and protein concentration was determined using the BCA protein assay (Pierce) and anti-actin (Sigma). The OCT pharmacophore was generated as follows: Tetrapentylammonium ion (TPA) was overlaid on decynium-22 using the Omega software package (OpenEye Scientific Software) and low-energy conformations of TPA and decynium-22 were generated. ROCS (Rapid Overlay of Chemical Structures; ref. 18) was then used to overlay TPA conformations on the lowest-energy decynium-22 conformer, based only on shape (Shape-Tanimoto scoring function). The best 1,000 TPA conformers from this filter were then compared with decynium-22 using shape and electrostatics, using the ET-Combo scoring function from EON. Finally, using this overlaid decynium-22/TPA pair, a four-point pharmacophore was generated using MOE (Chemical Computing Group). Subsequently, a pharmacophore search was performed for conformations of a set of 20 known nontransported OCT2 inhibitors (19) and for saracatinib. The MOE Wash function was used in combination with ACD/Labs (Advanced Chemistry Development, Inc.) to assign protonation states and tautomers of the library of compounds before conformer enumeration by Omega.

**Statistics**

Student's t test, two-way ANOVA, and IC₅₀ calculations were performed using Prism (GraphPad).

**Results**

**Saracatinib reduced the efficacy of oxaliplatin, but not cisplatin, in a schedule-dependent manner**

Because saracatinib is likely to be used to treat patients with metastatic CRC in combination with other standard of care drugs, the effect of saracatinib was assessed in two CRC cell lines treated with oxaliplatin or 5-FU. The cell lines

**Wound healing assay**

Scratches were made in confluent HCT116 cell monolayers grown in ImageLock 24-well plates using a Woundmaker (Essen); medium was removed and replaced with medium containing the specified drugs. Cells were placed in an Incucyte (Essen) and the wound was imaged every 2 hours for 48 hours. The percent wound closure after 48 hours was determined using Incucyte scratch wound assay software (Essen).

**ASP⁺ uptake assay**

HEK293 cells overexpressing OCT isoforms (10⁶) were warmed with warm HCO₃⁻-free Ringer's-like solution (RLS; 130 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L CaCl₂, 1 mmol/L MgSO₄, 1 mmol/L Na₂HPO₄, 20 mmol/L HEPES, 18 mmol/L glucose (pH 7.4)) and incubated in RLS containing the indicated concentration of saracatinib for 10 minutes at 37°C. 4-(4-Dimethylaminostyryl)-N-methylpyridinium (ASP⁺; Sigma) was added to a final concentration of 1 μmol/L and the cells were incubated for a further 30 minutes at 37°C. Cells were washed twice in ice-cold RLS and solubilized in isopropanol. Fluorescence was measured after 485 nm λₑₓ at 610 nm on a Fluostar Optima plate reader (BMG Labtech).
chosen for this study, HCT116 and WiDr, have low and medium levels of c-Src activity, respectively (20, 21). Saracatinib treatment for 24 hours reduced the levels of phosphorylated FAK, a target of SFKs, in a concentration-dependent manner (Fig. 1A), showing that saracatinib inhibited SFK activity in these cellular contexts. It is interesting to note that even low concentrations of saracatinib lead to increased levels of total FAK, although the mechanism by which this occurs is unclear. Saracatinib had little effect on the proliferation of HCT116 or WiDr cells; a 6-day treatment of 1 μmol/L saracatinib had minimal effect on either cell line (Fig. 1B), consistent with previously published data (11). To better mimic clinical exposure, cells were treated for 1 hour with oxaliplatin or for 6 days with 5-FU, both of which caused a concentration-dependent reduction in cell population (Fig. 1B; Supplementary Fig. S1). The addition of saracatinib had no effect on 5-FU efficacy in either cell line (Supplementary Fig. S1). However, if saracatinib and oxaliplatin were added simultaneously and saracatinib was replenished after oxaliplatin removal, there was a significant decrease in oxaliplatin efficacy in both cell lines [Fig. 1B; P < 0.001 for oxaliplatin versus oxaliplatin and saracatinib in HCT116 and WiDr (two-way ANOVA)]. The negative effect of saracatinib on oxaliplatin was schedule dependent; if saracatinib was added to

Figure 1. Saracatinib reduces oxaliplatin efficacy in a schedule-dependent manner. A, HCT116 (left) and WiDr (right) cells were exposed to the indicated concentrations of saracatinib for 24 h and the effect on total FAK or FAK phosphorylation on tyrosine 576 or 861 was assessed by Western blotting. Results are representative of three independent experiments. B to D, HCT116 (left) and WiDr (right) cells were exposed to the indicated concentrations of oxaliplatin (B and C) or cisplatin (D) for 1 h and/or saracatinib for 6 d (B–D). Where two drugs were used in combination (white squares), saracatinib was either added at the same time as oxaliplatin/cisplatin and more saracatinib added after the removal of oxaliplatin/cisplatin (B and D) or saracatinib was only added after the removal of oxaliplatin (C). Six days after the removal of oxaliplatin/cisplatin, cells were fixed and stained with SRB, and the absorbance relative to untreated (UnT) cells was determined as an approximation of cell population. Graphs show the mean of three independent experiments carried out in triplicate ± SEM.
cells after the 1-hour oxaliplatin exposure, there was no effect on oxaliplatin efficacy (Fig. 1C). Furthermore, concomitant saracatinib exposure did not affect cisplatin (Fig. 1D) or carboplatin (Supplementary Fig. S2) efficacy, suggesting that saracatinib does not reduce the efficacy of all platinating agents, but interacts with oxaliplatin specifically.

**Saracatinib reduced oxaliplatin-induced DNA cross-links**

The mechanism of action of oxaliplatin is thought to be predominantly via DNA damage induced by DNA-platinum-DNA interstrand cross-links (22). Therefore, the effect of saracatinib on oxaliplatin-induced DNA cross-links was investigated using the comet-X assay (23). Cells were treated with oxaliplatin or cisplatin for 1 hour in the presence (and sara) or absence (then sara) of saracatinib and then grown in the absence of the platinum agent, with saracatinib where indicated, for a further 8 hours to allow DNA interstrand cross-links to form (24). In the comet-X assay, reduced DNA in the comet tail is indicative of increased DNA interstrand cross-linking. The exposure of HCT116 or WiDr to oxaliplatin or cisplatin caused a significant reduction in the amount of DNA in the comet tail, whereas the presence of saracatinib during the 1-hour oxaliplatin exposure caused a significant increase in the comet tail relative to oxaliplatin only (Fig. 2A). Adding saracatinib after oxaliplatin exposure did not alter comet tail size, nor did addition of saracatinib

![Figure 2. Saracatinib inhibits oxaliplatin uptake. A to C, HCT116 (left) and WiDr (right) cells were not treated (white columns) or exposed to 120 μmol/L (HCT116) or 80 μmol/L (WiDr) oxaliplatin (black columns) or 90 μmol/L (HCT116) or 60 μmol/L (WiDr) cisplatin (gray columns) for 1 h in the presence (ox/cis and sara) or absence (ox/cis then sara) of 1 μmol/L saracatinib. Cells were either harvested (B and C, 0 h) or fresh medium with or without 1 μmol/L saracatinib (as indicated) was added and cells were harvested 8 h later [B and C (8 h) and A]. A, cells were processed and analyzed for comet-X assay as described in Materials and Methods. B and C, genomic DNA was extracted as described in Materials and Methods (B) or cells were lysed in cell lysis buffer (C) and the level of platinum in the samples was determined by inductively coupled plasma mass spectrometry. Platinum concentration was normalized to the equivalent oxaliplatin or cisplatin only treatment from the corresponding time point. Graphs show the mean of three independent experiments ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001, two-tailed unpaired t test.](cancerres.aacrjournals.org)
during cisplatin exposure. This suggests that saracatinib can cause a reduction in the amount of oxaliplatin-induced DNA interstrand cross-links, but only when present at the time of oxaliplatin treatment.

To formally test if the reduction in oxaliplatin-induced DNA interstrand cross-links caused by saracatinib was due to reduced platinum-DNA adducts, the level of DNA-associated platinum was measured using inductively coupled plasma mass spectrometry. Genomic DNA was isolated from cells treated with oxaliplatin or cisplatin in the presence or absence of saracatinib, either immediately after the removal of the platinum or 8 hours after platinum removal. Results shown in Fig. 2B are relative to the corresponding oxaliplatin or cisplatin only treatment. The presence of saracatinib during the 1-hour oxaliplatin exposure (ox and sara) reduced the amount of DNA-platinum adducts by ∼50% immediately after and 8 hours after oxaliplatin removal (Fig. 2B). If saracatinib was added after removal of oxaliplatin (ox then sara), it had no effect on DNA-platinum adduct level. Regardless of the schedule, saracatinib had no effect on DNA-platinum adducts in cisplatin-exposed cells. This confirmed that saracatinib reduced oxaliplatin-induced DNA-platinum adduct levels if present during the oxaliplatin exposure.

**Saracatinib reduced uptake of oxaliplatin**

There are at least two possible explanations for the change in oxaliplatin-induced DNA-platinum adducts caused by saracatinib. Either saracatinib causes an increase in the rate of removal of oxaliplatin-induced DNA-platinum adducts (if present during treatment) or saracatinib reduces the total level of oxaliplatin in the cell, either by inhibiting uptake or increasing efflux. Due to the fact that the relative level of DNA-platinum adducts did not change over time, the reduced oxaliplatin uptake explanation seemed the more plausible. Therefore, the level of soluble/protein-adducted platinum was determined from cells treated with the same drug combinations as in Fig. 2B. The level of soluble/protein-adducted platinum was reduced to 60% to 80% compared with oxaliplatin only when saracatinib was present during the 1-hour oxaliplatin exposure (ox and sara) and not in any of the other experimental conditions (Fig. 2C). This strongly suggested that the presence of saracatinib reduced the uptake of oxaliplatin into HCT116 and WiDr CRC cells.

**c-Src RNA interference did not phenocopy the effect of saracatinib on oxaliplatin efficacy**

The effect of c-Src RNA interference (RNAi) on oxaliplatin efficacy was assessed to determine whether the interaction between saracatinib and oxaliplatin was due to inhibition of c-Src by saracatinib. Transfection of siRNAs targeting c-Src mRNA into HCT116 cells consistently reduced the level of c-Src (Fyn levels were also reduced whereas levels of Yes were unchanged; Fig. 3A) and also reduced phosphorylated FAK levels, showing reduced c-Src activity. However, c-Src RNAi did not affect oxaliplatin efficacy (Fig. 3B; P > 0.05, two-way ANOVA), suggesting that c-Src activity is not necessary for oxaliplatin efficacy or uptake. Therefore, the observed interaction between saracatinib and oxaliplatin is unlikely to be due to inhibition of c-Src activity.

**The SFK inhibitor PP2 did not phenocopy the effect of saracatinib on oxaliplatin efficacy**

As well as inhibiting c-Src, saracatinib also inhibits other members of the SFK family (11). Therefore, to determine whether the interaction observed between saracatinib and oxaliplatin could be attributed to SFK inhibition, the effect on oxaliplatin efficacy of a second SFK inhibitor, PP2, was investigated. Unlike saracatinib treatment, 3-day exposure of HCT116 cells to PP2 leads to a reduction in cell number (data not shown), presumably due to inhibition of a non-SFK kinase by PP2 but not by saracatinib (25), which makes direct comparison between saracatinib/oxaliplatin and PP2/oxaliplatin combinations difficult over prolonged periods. Thus, the experimental design was altered to examine the effect of PP2 or saracatinib only during the 1-hour oxaliplatin exposure. Both saracatinib and PP2 inhibited SFK activity within 1 hour, as assessed by Western blotting for levels of phosphorylated FAK (Fig. 3C). When saracatinib was added to cells only during the 1-hour oxaliplatin exposure, oxaliplatin efficacy was reduced [Fig. 3D, top; P < 0.001 for oxaliplatin versus oxaliplatin and saracatinib (two-way ANOVA)] as predicted by the hypothesis that saracatinib inhibits oxaliplatin uptake. However, the addition of PP2 during a 1-hour oxaliplatin exposure had no effect on the efficacy of oxaliplatin [Fig. 3D, bottom; P = 0.98 for oxaliplatin versus oxaliplatin and PP2 (ANOVA)]. Overall, these data comparing two SFK inhibitors in conditions where SFKs are inhibited by both suggest that the effect of saracatinib on oxaliplatin uptake is independent of inhibition of SFK activity.

**Oxaliplatin did not affect the potency of saracatinib**

Because saracatinib inhibited oxaliplatin uptake, it is possible that both drugs enter the cell via the same transporter and that, reciprocally, the presence of oxaliplatin may also inhibit the uptake of saracatinib. To test this hypothesis, the effect of oxaliplatin on biomarkers of SFK inhibition in saracatinib treated cells was investigated. As the predominant effect of saracatinib in a range of cancer cell types (including CRC, breast, and lung) was to inhibit migration (11), combinations of saracatinib and oxaliplatin were assessed on their antimigratory properties. Saracatinib (1 μmol/L) alone caused a 74% reduction in the migration of HCT116 cells, 3 μmol/L oxaliplatin (a concentration whose efficacy was antagonized by saracatinib in constant challenge experiments; data not shown) had no effect, whereas 10 μmol/L oxaliplatin caused a 34% reduction in migration (Fig. 4A; P < 0.05, Student’s t test). When 3 μmol/L oxaliplatin was combined with 1 μmol/L saracatinib, it did not alter the ability of saracatinib to inhibit migration, whereas there was a further reduction in migration when 1 μmol/L saracatinib and 10 μmol/L oxaliplatin were combined (P < 0.05, Student’s t test). The effect of oxaliplatin on saracatinib-mediated reduction in phosphorylated FAK was also assessed, where HCT116 cells were treated for 2 hours with varying concentrations of saracatinib and oxaliplatin.
oxaliplatin. A 2-hour time point was chosen, as oxaliplatin can provoke an adaptive increase in SFK activity, but it takes longer than 2 hours for this to occur (ref. 26 and confirmed by data not shown). Irrespective of the presence of oxaliplatin, saracatinib reduced FAK phosphorylation over the same concentration range (Fig. 4B). These data suggest that oxaliplatin does not impair the ability of saracatinib to inhibit SFK activity, and thus, that it is unlikely that saracatinib uptake is inhibited by oxaliplatin.

**Saracatinib inhibited uptake by OCTs**

The observation that saracatinib inhibited the uptake of oxaliplatin but not cisplatin implies that saracatinib inhibits a transporter that distinguishes between these two platinum drugs. Members of the OCT family of transporters can influx oxaliplatin more effectively than cisplatin (27, 28), whereas OCT3 expression has been shown to correlate with oxaliplatin sensitivity in CRC (29). OCT1, OCT2, and OCT3 are expressed in HCT116 and WiDr cells (30, 31), and therefore...
the effect of saracatinib on uptake via OCT1, OCT2, and OCT3 was assessed. HEK293 cells that overexpress OCT1, OCT2, or OCT3 (15) were incubated in saracatinib for 10 minutes before Asp+, a fluorescent substrate of OCTs, was added to the cells. The effect of saracatinib on Asp+ uptake was then determined. Saracatinib was able to inhibit uptake of Asp+ in all three cell lines (IC50 for OCT1, 27.1 μmol/L; OCT2, 0.46 μmol/L; and OCT3, 10.3 μmol/L), although it was over an order of magnitude more potent against cells overexpressing OCT2 (Fig. 5A; Asp+ uptake in parental HEK293 cells was negligible). Thus, saracatinib inhibited uptake via OCT2 at concentrations that inhibit oxaliplatin uptake. Furthermore, PP2 had no significant effect on Asp+ uptake in cells overexpressing OCT2 [Supplementary Fig. S3; P > 0.05, PP2 versus DMSO (two-way ANOVA)], consistent with PP2 not causing oxaliplatin resistance.

To test further the causal relationship between OCT transporter function and saracatinib-mediated antagonism of oxaliplatin, the effect of combining oxaliplatin and saracatinib was determined in OCT-overexpressing cells. All the HEK293 cells, including parental cells, exhibited a concentration response to oxaliplatin in the absence of saracatinib; however, cells overexpressing OCT2 were >20-fold more sensitive to oxaliplatin than any of the other cells (Fig. 5B; oxaliplatin IC50: OCT2, 14 nmol/L; parental, 320 nmol/L; OCT1, 520 nmol/L; OCT3, 910 nmol/L). Furthermore, whereas the addition of saracatinib reduced the oxaliplatin efficacy in all cell lines, this was most pronounced in OCT2-overexpressing cells (Fig. 5C). Specifically, 1 μmol/L saracatinib caused the IC50 of oxaliplatin to increase 1.7-, 1.6-, and 2.4-fold in parental, OCT1, and OCT3 cells, respectively, and 5.8-fold in OCT2-overexpressing cells. Taken together, these data implicate saracatinib-mediated modulation of OCT2 as the mechanism underlying reduced uptake of oxaliplatin in CRC cell lines.

Saracatinib fits the pharmacophore of known OCT inhibitors

To explore the molecular basis for saracatinib (Fig. 6A) as an OCT2 inhibitor, a pharmacophore was derived identifying key molecular features required for recognition by OCT2. Using two potent OCT2 inhibitors, decynium-22 and TPA (Supplementary Table S1), a four-point pharmacophore was generated, consisting of a central feature satisfied by a cationic or hydrogen bond donor group, surrounded by three hydrophobes in an approximately T-shaped configuration (Fig. 6B). This pharmacophore was then able to identify 19 compounds from a set of 20 known nontransported OCT inhibitors (ref. 19; Supplementary Table S1). Interestingly, the pharmacophore derived here bears considerable similarity in orientation and nature to a previously reported four-point OCT1 pharmacophore (32). However, their central pharmacophore point differs in requiring solely a cationic group, which would lead to exclusion of a small number of compounds from the OCT inhibitor set considered here.

Saracatinib was found to adopt low-energy conformations, which also satisfy all four features of this OCT pharmacophore. One such conformation of saracatinib satisfies the central polar feature via a cationic amine in the piperazine ring; ligand methylene and cyclic groups also satisfy the hydrophobicity requirements of the OCT pharmacophore (Fig. 6C). The second distinct saracatinib conformation maps to the polar feature of the pharmacophore via an NH group that links the benzodioxolane and quinazoline rings. For this conformation, two aromatic rings and one aliphatic six-membered ring satisfy the hydrophobicity requirements of the OCT pharmacophore (Fig. 6D). Both these conformations...
lie within 0.2 kcal/mol of the lowest-energy pose. Therefore, saracatinib possesses the shape and polarity necessary for OCT inhibition. Furthermore, PP2, which does not cause resistance to oxaliplatin or inhibit uptake via OCT2, does not satisfy the OCT pharmacophore.

Discussion

Because cancer is a multi-lesion group of diseases that will usually require a combination therapy approach, it is imperative that drug interactions are understood to optimize the chances of treatment success. Drug resistance is a predominant obstacle for the treatment of cancer and can occur at a variety of loci proximal and distal to drug targets. Changes in drug transport in and out of cancer cells have been a recognized mechanism of drug resistance for a variety of anticancer drugs. This can occur at the biological level, where, for instance, drug efflux pumps are overexpressed in tumor cells, or via competition of two drugs for the same uptake mechanism. A plethora of oncogenic signal transduction interrupting drugs have entered oncology clinical trials, and thus far, very few have achieved single agent status. Because current standard-of-care therapies show efficacy in most tumor types, combinations with conventional cytotoxic agents are common, not only following ethical considerations but also in a hope that efficacy can be increased by combination therapy. The rationale for investigating the combination of saracatinib with CRC standard-of-care chemotherapeutics was to predict whether saracatinib might enhance or detract from the chemotherapeutic agent if the two were combined in the clinic—a likely scenario if saracatinib were used to treat patients with advanced metastatic CRC.

The main findings of this study are that the SFK inhibitor saracatinib can antagonize the effects of oxaliplatin in human CRC cells in vitro. This antagonism was highly schedule dependent, with the presence of saracatinib during oxaliplatin treatment being essential for antagonism. Furthermore, saracatinib did not antagonize cisplatin or carboplatin efficacy, demonstrating oxaliplatin specificity not common to all DNA platinating agents. The oxaliplatin antagonism was

Figure 5. Saracatinib inhibits uptake through OCT2. A, HEK293 cells overexpressing the indicated OCT isoforms were incubated in the presence of the indicated concentration of saracatinib for 10 min and then 1 μmol/L Asp⁺ and saracatinib for 30 min at 37°C. The level of Asp⁺ uptake was measured and normalized to the Asp⁺ uptake in cells not exposed to saracatinib (100%) or Asp⁺ (0%). B and C, parental HEK293 and HEK293 overexpressing the indicated isoforms of OCT were exposed to the indicated concentrations of oxaliplatin and saracatinib for 3 d and processed as in Fig. 1B to D. All graphs show the mean of three independent experiments carried out in triplicate ± SEM.
due to reduced uptake of oxaliplatin in the presence of saracatinib and was independent of SFK inhibition. Finally, saracatinib was also shown to inhibit uptake of OCT substrates, including oxaliplatin, in cells engineered to over-express OCT2, consistent with a fit of saracatinib as an OCT inhibitor pharmacophore.

The data presented here differ from a work reporting a CRC cell line–dependent synergy between the SFK inhibitor dasatinib and oxaliplatin (33). In this report, the synergy is dependent on SFK activation after oxaliplatin exposure due to reactive oxygen species generation. Whereas an increase in SFK activity is observed in both of the cell lines described in the present study after oxaliplatin exposure (data not shown), there is no observed synergy with saracatinib. However, it has been shown that dasatinib is not a substrate for OCT (34) and therefore is unlikely to inhibit oxaliplatin uptake, potentially explaining these contrasting results. Another report has shown that combining oxaliplatin and saracatinib in vivo had a beneficial effect in an orthotopic mouse model of liver metastasis.3 Indeed, data in the present study (Fig. 4A) show that combining oxaliplatin and saracatinib has at least an additive effect on inhibiting cell migration.

Therefore, it is possible that combining oxaliplatin and saracatinib may prove clinically beneficial in appropriate settings, which may be even more pronounced if scheduling regimens are used that avoid coadministration of the two drugs.

There are two likely mechanisms by which saracatinib could inhibit uptake via OCT2: Either saracatinib directly binds to and inhibits OCT2 or it inhibits a factor, such as a kinase, which is essential for OCT2 function. Several kinases have been implicated in regulating OCT2 activity, including protein kinase A (PKA), protein kinase C (PKC), calmodulin-dependent kinase II (CaMKII), and the SFK member Lck (35, 36). Saracatinib can inhibit Lck activity (11); however, PP2 also targets Lck (25) yet does not affect oxaliplatin efficacy, making it unlikely that saracatinib inhibition of OCT2 activity is due to Lck inhibition. Furthermore, saracatinib did not inhibit serine/threonine protein kinases in a kinase screen (11), reducing the probability that saracatinib inhibits PKA, PKC, or CaMKII. The fact that saracatinib fits a pharmacophore of known OCT inhibitors, many of which are also transported by OCT, suggests that saracatinib may bind to OCT2 and thus directly inhibit OCT2.

Previously, schedule-dependent interactions of the vascular endothelial growth factor receptor and EGFR protein kinase inhibitor vandetinib (Zactima; ref. 37) and the EGFR inhibitor gefitinib (Iressa; ref. 38) with oxaliplatin were attributed to mechanistic interactions between DNA damage and growth/survival signaling pathways. Oxaliplatin was

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shown to influence the sensitivity of cells to treatment with gefitinib as a result of increasing or decreasing EGFR phosphorylation (39), and has also been shown to activate SFKs via the generation of intracellular reactive oxygen species (33). In addition to a mechanism-based interaction, vandetanib, gefitinib, and saracatinib share an anilinoquinazoline backbone that fits the pharmacophore generated from known OCT inhibitors, suggesting a common mechanism by which these inhibitors may interact with oxaliplatin. Indeed, recent work has shown that gefitinib can inhibit OCT1 and OCT2 functions (40), whereas uptake of the Abl inhibitor imatinib is OCT dependent and downregulation of OCTs in chronic myeloid leukemia has been proposed as a mechanism for imatinib resistance (41). Interaction with OCT function may be a more generalized phenomenon that requires consideration when potential novel drug combinations are undertaken.

One of the major physiologic roles of OCTs is the excretion of organic cations, including antibiotic, antiviral, antidiabetic, and cancer chemotherapeutics, via the liver and kidney (19). There are several examples of drugs that inhibit OCT, altering the pharmacokinetics-pharmacodynamics of other OCT substrates, such as the antiretroviral drugs lamivudine and zalcitabine, which interact with each other (42), or the histamine H2 receptor antagonist cimetidine, which increases the plasma concentration of the antidiabetic drug metformin (43). Therefore, it is highly plausible that novel agents sharing the OCT pharmacophore could alter the pharmacokinetic-pharmacodynamic relationship of coadministered drugs, and the data presented here for oxaliplatin and saracatinib suggest that this possibility should be anticipated and incorporated into schedule planning. In conclusion, this study has shown that saracatinib inhibits uptake of oxaliplatin in CRC cells where uptake via OCT2 is strongly implicated as the underlying mechanism. Follow-up studies where OCT2 is knocked down by RNA interference will allow this hypothesis to be tested; however, this will first require the generation of specific antibodies to access efficient downregulation of OCT2.

Disclosure of Potential Conflicts of Interest

C. Dyer: commercial research grant, AstraZeneca. C. Smith, D.M. Hickinson, and T.P. Green were employees of AstraZeneca at the time of this work. The other authors disclosed no potential conflicts of interest.

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