Combination of an Src Kinase Inhibitor with a Novel Pharmacological Antagonist of the Urokinase Receptor Diminishes in Vitro Colon Cancer Invasiveness

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ABSTRACT

Purpose: The urokinase-type plasminogen activator receptor (u-PAR) contributes to colon cancer invasion and metastases. We have shown previously that u-PAR expression in colon cancer is driven by the Src tyrosine kinase. In the current study, we determined the ability of PP2 (4-amino-5-(4-chlorophenyl)-7-((t-butyl)pyrazolo[3,4-d]pyrimidine), a Src kinase inhibitor, to reduce u-PAR expression and colon cancer invasion.

Experimental Design: Western blotting, Northern blotting, and u-PAR promoter-reporter assays were performed to determine whether PP2 represses u-PAR expression. In vitro invasion assays were used to determine whether this kinase inhibitor, with or without a novel u-PAR antagonist, diminished cultured colon cancer invasiveness.

Results: A constitutively active c-Src increased in vitro invasiveness of SW480 cells, whereas HT-29 cells expressing antisense c-Src showed diminished invasiveness, validating c-Src as a target for low molecular weight compound(s). The Src inhibitor PP2 reduced u-PAR transcription in HT-29 cells over the concentration range that blocked Src kinase activity. PP2 also reduced u-PAR protein amounts in three other colon cancer cell lines with modest to high constitutive Src activity. Treatment of HT-29 cells and 2C8 cells (a SW480 clone expressing a constitutively active Src) with PP2 diminished their in vitro invasiveness. Furthermore, combination of the Src inhibitor with a novel u-PAR peptide antagonist (NI-5.12) proved superior to the individual agents in suppressing invasiveness.

Conclusions: A c-Src kinase inhibitor represses u-PAR expression and, alone or in combination with a u-PAR antagonist, diminishes colon cancer invasiveness. Thus, concurrent targeting of c-Src expression and pharmacological blockade of the u-PAR may represent a novel means of controlling colon cancer spread.

INTRODUCTION

The urokinase-type plasminogen activator receptor (u-PAR), a M, 45,000–60,000 glycosylated cell surface receptor (1) linked to the cell surface via a glycolipid chain (2, 3), promotes cell migration, adhesion, extracellular matrix degradation, and epidermal growth factor signaling (4–7). The u-PAR contributes to these cellular functions via different mechanisms. First, the serine protease urokinase bound to this receptor activates plasminogen at a much faster rate than fluid-phase plasminogen activator, thereby augmenting extracellular matrix degradation (8). Second, the binding site clears urokinase-inhibitor complexes from the extracellular space (9, 10) via an α2 macroglobulin receptor-dependent mechanism. Third, the u-PAR interacts with the extracellular domain of integrins, thereby mediating cell adhesion and migration (11). Fourthly, it has been shown that the seven-trans-membrane receptor formyl peptide receptor-like receptor-lipoxin A4 receptor, a G protein-coupled receptor, directly interacts with a soluble cleaved form of u-PAR to induce chemotaxis (12).

Several previous observations indicate a prominent role for u-PAR in tumor progression. For example, gene expression profiling revealed enhanced expression of this binding site in various malignancies (13, 14). More importantly, intervention studies targeting u-PAR with antibodies, peptide antagonists, and antisense vectors have demonstrated the efficacy of such strategies in countering the invasiveness and metastasis of divergent malignancies (15–19). For colon cancer, a high u-PAR protein level in resected tumors is predictive of shortened patient survival (20–22). Furthermore, in cultured colon cancer, u-PAR protein levels correlate well with the ability of cells to degrade laminin and invade through Matrigel (23, 24).

How then can u-PAR expression be repressed to achieve a more indolent phenotype? We previously reported several lines of evidence for a role of the Src protein tyrosine kinase in driving u-PAR expression in colon cancer. Thus, stable transfection of Src up-regulated u-PAR transcription and protein levels (25). Furthermore, Src activity, which is predictive of shortened survival of colon cancer patients, correlated well with u-PAR protein amounts in resected colon tumors (25, 26). Considering these findings, we undertook a study to determine the ability of a Src kinase inhibitor, PP2 (4-amino-5-(4-chloro-
phenyl)-7-((t-butyl)pyrazolo[3,4-d]pyrimidine; Ref. 27), (a) to repress u-PAR expression and (b) to suppress the in vitro invasiveness of cultured colon cancer when used alone or in combination with a novel u-PAR antagonist, NI-5.12 (cyclo[21,29][D-Cys<sup>21</sup>Cys<sup>29</sup>]-uPA<sub>21-30</sub>).

**MATERIALS AND METHODS**

**Cell Lines.** The 2C8 cell line was derived by stably transfecting SW480 colon cancer cells with a constitutively active c-Src as described elsewhere (25). These cells, as well as SW620 cells, were maintained in DMEM/F-12 culture medium.
supplemented with 10% fetal bovine serum and, in the case of the 2C8 clone, G418 (500 \( \mu \)g/ml) as well. The AS15 and AS33 clones were derived by stable transfection of HT-29 colon cancer cells with an antisense c-Src expression vector as reported previously (28). The HT-29 and AS clones were grown in DMEM supplemented with 10% fetal bovine serum. For the AS clones, the culture medium also contained 500 \( \mu \)g/ml G418. HCT 116 and RKO cells (29) and DiFi cell lines were routinely cultured in McCoy’s 5A culture medium containing 10% fetal bovine serum (30, 31).

**Invasion Assays.** These were carried out as described previously by this laboratory (23) using nonenzymatically dissociated cells and Matrigel-coated porous filters as described by the manufacturer (BD Biosciences, Bedford, MA). After 24–72 h, cells on the upper aspect of the membrane were removed, and invasive cells on the lower aspect were stained with Diff-Quik and enumerated.

**Immune Complex Kinase Assays for Determination of c-Src Activity.** These assays were performed as described by us elsewhere (25). Cell lysates in radioimmunoprecipitation assay buffer (250 \( \mu \)g of protein) were reacted with anti-c-Src monoclonal antibody 327 (Oncogene Science Inc., Cambridge, MA). Immune complexes were formed with rabbit antimouse IgG and formalin-fixed Pansorbin. The kinase reaction was initiated with 10 \( \mu \)Ci of \( [\gamma-^{32}P]ATP, 10 \text{ mm} \text{ Mg}^{2+}, 10 \mu \text{g} \text{ of rabbit muscle enolase (Sigma), and 100} \mu \text{m sodium orthovanadate.}}

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**Fig. 2** Stable expression of an antisense c-Src reduces in vitro invasiveness of HT-29 cells. Invasion assays were carried out as described in the Fig. 1 legend. Magnifications are \( \times40, \times100, \) and \( \times400 \) for the left, middle, and right panels, respectively.
date in 20 mm HEPES. After 10 min, reactions were terminated with SDS. Products were separated in an 8% polyacrylamide gel.

Northern Blotting. This method was carried out as described by us previously (32). Purified RNA was electrophoresed in a 1.5% agarose-formaldehyde gel and transferred to Nytran-modified nylon. The Northern blot was probed at 42°C with random-primed radiolabeled cDNAs. Blots were washed at 65°C using 0.25X SSC/1.0% SDS. Loading efficiencies were checked by reprobing the blot with a radioactive glyceraldehyde-3-phosphate dehydrogenase cDNA.

Reporter Assays. A u-PAR gene fragment spanning -1469/+52 was PCR amplified from human genomic DNA and cloned into the SrfI site of the PCR-Script vector. The resulting vector was digested with BamHI/NotI, and the -1469/+52 fragment was purified, blunt-ended, and inserted into pGL3 at the SmaI site. This construct was designated 1469 u-PAR Luc.

HCT 116 colon cancer cells were cotransfected with 1469 u-PAR Luc and pcDNA3.1 in a 10:1 ratio. G418-resistant clones were selected and pooled. Reporter assays were performed as described previously by us (33) after treatment of the cells with or without PP2 for 24 h.

Western Blotting for u-PAR. Western blotting for u-PAR was done as described previously (25). Cell extract (in a Triton X-100 buffer with protease inhibitors) was immunoprecipitated with a polyclonal anti-u-PAR antibody. The immunoprecipitated material was then subjected to Western blotting, and the blot was probed with 5 μg/ml anti-u-PAR monoclonal antibody (3931; American Diagnostica, Greenwich, CT) and a horseradish peroxidase-conjugated goat antimouse IgG. Bands were visualized by enhanced chemiluminescence.

Statistical Analysis. Statistical analysis (Students’ t test) was carried out using the Prism software package (v3; GraphPad Software, San Diego, CA).

RESULTS

We showed previously that u-PAR expression was upregulated by Src overexpression (25). To confirm that the Src-mediated increase in u-PAR expression was associated with enhanced in vitro invasiveness of cultured colon cancer, two experiments were undertaken. First, the in vitro invasiveness of SW480 parental cells and a SW480-derived clone (2C8) made to overexpress a constitutively active c-Src (25, 34) was compared. 2C8 cells demonstrated a dramatic enhancement (6-fold) in in vitro invasiveness when compared with the parental SW480 cells (Fig. 1). Second, HT-29 cells (which contain a constitutively active c-Src) and two derived clones (AS15 and AS33) expressing an antisense c-Src (28) were compared for in vitro invasiveness. Both clones show a marked decrease in pp60c-Src autophosphorylation, reflecting reduced amounts of this protein tyrosine kinase (28). Of the two clones, suppression is slightly more effective with clone AS15 (28). In contrast, the expression and activity of the Src family kinase pp62c-Yes in both clones are comparable with those of the parental HT-29 cells (28). The parental HT-29 cells penetrated the extracellular matrix-coated filter, consistent with a previous report (35) characterizing the u-PAR-dependent ex-
tracellular matrix-degrading ability of this cell line. More importantly, the Src antisense-expressing clones failed to demonstrate an invasive phenotype (Fig. 2). Taken together, these data rationalize our study to target Src using a low molecular weight compound (PP2), thereby repressing u-PAR expression and attenuating in vitro invasiveness.

**Treatment of HT-29 Cells with the Src Kinase Inhibitor PP2 Reduces u-PAR Protein.** To first determine the efficacy of PP2 in targeting u-PAR expression, HT-29 cells were treated with PP2 for 48 h and assayed for u-PAR protein by Western blotting (Fig. 3A). PP2 caused a dose-dependent reduction in u-PAR protein that paralleled the inhibition of c-Src activity as seen in Fig. 4PP2 reduces steady-state urokinase-type plasminogen activator receptor (u-PAR) mRNA levels and promoter activity. A, HT-29 cells were treated for 18 h with the indicated PP2 concentration. Total RNA was extracted and subjected to Northern blotting using cDNAs corresponding to u-PAR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). B, HCT 116 cells containing an integrated luciferase reporter regulated by 1469 bp of u-PAR promoter sequence were treated for 24 h with the indicated concentration of PP2. After this time, cells were harvested, and equal amount of protein was analyzed for luciferase activity. Data are shown as average values ± SD of four separate determinations. *, *P < 0.0001.

**Fig. 5** Matrigel invasion by HT-29 cells is inhibited by PP2. A, HT-29 cells (25,000 cells) dissociated with EDTA were plated on Matrigel-coated porous filters. PP2 was added after cell attachment (~6 h later). After 48 h, cells on the upper aspect were removed, and invasive cells were stained with Diff-Quik and enumerated. Data are shown as average ± SD of three independent experiments. *, *P < 0.05; **, *P < 0.01. B, growth curve of HT-29 cells treated with or without 30 μM PP2.
shown by immunocomplex kinase assay (Fig. 3B). The suppression of u-PAR synthesis by PP2 was time dependent, with a reduction in u-PAR protein evident as early as 6 h (Fig. 3C).

Reduction of u-PAR Gene Expression by PP2. We conducted Northern blotting to determine whether the reduction of u-PAR protein levels evident by Western blotting was due to an inhibition of gene expression. HT-29 cells were treated with PP2 for 18 h, and total RNA was extracted and subjected to Northern blotting using a u-PAR cDNA (Fig. 4A). PP2 diminished steady-state u-PAR mRNA levels in a dose-dependent manner, with the highest concentration (30 μM) repressing the transcript level by >75% as determined by densitometry.

To confirm that the reduction in steady-state u-PAR mRNA levels was due to an effect at the transcriptional level, colon cancer cells containing an integrated 1469 u-PAR Luc reporter were treated with PP2 and analyzed for reporter activity. The Src kinase activity proved a potent inhibitor of u-PAR promoter activity (P < 0.0001) over an identical concentration range used in the Northern and Western blotting (Fig. 4B). Thus, PP2 reduces u-PAR gene expression.

Targeting of c-Src with PP2 Reduces in Vitro Invasion. We then determined the effect of inhibiting c-Src with PP2 on the in vitro invasiveness of cultured colon cancer. HT-29 cells were plated on Matrigel-coated filters and treated with concentrations of PP2 that repressed u-PAR expression. After 72 h, the number of invasive cells were enumerated. The Src kinase inhibitor caused a dose-dependent reduction in in vitro invasion of the HT-29 cells (Fig. 5A). At the highest concentration (30 μM), invasion was inhibited >60% (P < 0.0001). Whereas PP2 did also have a growth-retarding effect on HT-29 cells (Fig. 5B), the effect was modest and could not entirely account for the attenuated invasiveness.

We also investigated the effect of PP2 on the invasiveness of 2C8 cells, the clone derived from the stable transfection of SW480 colon cancer cells with a constitutively Src (25). Untreated 2C8 cells were highly invasive (Fig. 6A, arrows in vitro), whereas the addition of PP2 concentrations that blocked Src activity yielded reduced invasion (Fig. 6, A and B). These differences were statistically significant (P < 0.0002). Importantly, whereas PP2 had a dramatic effect on invasion, prolifer-
ation was only marginally reduced (Fig. 6C). Thus, the attenuation in in vitro invasiveness of the 2C8 clone cannot be accounted for by a mere reduced proliferation rate.

**PP2 Reduces u-PAR Protein Levels in Several Colon Cancer Cell Lines.** Considering the heterogenous nature of colon cancer, we then wanted to determine the efficacy of PP2 in repressing u-PAR expression in other cultured colon cancer cell lines characterized by their elevated Src activity. To this end, SW620, DiFi, and RKO cells, which have modest to high constitutive levels of c-Src (36), were treated with PP2 concentrations that block Src kinase activity and assayed for u-PAR protein by Western blotting. It is apparent from Fig. 7 that the three independent colon cancer cell lines responded to PP2 with a reduction in u-PAR protein amounts, although the attenuation with the RKO cell line was modest. Thus, interfering with Src activity represents a potential means of repressing u-PAR expression in some of the different tumor cell types that constitute colon cancer.

**Combination of PP2 and a Novel u-PAR Antagonist Is Superior to Individual Agents in Suppressing in Vitro Invasion.** Because Src may not be the sole regulator of u-PAR expression in colon cancer, we undertook experiments to determine whether combining a u-PAR antagonist with PP2 would further suppress the invasiveness of cultured colon cancer. To this end, we used NI-5.12, which is a cyclized substituted peptide derived from the receptor binding portion of urokinase (37, 38). This peptide is a potent antagonist of urokinase binding with u-PAR (37) and is resistant to proteolytic degradation in plasma and serum. NI-5.12 inhibited the in vitro invasion of 2C8 cells by 65% ($P < 0.01$), whereas peptide NI-5.16 (cyclo[21,29][Cys21–Ile28Cys29–uPA21–30]), which does not interfere with the binding of urokinase with u-PAR (37), had little effect on this parameter (Fig. 8, A and B).

Subsequently, 2C8 cells were assayed for in vitro invasion using suboptimal concentrations of NI-5.12 (1 μM) and PP2 (3 μM). As expected, both agents attenuated the in vitro invasiveness of 2C8 cells (Fig. 8, C and D), albeit to a lesser extent than that achieved with optimal concentrations. However, combination of the u-PAR antagonist with the Src inhibitor proved more efficacious than the single agents. Thus, whereas NI-5.12 (1 μM) or PP2 (3 μM) individually diminished in vitro invasiveness between 45% and 47%, the combination of the agents yielded >75% inhibition. The difference between in vitro invasiveness achieved with the single agents and the combination was statistically significant ($P < 0.0001$).

**DISCUSSION**

The activity of c-Src, which is predictive of disease progression and shortened colon cancer patient survival (26), is a potent inducer of u-PAR expression (25). The u-PAR, in turn, promotes tumor invasiveness and metastases (4, 5, 16). Accord-
ingly, we undertook a study to determine the efficacy of a Src kinase inhibitor in reducing u-PAR expression and cultured colon cancer invasiveness. The current study clearly demonstrates the ability of PP2, a Src kinase inhibitor, to repress u-PAR expression and the invasiveness of cultured colon cancer. Furthermore, combination of the Src kinase inhibitor with a novel pharmacological antagonist of the u-PAR was superior to the individual agents in diminishing in vitro invasion. To our knowledge, this is the first report demonstrating the efficacy of combining a compound that targets a signaling protein tyrosine kinase with a u-PAR antagonist in attenuating the in vitro invasiveness of colon cancer cells.

At the present time, we do not know whether PP2 is targeting c-Src or other Src kinase family members such as c-Lyk, c-Fyn, or c-Yes. Indeed, in vitro, PP2 effectively inhibits the activity of all of these Src kinase family members (27). Moreover, like c-Src, c-Yes is also constitutively activated in colon cancer (28). Therefore we cannot exclude the possibility that the anti-invasive activity of PP2 reflects the targeting of one or more of the Src kinase family members. However, a previous report argues in favor of the contention that the anti-invasive effect of PP2 is mediated via c-Src. Thus, Staley et al. (28) demonstrated that the stable expression of a c-src antisense expression vector in HT-29 cells that specifically reduced the amount and activity of this protein tyrosine kinase, but not of c-Yes, resulted in a clear attenuation of tumor progression in vivo.

Whereas we observed that PP2 was effective in repressing u-PAR expression in various colon cancer cell lines, the extent to which u-PAR was repressed depended on the cell line. For example, whereas HT-29, SW620, and DiFi cells showed a dramatic reduction in u-PAR protein, this parameter was less affected by PP2 in RKO cells. This reduced sensitivity in RKO cells would suggest that u-PAR expression in some colon cancer cells is dependent not just on c-Src but on other stimuli as well. This contention is supported by our earlier statistical analysis.

Fig. 8 Combination of PP2 and NI-5.12 is superior to individual agents in suppressing in vitro invasiveness. A and B, invasion assays with 2C8 cells were performed as described in the Fig. 5 legend. The peptides were added at the indicated concentration 2 h after cell plating. In the control, the reconstituting solvent was used. Magnification, ×40. Data in B are average values ± SE for three separate determinations. *, P < 0.01.
(25) showing that whereas c-Src activity correlated well with u-PAR protein amounts in resected colon cancer, a small but positive u-PAR value was predicted at zero Src activity. If, indeed, u-PAR expression in some colon cancer cells is dependent on stimuli other than the Src kinase, pharmacological blockade of the binding site may represent a means of targeting u-PAR that has escaped the countering effects of PP2.

Considering the wealth of studies implicating u-PAR in tumor progression, considerable attention has been spent by various groups on developing strategies to antagonize this molecule. For example, using a bacteriophage peptide display library, Goodson et al. (39) identified a number of peptides capable of blocking u-PAR. In separate studies, other investigators have shown that peptides derived from different regions of the urokinase molecule perturb the urokinase-u-PAR axis (17, 40, 41), thereby reducing growth and metastasis of divergent cancers including glioblastoma and breast cancers. Furthermore, Ploug et al. (42) used combinatorial chemistry to design a 9-amino acid, non-natural, linear peptide that disrupted urokinase-u-PAR interactions and inhibited invasion of Hep-3 cells into the chorioallantoic membrane. Immunological strategies such as using an anti-u-PAR antibody have also proven efficacious in blocking the invasiveness of rat mammary adenocarcinoma (18). Likewise, using a recombinant approach, Wilhelm et al. (43) reported that a soluble form of u-PAR, which scavenged urokinase, inhibited Matrigel invasion by ovarian cancer cells (43). In addition to physically antagonizing u-PAR, other investigations have attempted to block expression of this molecule. Notably, Zannetti et al. (44), observing that u-PAR expression is trans-activated, at least in part, via Sp1 binding (25, 44), reported that mithramycin, which inhibits binding of this transcription factor to the promoter, represses u-PAR expression.

Although we clearly showed a repression of u-PAR expression by PP2, we cannot be certain that the countering effect of this kinase inhibitor is mediated entirely via repression of the urokinase binding site. Indeed, interfering with Src activity could also result in the repression of other molecules required for tumor cell invasion. For example, the synthesis of the Mr 92,000 type IV metalloproteinase (MMP-9), as well as urokinase, has been reported previously to be Src dependent (45, 46).

**Fig. 8** Continued. C and D, invasion assays with 2C8 cells were performed as described in the Fig. 5 legend, with the exception that the cells were cultured on the Matrigel-coated filter for 72 h. In the control, the reconstituting solvent was used. The agents were added 2 h after cell plating. Magnification, ×40. Data in D are average values ± SE for nine separate determinations. *, P < 0.005 compared with control; **, P < 0.0001 compared with individual N15.12 or PP2 treatments.
Furthermore, vascular endothelial growth factor expression (47) and extracellular signal-regulated kinase activation (48), two events associated with tumor progression, are also Src dependent. Nevertheless, because the extent of u-PAR repression (~75%) was quantitatively similar to the reduction of in vitro invasion (60–80%), it is likely that the diminished in vitro invasion achieved by PP2 is largely due to attenuated u-PAR expression.

In conclusion, we have demonstrated the ability of a Src kinase inhibitor, PP2, to repress u-PAR expression and in vitro invasion in some of the different tumor cell types that make up colon cancer. Additionally, combination of the Src kinase inhibitor with a u-PAR antagonist proved superior to the individual agents in reducing in vitro invasion. These studies rationalize further investigations to determine the efficacy of this Src kinase inhibitor, or congeners, either alone or in conjunction with u-PAR antagonists, in minimizing the spread of colon cancer in vivo.

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