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Pyrazolo[3,4-*d*]pyrimidines c-Src inhibitors reduce epidermal growth factor-induced migration in prostate cancer cells

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ABSTRACT

During its biological progression, prostate cancer frequently develops dependence on growth factor receptors and their downstream signalling messengers, including c-Src. Evidence for this supports the choice of c-Src as a therapeutic target in the prevention of tumour spreading. Two new pyrazolo[3,4-*d*]pyrimidines c-Src inhibitors, SI35 and SI40, were used to investigate the role of c-Src in the control of the aggressive phenotype of prostate carcinoma cell line, PC3. SI molecules reduced the proliferation of PC3 cells in a time- and dose-dependent manner, with an IC50 of approximately 50 μM. PC3 cells responded to the presence of epidermal growth factor (EGF) by increasing their migratory ability, and this effect was strongly reduced by the addition of SI at concentrations less than IC50. Further observations demonstrated that SI molecules modulated cell morphology and their adhesive capacity on different physiological substrates. The action of SI molecules appeared to involve, in parallel with c-Src inhibition, the down-modulation of the active forms of paxillin and extracellular signal-regulated kinase (ERK). Our data suggest a promising role for pyrazolo[3,4-*d*]pyrimidines c-Src inhibitors in the control of a highly invasive tumour phenotype.

1. Introduction

The blockade of key molecules in the signalling pathways regulating proliferation and diffusion of tumour cells is one of the most promising strategies in modern cancer therapy. In the last few years many molecular targets promoting malignant behaviour of prostatic cells have been identified.[1] However, the androgen-dependent origin of prostatic carcinoma (PCa) has initially induced scientists to focus their studies on the role of androgen receptors in tumour progression. In fact, apart from prostatectomy, androgen ablation represents the 'gold standard' therapeutic approach in PCa. Because of the frequent failure of hormonal therapy, as demonstrated by the high number of refractory patients progressing toward the incurable metastatic phase, several authors have reconsidered molecular pathways other than the androgen axis as potential therapeutic targets.

Although c-Src was the first proto-oncogene described, its role in cancer determination is still not completely understood. According to an initial hypothesis, the over-expression

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of a specific mutated form of c-Src is associated with cell transformation.[2] Further studies showed that the wild-type form of c-Src was also able to stimulate cell division when hyper-activated.[3] However, the discovery of an increasing number of molecular partners implicated in several transduction pathways has amplified the potential roles of c-Src. New evidence has suggested that the activation of c-Src not only promotes growth, but also regulates adhesion and migration of tumour cells during their progression.[4] c-Src is involved in the control of cellular adhesiveness and migration, functioning as a key molecule regulating the signal transduction pathways triggered by various surface molecules, such as growth factor receptors and integrins (see review[4]).

There is increasing evidence that the control of adhesion turnover and cell migration by c-Src is exerted through the formation of active complexes containing focal adhesion kinase (FAK).[5] The elevated c-Src activity and increased FAK expression, which frequently occurs in human cancer cells, may account for the enhanced invasive potential of these cells. A recent study has suggested that FAK/Src exerts the role of decoy complex, recruiting other kinases that can phosphorylate key substrates.[6] One of these proteins is paxillin, a scaffold protein in integrin signalling, which has been implicated in the metastatic potential of human cells.[7,8] Several studies have shown that different growth factors, including epidermal growth factor (EGF), induce tyrosine dephosphorylation of paxillin in human carcinomas.[9,10] Moreover, EGF-induced paxillin dephosphorylation leads to c-Src activation and is involved in EGF-stimulated extracellular signal-regulated kinase (ERK) activation and cell migration.[11]

Many c-Src inhibitors with different heterocyclic structures have been described and reviewed in the literature. As an example, we describe here the pyrrolo-pyrimidine CGP-7767,[12] the recent phenylaminothieno-pyridine 1,[13] and the pyrazolo-pyrimidines PP1 and PP2 [14] (Fig. 1). Other pyrazolo-pyrimidines as selective inhibitors of c-Src family kinase were reported by Shokat [15] and Hirst.[16] On the basis of this knowledge, we synthesised several new 4-aminopyrazolo-[3,4-*d*]pyrimidines, among these SI35 and SI40, structurally related to PP1, but possessing different substitutions; in particular, on the N-1 of the pyrazole ring, a 2-chloro-2-phenylethyl or a styryl chain is present, in position 6 of the pyrimidine ring a thiomethyl group is present, and in position 4 a phenylethylamino substituent. These molecules are good inhibitors of c-Src, as demonstrated recently.[17,18]

To date, few studies have examined the impact of c-Src inhibitor on PCa cell lines. Pyrrolopyrimidine c-Src inhibitors dose dependently decrease the proliferation rate of PC3 cells.[19] Moreover, these inhibitors were effective in the reduction of the ability of PC3 cells to bind and invade Matrigel. A previous study has likewise shown that PP2 slows the migration of several PCa cell lines, suggesting a leading role of active c-Src in the activation of FAK.[20]

2. Material and methods

2.1. *Reagents and cell cultures*

Synthesis of SI35 has been reported previously.[17] Synthesis of SI40 was performed by treatment of SI35 with 4 M NaOH solution in ethanol under reflux for 4 h. The crude product was filtered and recrystallised from absolute ethanol to give SI40 as a white solid, with a melting point of 121–122 °C, and yield of 70%. The structure was confirmed by elemental analysis,

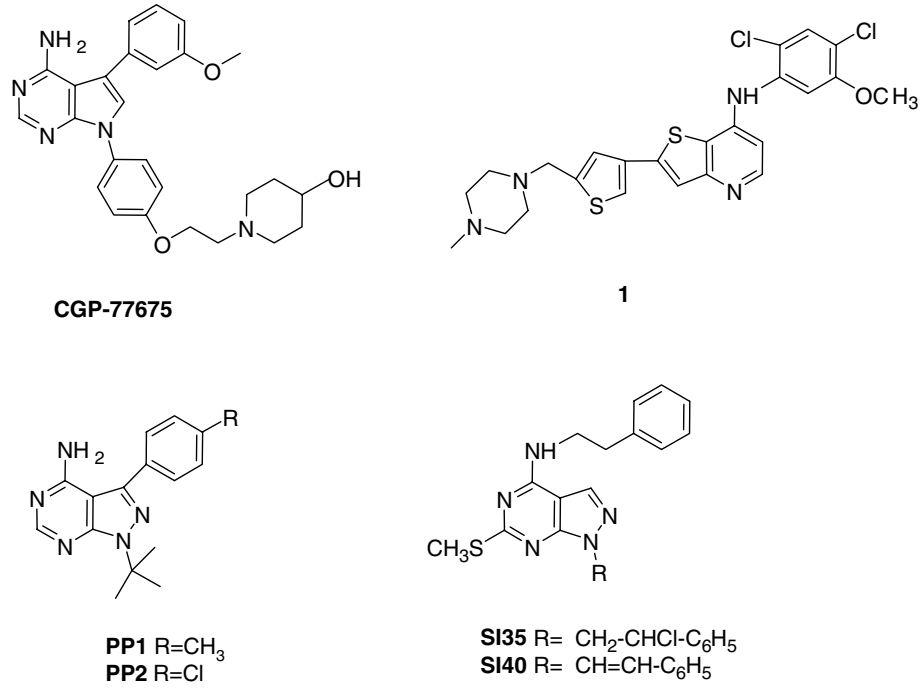

Fig. 1 – Representative structures of principal Src inhibitors used in preclinical studies, the pyrrolo-pyrimidine CGP-7767,[12] the phenylaminothieno-pyridine 1,[13] the pyrazolo-pyrimidine PP1 and PP2. The inhibitors used in our work are SI35 and SI40 that are structurally related to PP1.

^{1}H-nuclear magnetic resonance (NMR) and infra-red (IR) data (not shown). For *in vitro* experiments, a stock solution in dimethyl sulphoxide (DMSO) was prepared and appropriate dilutions were made in foetal calf serum (FCS). The human prostatic cell line PC3, derived from a bone metastasis, and normal foreskin fibroblasts, Hs27, were obtained from ATCC (Rockville, MD, United States of America (USA)) and were passaged in 10% FCS v/v supplemented DMEM (Dulbecco's Modified Eagle medium from Sigma, St Louis, CA, USA). Antibodies anti-c-Src (src2), anti-phospho-c-Src, anti-phospho-ERK (E-4), and anti-actin were purchased from Santa Cruz Biotechnology (Delaware, CA, USA), while antibodies anti-paxillin and anti-paxillin-phosphorylated (Tyr118) were from Biolegend (Camino Santa, CA, USA). Recombinant human EGF, laminin and collagen I were purchased from Sigma, matrigel was from BD Biosciences (Bedford, MA, USA). ZD1839 (Gefitinib) was kindly provided by AstraZeneca Italy (Basiglio, Milan, Italy).

2.2. Growth and inhibitory curves

A classical growth curve analysis was used to determine the effects of different concentrations of EGF. Cells were cultured in DMEM plus 5% FCS v/v, then trypsinised, counted using a haemocytometer, and adjusted to 2×10^4 cells per 50-mm diameter Petri dish in serum-free DMEM. After 24 h, three dishes were sacrificed for cell counting (time 0) to measure the baseline cell number. The remaining dishes received fresh medium containing 50 ng/ml or 80 ng/ml EGF. For cell counting, cells were trypsinised, resuspended in 20 ml saline, and counted in a Coulter Counter (LabRecyclers, Gaithersburg, MD, USA). Five independent counts were made from each dish. Inhibitory effect of SI molecules on cell proliferation were tested by treating cells with increasing concentrations of SI molecules, in the presence of 50 ng/ml EGF, and counting cells after 72 h. IC50 was calculated using Kaleidagraph 3.6 (Synergy Software).

2.3. Adhesion assay

Cells were seeded at 5×10^5 cells/cm^2 in a 96-well plate coated with 10 μg/ml of extracellular matrix protein and incubated for 1 h at 37 °C in a 5% CO_2 humidified atmosphere. Thirty minutes after seeding, adherent cells were fixed with cold methanol for 10 min, washed with phosphate-buffered saline (PBS) and air-dried. Adherent cells were stained with 100 μl of 0.5% crystal violet w/v for 15 min at room temperature. Then cells were rinsed with PBS and lysed with 2% sodium dodecyl sulphate (SDS) w/v, 0.05% sodium azide w/v in water for 1 h with gentle agitation. Lysed cells were transferred to 96-well plates and absorbance was measured at 595 nm in a Bio-Rad Multiscan plate reader (Hercules, CA, USA).

2.4. Wound healing assay

Cells were cultured in 50-mm Petri dishes in normal culture conditions and allowed to reach maximum confluence. A round-tip steel needle, previously sterilised, was used to draw several wounds of approximately 0.2 mm in the cellular stratum. Culture dishes were washed three times with DMEM and then cells were cultured in medium containing 50 ng/ml EGF. The status of wounds was monitored by contrast phase microscopy and representative images were collected.

2.5. Migration assay

PVPF 8 μm polycarbonate filters (Nucleopore, Concorezzo, Milan, Italy) were coated on one side with 0.01% gelatine w/v, rinsed once with PBS and then placed in contact with the lower chamber containing chemo-attractants. Cells (1×10^5 per chamber) were trypsinised, washed twice with PBS, rinsed in complete medium and incubated at 37 °C for 30 min to reconstitute the membrane structures and then added to the upper compartment of each chamber in medium without FCS. Cells were allowed to migrate through coated filters for 6 h. The cells attached on the lower membrane surfaces were stained with 0.1% crystal violet w/v in 0.1 M borate, pH 9.0 and 2% ethanol v/v for 20 min at room temperature. Cells were counted at ×400 magnification in standard optical microscopy and the average number of cells per field in 5 random fields was recorded. Triplicate filters were used and the experiments were repeated three times.

2.6. Western blotting

Total cell lysates were obtained by resuspending the cells in buffer containing 1% Triton v/v, 0.1% SDS w/v, 2 mM $CaCl_2$, 100 μg/ml phenylmethyl sulphonyl fluoride. Protein content was determined using the Protein Assay Kit 2 (Bio-Rad Laboratory, Hercules, CA, USA). 80 μg of proteins were electrophoresed in 10% SDS-polyacrylamide gel and then electrotransferred to nitrocellulose membrane (Schleicher & Schuell BioScience, Inc., USA), which was then blocked overnight with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20 v/v (TBS-T) containing 10% non-fat dried milk w/v. The membrane was then incubated with 1 μg/ml primary antibody in TBS-T and with specific horseradish peroxidase-conjugated secondary antibodies in TBS-T. Protein bands were visualised using a chemiluminescent detection system (Amersham Biosciences, NJ, USA).

2.7. Statistical analysis

Results are expressed as means ± standard deviation (SD) for at least three distinct experiments. Demonstration of significant differences among means was performed by Student's analysis with 0.01 as the threshold value of *P*. All statistical analyses were performed using Kaleidagraph 3.6 (Synergy Software).

3. Results

3.1. EGF is a potent inducer of migration in PC3 cells

The addition of EGF to PC3 cells cultured in serum-free medium for 24 h determined an early activation of c-Src through the phosphorylation in Tyr-418 (Fig. 2(a)). The enhancement in the phosphorylated state of c-Src was detected within 1 h after EGF matching. The prolonged presence of EGF in culture medium was able to stimulate different cellular activities, including cell proliferation and migration (Fig. 2(b and c)). Maintaining PC3 cells for 4 d in the presence of EGF determined

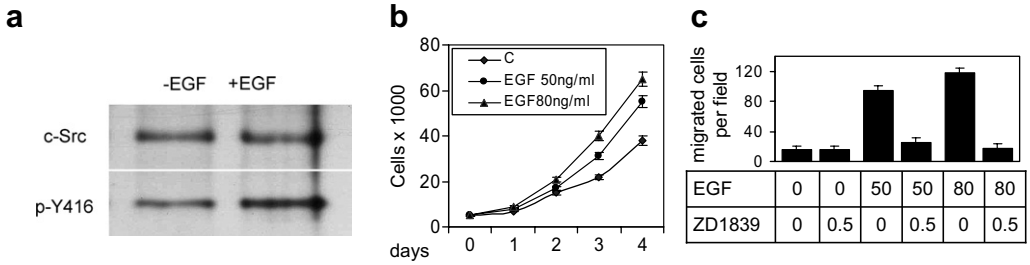

Fig. 2 – Effects of epidermal growth factor (EGF) in PC3 cells. (a) Expression levels of total and phosphorylated c-Src by Western blot from whole cell lysates. Cells were treated with 50 ng/ml EGF for 1 h. (b) Growth curves in the presence of two concentrations of EGF. Cells were cultured in serum-free medium for 5 d and values represent the mean of three measurements performed each day (± SD). (c) Migration ability of PC3 cells was evaluated allowing cells to migrate for 6 h through a gelatine-coated filter and using as chemo-attractant two concentrations of EGF (50 and 80 ng/ml). The specific epidermal growth factor receptor (EGFR) inhibitor, ZD1839 (0.5 μg/ml) was added to cells 1 h prior to the test and during the migration.

a dose-dependent increase in cell number with respect to control, but the stimulation was significantly evident only after 48 h (Fig. 2(b)). Moreover, PC3 cells were attracted by EGF and crossed the 8 μm pore size membrane at an increment of approximately 6-fold with respect to the number of cells in basal conditions (Fig. 2(c)). The migration was evaluated after 6 h and the effect of EGF appeared to be dose-dependent. In order to verify the implication of epidermal growth factor receptor (EGFR) in EGF stimulation, we used the tyrosine kinase inhibitor ZD1839, treating cells 1 h before the migration test. After the treatment with 0.5 μM ZD1839 we observed a complete repression of EGF-mediated migration and the number of cells able to cross the membrane was no longer influenced by the presence of EGF (Fig. 2(c)).

3.2. *SIs modulate EGF action*

We focused our attention on the role of two pyrazolo[3,4-*d*]pyrimidines inhibitors, SI35 and SI40. First we evaluated the effectiveness of SIs by analysing the influence on the modulation of transduction pathways. After 24 h starvation PC3 cells were treated for 1 h with EGF in the presence or absence of two different concentrations of SIs and the expression of p-Tyr-416 c-Src in total cell lysates by specific antibody was evaluated (Fig. 3(a)). While the level of total c-Src remained the same, SIs were able to decrease its phosphorylated form. The dephosphorylation effect of PP2 was included as a positive control. We then evaluated the effect of SI molecules on the proliferation of PC3 cells, counting viable cells after 72 h of treatment with different concentrations of SIs. In Fig. 3(b) we show the percentage of viable cells with respect to control (100%) after matching cells with increasing doses of SI35, SI40 and PP2. The decrease in viable PC3 cell number was evident with at least 5 μM of all inhibitors and was progressively more evident according to the inhibitor concentration. SI molecules were ineffective, at the same concentrations, in affecting the proliferation of normal fibroblasts, Hs27.

3.3. *Inhibition of c-Src activation reduces cell adhesion*

In parallel with the reduction in cell proliferation, we observed that the presence of SI molecules determined an early modification in cellular morphology. The cells, almost within the time of our observations, remained viable, but assumed a completely different round shape, determining marked

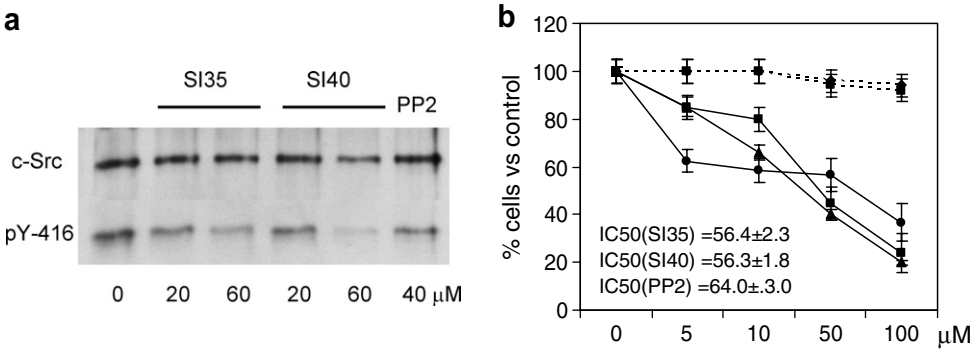

Fig. 3 – PC3 cell response to SI molecules. (a) Expression levels by Western blot of total and phosphorylated c-Src in the presence of two concentrations of SIs inhibitors (20 and 60 μM) and 40 μM PP2. (b) Inhibition curves for PC3 and Hs27 (*dotted lines*) cells obtained using four concentrations (5, 10, 50, 100 μM) of SI35 (■) SI40 (◆) and PP2 (▲) and counting cells after 72 h of treatment. Calculated IC50 for PC3 cells are indicated. Each value is a mean of three different measurements (± SD).

increase of susceptibility in their detachment. This phenomenon was induced by concentrations lower than IC50 of SI (5–10 μM) and within 24 h of treatment, and was not observed in the presence of equivalent doses of PP2 (data not shown). Cells treated with SI molecules were detached by mechanical procedure and were counted, and their viability evaluated (Fig. 4(a)). Results showed that, after 72 h of incubation, SI35 and SI40 were able to induce the detachment of approximately 20% and 40% of total cells, respectively. We then evaluated the adhesive affinity of PC3 cells for different physiological substrates in the presence of SI molecules. We pre-treated cells for 24 h before the test and plated them on laminin, collagen I and Matrigel. The adhesive capacity was determined by the number of cells that remained adherent on the substrate after 30 min of incubation. Pre-treatment with SI inhibitors determined a generalised decrease in the adhesive capacity of cells, with a significant reduction especially on laminin and collagen, with respect to control (Fig. 4(b)).

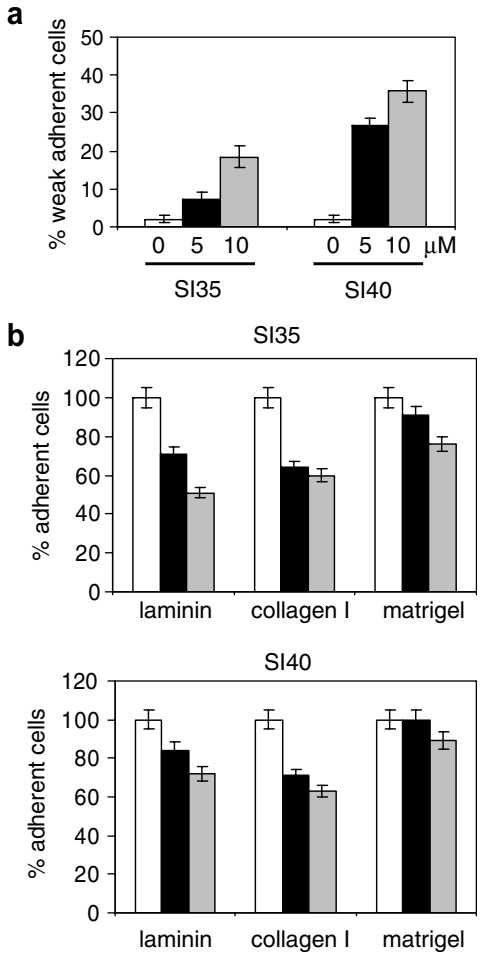

Fig. 4 – Adhesion inhibition by SI molecules. (a) PC3 cells were cultured for 48 h with two concentrations of SI35 and SI40 (5, 10 μM) and then cells with weak adhesive binding to substrate were recovered by repeated medium flushing. The cells so obtained, were counted and the result was expressed as a percentage with respect to the total number of cells (*white bar*). The values are means of three different measurements (± SD). (b) Adhesion assay performed by plating PC3 cells on three different physiological substrates (laminin, collagen I and Matrigel) for 30 min in the presence of SI35 (*upper panel*) and SI40 (*lower panel*). SI molecules were added to cells 1 h prior the assay at two concentrations (5 μM, *black bar*, and 10 μM, *grey bar*). The values are expressed as mean percentage with respect to control (*white bar*) of three different experiments (± SD).

3.4. *Migration is strongly reduced by Sis*

The migratory capacity of cells was evaluated by the 'wound healing' assay. PC3 cells were allowed to reach the maximum confluence degree using standard culture conditions and, after 24 h of starvation, a 'wound' of approximately 0.2 mm was produced in several areas of different culture plates. Fig. 5 shows pictures of representative wounds stained after 12 h of incubation. In the presence of 50 ng/ml EGF, all wounds at this time were almost completely closed, but when we added 50 μM SI inhibitors the wounds appeared to be of the same size as that of time zero (Fig. 5). The migration ability was evaluated also by chemo-attraction assay (Fig. 6(a)). The mean number of PC3 cells able to cross the gelatine-coated filter within 6 h in the absence of a chemo-attractant gradient was approximately 18 cells. This value was due to the random scattering of PC3 cells. When we pre-treated cells with SI35 or SI40 for 24 h before the migration test, the random scattering was significantly reduced by the concentration of 10 μM for both drugs (Fig. 6(a)) with a reduction of up to 83% (SI35 10 μM) with respect to control. At a concentration of 5 μM, although both SI inhibitors determined the reduction in the mean number of migrated of cells, only SI35 produced a statistically significant decrement with respect to the basal value ($P < 0.01$). The same experiments were performed using 50 ng/ml EGF as chemo-attractant. The pre-treatment with two concentrations of the inhibitors reduced by up to 85% (10 μM SI35) the number of cells migrated after 6 h (Fig. 6(a)). We then investigated the role of some key molecules implicated in EGF-induced migration. We considered the expression levels of total paxillin, p-Tyr-118 paxillin and p-ERK (Fig. 6(b)). PCa cells were cultured in serum-free medium for 24 h and then treated with 50 ng/ml EGF for 1 h. EGF addition determined an early up-modulation of p-ERK while phospho-paxillin level remained substantially unchanged. The addition of 10 μM SI35 determined a decrease in phospho-paxillin and in p-ERK, in both the presence and absence of EGF. The total amount of paxillin and of the loading control, actin, was unchanged in each condition.

4. Discussion

Several studies have suggested that c-Src activation is a key event in signal transduction initiated by EGFR and leading to cell proliferation and invasion, and that this might be particularly relevant in tumours in which both proteins are frequently up-regulated. PCa develops from an androgen-dependent disease and, in the late stages of its progression, frequently becomes insensitive to androgen deprivation. However, prostatic epithelium, which potentially generates PCa, normally shows dependence on its homeostasis from various growth factors, including EGF, produced by stromal

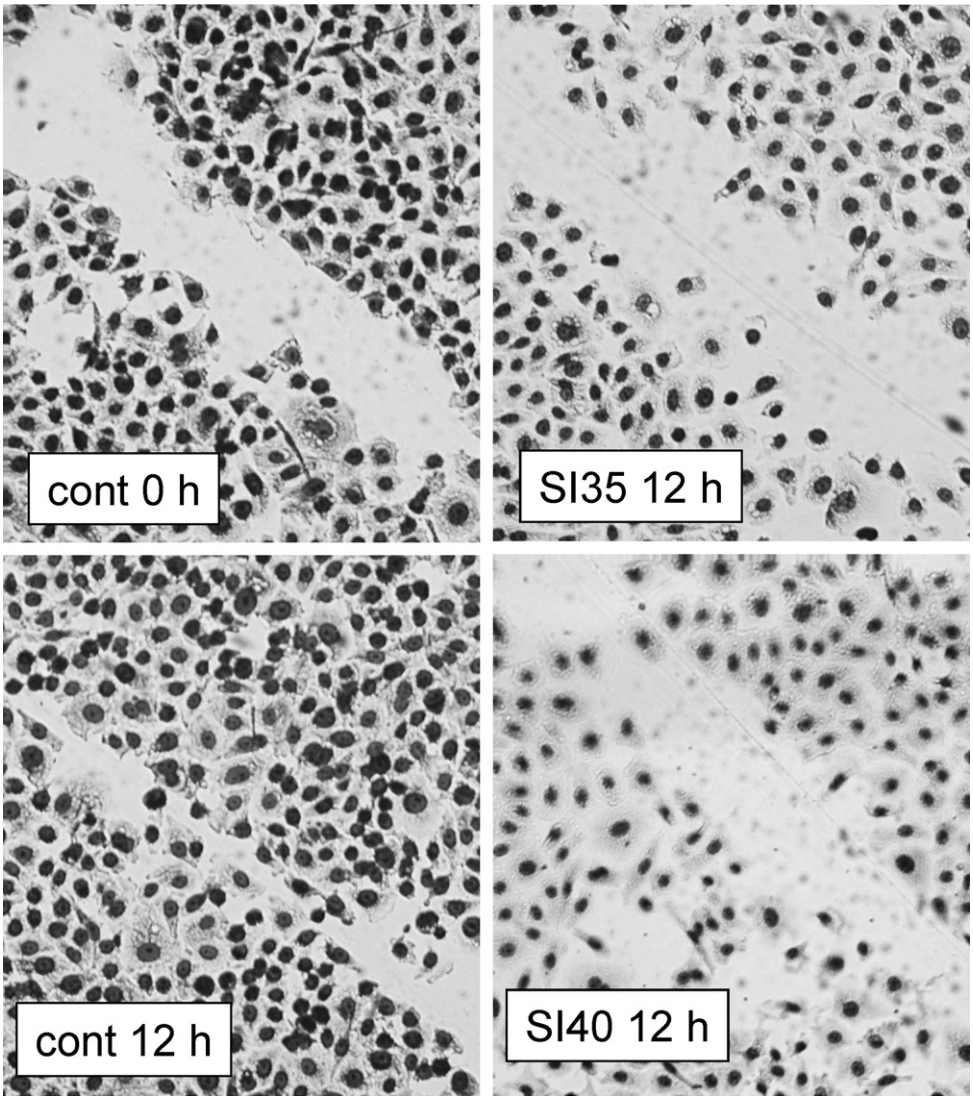

Fig. 5 – Wound healing assay. PC3 cells were allowed to reach maximum confluence and several wounds were traced in the cell monolayer (cont 0 h). In the presence of 50 ng/ml EGF wounds were healed within 12 h (cont 12 h, a representative wound) while the addition of 10 μM SI35 and SI40 blocked this phenomenon (*right-hand panels*). Cells were stained by haematoxylin and eosin (H&E) procedure and observed standard optical microscopy (×400).

cells.[21] Androgen-dependency escape is thought to be caused by several mechanisms, one of which is the overexpression of growth factor receptors. Several PCa cell lines express functional EGFR and share a common sensibility to EGFR inhibition in their proliferative and migratory ability.[22] PC3 cells are the most used cellular prototype of advanced, lethal stage of PCa progression, and their invasive potential in the presence of EGF has been extensively described.[23,24] However, the real mechanisms underlying the EGF-induced migration in PCa are largely unknown. At the same time, PC3 cells exhibit intrinsic and high migratory ability that depends on the presence of elevated levels of active c-Src.[20]

Our interest in this study was focused on investigation of the relationship between the EGF-induced migration and the activation of c-Src. The main tools we used were new pyrazolo[3,4-*d*]pyrimidines derivatives that have already been demonstrated to be effective in the inhibition of c-Src activation and of cellular proliferation. In fact, 4-amino-6-methylthio-pyrazolo[3,4-d]pyrimidine N1 substituted SI35 and SI40 showed anti-proliferative activity toward human epidermoid carcinoma A431 cells and inhibition of c-Src phosphorylation with almost the same efficacy as that of PP2, used as a reference compound; moreover the new derivatives induced apoptotic cell death in PARP (poly-ADP-ribose-polymerase) assay, probably as a consequence of the inhibition of the tyrosine kinase signalling pathways.[18] The same effects have been observed on breast ductal infiltrating carcinoma 8071-BC cells.[17] In our cell system we observed IC50s similar to those measured in A431, in 8071-BC,[17] and in other PCa cell lines, including LNCaP (our observation, not shown). On the contrary, the adhesive modification induced in PC3 cells by SI molecules was a new observation, especially in comparison with data obtained with PP2. A similar observation has been made in the presence of new pyrrolopyrimidine inhibitors, when PC3 cells modified their spreading, motility and invasive capacity.[19] SI concentrations used in adhesion and

a

pre-treatment	chemoattractant	
	SFM	EGF
Null	18±5	79±8
SI35 5μM	8±2*	30±5°
SI35 10μM	3±1*	12±5°
SI40 5μM	12±4	32±7°
SI40 10μM	5±2*	15±5°

b

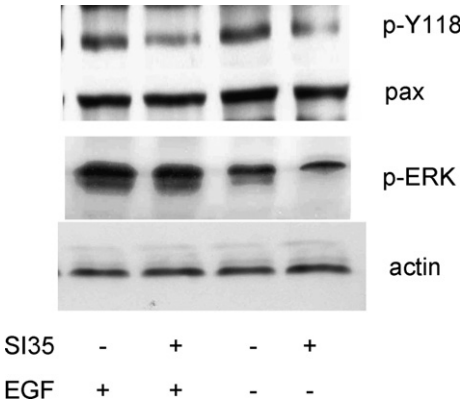

Fig. 6 – Inhibition of migratory ability by SI molecules. (a) PC3 cells were allowed to cross a gelatine-coated filter in response to serum-free medium (SFM) or 50 ng/ml epidermal growth factor (EGF) for 6 h. Cells were treated 1 h prior to the assay at the indicated concentrations of SI molecules. Results are expressed as number of cells able to cross the filter in an optical field (×400). Each value represents the mean of five counts per filter in triplicates (± SD). *P < 0.01 with respect to SFM control, °P < 0.01 with respect to EGF control (Student's t-test). (b) Expression level by Western blot of paxillin (total and phosphorylated form), phospho- extracellular signal-regulated kinase (ERK) and actin. Cells were treated with 10 μM SI35 in SFM for 1 h in the presence or absence of 50 ng/ml EGF.

migration assays were considerably lower than anti-proliferative IC50s and cells were incubated with these drugs for a few hours. In these conditions we did not observe any evidence of cell death. For these reasons we suppose that PC3 cells are extremely dependent on c-Src in their adhesive and migratory ability, and that these effective and specific new inhibitors are potentially able to block the highly invasive phenotype of PCa cells. In agreement with this hypothesis, other authors demonstrated that, by inhibiting the c-Src signal transduction pathway emanating from FAK complexes by c-Src family kinase inhibitors, we could significantly inhibit the migration of highly invasive PCa cell lines, including PC3 cells.[20]

One of the possible mechanisms underlying the EGF-induced activation of c-Src depends on the phosphorylation status of paxillin.[11] According to this finding, EGF is able to induce the dephosphorylation in paxillin that, in turn, permits the activation of c-Src. At the same time, the enhanced activity of c-Src and the overexpression of hyper-phosphorylated paxillin may contribute to the highly metastatic potential of human tumours.[8] This apparent contradiction depends on the scarce knowledge we have about the real functioning of the migratory mechanism and of its deregulation in tumours. In our cellular model we observed that the addition of SI35 also determined, apart from a strong reduction in EGF-induced c-Src activation, a reduction in paxillin phosphorylation. Since several studies indicated that members of the c-Src family are able to phosphorylate paxillin, the effect of SI on paxillin may be modulated indirectly by the inhibition of c-Src.

Considering the net effect induced by c-Src in our cellular system we suggest that paxillin activation by c-Src may play a central role in the modulation of c-Src activity. Moreover, our data, in agreement with the results of other authors,[11] demonstrated that ERK is also implicated in EGFR/src induction of cell migration. Although it is well known that serum-induced activation of the ERK pathway is also mediated by FAK-Src-Paxillin complex activation,[25] how this mechanism can regulate cell migration is not completely understood.

In conclusion, our data demonstrate that EGF-induced migration in an invasive PCa cell line is highly dependent on c-Src activation. The use of new c-Src inhibitors described in this work demonstrated a strong effectiveness in blocking tumour cell adhesion and migration, in addition to their anti-proliferative action.

Conflict of interest statement

None declared.

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