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Pyrrolopyrimidine c-Src inhibitors reduce growth, adhesion, motility and invasion of prostate cancer cells *in vitro*

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Abstract

Two *bona fide* c-Src inhibitors (Bone 24(1999) 437; Bioorg Med Chem Lett 10 (2000) 945)), denominated CGP77675 and CGP76030, reduced in a time- and concentration-dependent manner (i) the proliferation of the PC3 prostate carcinoma cell line, as assessed by the [^{3}H]-thymidine incorporation test, (ii) the capacity of PC3 cells to adhere and spread on Matrigel substrate, as determined by crystal violet staining, (iii) the ability of PC3 cells to migrate through a gelatine boundary and invade a Matrigel substrate. The latter effect was not due to a decrease of urokinase-type plasminogen activator (uPA), nor of metalloproteinase-2 (MMP-2) activities. The MMP-9 activity, along with the expression of the Tissue Inhibitor of Metalloproteinases (TIMP)-1 and TIMP-2, were reduced by the two inhibitors, consistent with the ability of c-Src to enhance MMP-9 and TIMP expression levels. Collectively, these data demonstrate that the pyrrolopyrimidine-derived c-Src inhibitors significantly reduced PC3 cell activities associated with their malignant phenotype.

Keywords: Prostate cancer; c-Src; c-Src inhibitors; Bone metastases

1. Introduction

Prostate cancer generally starts as an androgen-dependent tumour, and progresses to an androgen-independent state following the initial response to androgen ablation [1]. In order to have a realistic chance of tackling this poor outcome, the identification of alternative therapeutic approaches is necessary. In particular, the understanding of the signal transduction pathways involved in the increased cellular growth and aggressiveness could make it possible to develop selective inhibitors able to target the tumour and prevent it spreading.

Although the mechanisms whereby androgen-independence develops are not yet fully clarified [2], it is known that malignant progression of prostate cancer involves upregulation of autocrine growth factors and their receptors [3]. The process of autocrine reprogramming facilitates autonomous growth and metastasis of the tumour cells. For this reason, many of the major novel therapeutic approaches for prostate cancer, currently in clinical trials, are directed against growth factor signalling pathways involving tyrosine kinase receptors and their downstream signalling messengers. Among these, recent evidence suggests a central role for the non-receptor tyrosine kinase, c-Src, in the development, growth and metastasis of many human cancers [4], including prostate carcinomas, where c-Src and its substrate Focal Adhesion Kinase (FAK) have been found to be involved in neuropeptide-induced androgen-independence [5].

Collectively, these data support the choice of c-Src as a *bona fide* therapeutic target for the prevention of the spread of cancer and development of distant metastases [6,7]. Several classes of inhibitors are currently used to inhibit the activity of c-Src in a number of cell types. However, they often show poor selectivity within the

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c-Src family, which in mammals comprises at least eight members involved in many key functions of the cell [8]. Recently, we have shown that c-Src inhibitors of the pyrrolopyrimidine class exhibit a powerful inhibitory activity and a several-fold greater selectivity for c-Src against most tyrosine kinases [9–11]. In this study, we show their potent effect in inhibiting prostate cancer cell activity *in vitro*.

2. Materials and methods

2.1. Cells

The human prostate cancer cell line PC3 and NIH3T3 fibroblasts were obtained from the American Tissue Culture Collection (Rockville, MD, USA) and grown in Dulbecco's modified Minimum Essential Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine.

2.2. Treatments

c-Src inhibitors, CGP77675 and CGP76030, were synthesised in the Chemistry Research Laboratories of Novartis Pharma [9,10], dissolved in dimethyl sulphoxide (DMSO) at 10 mM and diluted in cell culture medium prior to use. Controls were carried out with DMSO concentrations corresponding to the highest dose of test compounds. The final DMSO concentration did not exceed 0.2% (v/v) and did not affect the parameters analysed.

2.3. Cell viability and apoptosis

Cell viability was evaluated by the trypan blue exclusion test. PC3 cells were plated in 24-well multiplates and grown in DMEM plus 10% FBS (v/v). Cells were treated with increasing concentrations of the c-Src inhibitors for different times, to obtain concentration- and time-dependent curves. At the end of treatment, cells were collected, stained with 0.04% trypan blue (w/v) and observed by conventional light microscopy. The number of trypan blue-positive cells was expressed as a percentage of the total number of cells counted.

To evaluate apoptosis, cells were grown as above, treated with vehicle or 20 μM c-Src inhibitors, fixed in Carnoy's fixative (methanol–glacial acetic acid, 3:1), then incubated for 30 min in 0.5 μg/ml bisbenzimide, a fluorochrome that specifically binds the Adenine-Thymine sites. Cells were rinsed twice in distilled water and observed by conventional epifluorescence microscopy. Nuclei showing condensed chromatin or apoptotic bodies were enumerated and expressed as a percent of the total number of nuclei counted.

2.4. Cell proliferation

PC3 cells were plated in 24-well multiplates at 5000 cells/well and grown for 24 h in DMEM plus 10% FBS. Cells were then serum-starved for 30 h in DMEM plus 0.2% Bovine Serum Albumin (BSA), and subsequently treated with the test compounds for 30 min in DMEM plus 0.2% BSA (w/v), followed by 24 h in DMEM plus 20% FBS (v/v), which included 14 h with 2 μCi/ml [^{3}H]-thymidine (specific activity 25 Ci/mmol). At the end of the incubation, cells were dissolved in 0.5 ml of 0.1% Sodium Dodecyl Sulphate (SDS) (v/v) to which 10 μl of 100 mg/ml BSA was added as a carrier protein, and precipitated by addition of 100 μl of 100% Tri-Chloroacetic Acid (TCA) (v/v). After incubation for 30 min at 4 °C, the TCA-precipitable material was pelleted by centrifugation at 775 *g* for 15 min, re-dissolved in 500 μl of 0.1% SDS (v/v), and counted in a scintillation spectrophotometer after the addition of 5 ml scintillation fluid.

2.5. Matrix coating of plastic-ware and glass coverslips

Matrix coatings were performed by incubating for 18 h round glass coverslips or plastic culture dishes with Matrigel (35 μg/cm^2, kindly provided by Dr A. Albini, Advanced Biotechnology Center, "Istituto Nazionale per la Cura dei Tumori", Genova, Italy) in DMEM at 4 °C. Residual protein binding sites were blocked by saturating with 1% BSA (w/v) in phosphate-buffered saline (PBS) for 1 h at 37 °C. Coated surfaces were then washed three times in PBS and once in DMEM. The solutions were then replaced with 60% methanol (v/v), and the wells were further incubated for 1 h at 4 °C. Methanol was removed and the wells were incubated with a buffer containing 50 mM Tris/HCl pH 7.8, 110 mM NaCl, 5 mM $CaCl_2$, 1% BSA, and 0.1 mM polymethylsulphonylfluoride (PMSF) for 30 min at room temperature. Finally, wells were washed three times with DMEM supplemented with 0.2% BSA (w/v) and immediately used for the experiment.

2.6. Cell morphology, adhesion and spreading

Confluent PC3 cells were trypsinised and treated for 30 min in suspension with 2 μM CGP77675, CGP76030 or vehicle alone, in DMEM supplemented with 10% FBS (v/v). Cells were plated on glass coverslips at 5×10^4 cells/well and incubated for 2 h in the presence of the inhibitors. Cells were then fixed with 3.7% formaldehyde (w/v) in PBS and observed using a Nomarski microscopy.

For quantitative analysis, confluent PC3 cells were trypsinised and treated for 30 min in suspension with inhibitors or vehicle alone in DMEM supplemented with 10% FBS (v/v). Cells were then plated in 96-well

multiplates and incubated at different times in the presence of the inhibitors. At the end of incubation, wells were rinsed three times with PBS to remove non-adhering cells. Cells were fixed with 80% methanol (v/v) for 30 min, then stained with 0.5% crystal violet (w/v) in 20% methanol (v/v) for 5 min before rinsing with distilled water and air drying for 15 min. Crystal violet was then dissolved in 100 μl of 0.1 N sodium citrate in 50% ethanol (v/v) and the absorbance, linearly proportional to the number of attached cells, evaluated at 595 nm in an enzyme-linked immunosorbent assay (ELISA) plate reader.

To evaluate the effect of the c-Src inhibitors on cell spreading, confluent PC3 cells were subjected to trypsin release, kept for 30 min in suspension in DMEM plus 10% FBS (v/v), then gently centrifuged, re-suspended in serum-free–DMEM and plated on Matrigel-coated coverslips at 0.5×10^6 cells/well. Two hours later, cells were treated with 2 μM c-Src inhibitors or vehicle alone, incubated for 6 h, then fixed and observed by Nomarski microscopy. To evaluate the ability of the inhibitors to induce cell detachment, PC3 cells were allowed to adhere for 2 h, then treated with the inhibitors and stained 6 h later with crystal violet.

2.7. Migration and invasion assays

Invasion through reconstituted Matrigel was performed by the modified Boyden-chamber method [12]. PC3 cells were grown to confluence, detached by trypsinisation and washed three times in serum-free DMEM. Cell suspension (4×10^4 cells) was added to 12 μm polycarbonate filter coated with Matrigel (35 μg/cm^2) in the upper compartment of transwell invasion chambers, with serum free-DMEM in the lower compartment. Two hours later, the upper medium was substituted with DMEM containing c-Src inhibitors or vehicle alone, and the lower compartment with the chemo-attractant NIH3T3 cell-conditioned serum-free medium. Cells were incubated at 37 °C for 6 h, and those that did not migrate were then removed by wiping the top of the membrane with a cotton swab. Filters were stained with 0.1% toluidine blue (w/v) and cells counted at a 200× magnification in five randomly chosen fields/filter. Triplicate filters were used for each point and the results were averaged. Migration assays were performed in a similar manner, except that the filters were coated with 4.5 μg/cm^2 gelatine.

2.8. Zymograms for protease activities

PC3 cells at 2×10^6/dish were plated in serum free-DMEM into 6 cm diameter culture dishes coated with Matrigel. Cells were allowed to adhere, then treated with increasing concentration of c-Src inhibitors, in serum free-DMEM. Forty-eight-hour conditioned media were recovered and stored in aliquots, at −20 °C, until use.

For the detection of urokinase-type plasminogen-activator (uPA) activities, PC3 cell-conditioned media were subjected to non-reducing SDS-polyacrylamide gel electrophoresis (PAGE), in gels co-polymerised with 0.1% casein (w/v) plus 15 μg/ml human plasminogen. For the detection of metalloproteinase (MMP) activities, conditioned media were concentrated 20-fold with a 10 000 MW cut-off centrifugal filter devices, and electrophoresed under non-reducing conditions in a 7.5% acrylamide gel (w/v) containing 0.1% gelatine (w/v). Gels were washed three times for 15 min in 10% Triton X-100 (v/v) under shaking at room temperature, then incubated at 37 °C overnight in collagenase buffer (50 mM Tris/HCl pH 7.4, 200 mM NaCl, 5 mM $CaCl_2$) for MMPs, or for 4 h in Tris/HCl pH 7.4 for uPA. Gels were fixed and stained for 30 min at room temperature with 0.1% Coomassie blue (w/v) in a mixture of acetic acid:methanol:water (1:4:5) and de-stained in the same solution without dye. Proteolytic activities were identified for the appearance of clear bands.

2.9. Western blotting

Tissue Inhibitor of Metalloproteinase (TIMP)-1 and TIMP-2 expression levels were evaluated by Western blotting in PC3 cell-conditioned media. Briefly, 15 μg proteins of 20-fold concentrated samples were subjected to 10% SDS-PAGE (w/v) under reducing conditions, then transferred to Hybond nitrocellulose. After blocking in 10% non-fat milk (w/v) in PBS, primary polyclonal antibodies (produced in our laboratory) were incubated with the blots at a dilution of 5 μg/ml overnight at 4 °C. Species-specific HorseRadish Peroxidase (HRP)-conjugated secondary antibodies were incubated at 1:5000 dilution for 60 min at room temperature prior to Enhanced ChemiLuminescence (ECL) detection of the proteins. Inhibition of c-Src activity by CGP77675 and CGP76030 was also assessed by western blotting. For sample preparation, confluent PC3 cells were detached with 1 mM ethylene diamine tetraacetic acid (EDTA), treated with 2 μM c-Src inhibitors or vehicle alone for 45 min in suspension, then plated in 10 cm diameter culture dishes, coated with 20% FBS (v/v). Cells were allowed to adhere for 30 min to activate c-Src, then lysed in extraction buffer (Tris/HCl 50 mM pH 7.4, 1% Nonidet P-40 (v/v), 0.25% sodium deoxycholate (w/v), 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate). Three hundred microgrammes protein were electrophoresed as described above. Anti-pY-Src416 (cat. #2101, Cell Signaling Technology, Inc., Beverly, MA, USA) antibody was incubated with blot, at a dilution of 1:500, overnight at 4 °C. After Enhanced

Chemiluminescence (ECL) detection, the filter was stripped and re-probed with anti-v-Src antibody (cat. #OP07, Oncogene Research Laboratory, Boston, MA, USA) at a dilution of 1:80. For the detection of total tyrosine phosphorylation, 100 μg protein were electrophoresed and the filters incubated with anti-pY antibody (cat. # sc-7020, SantaCruz Biotechnology, Inc. Heidelberg, Germany), at a dilution of 1:2000.

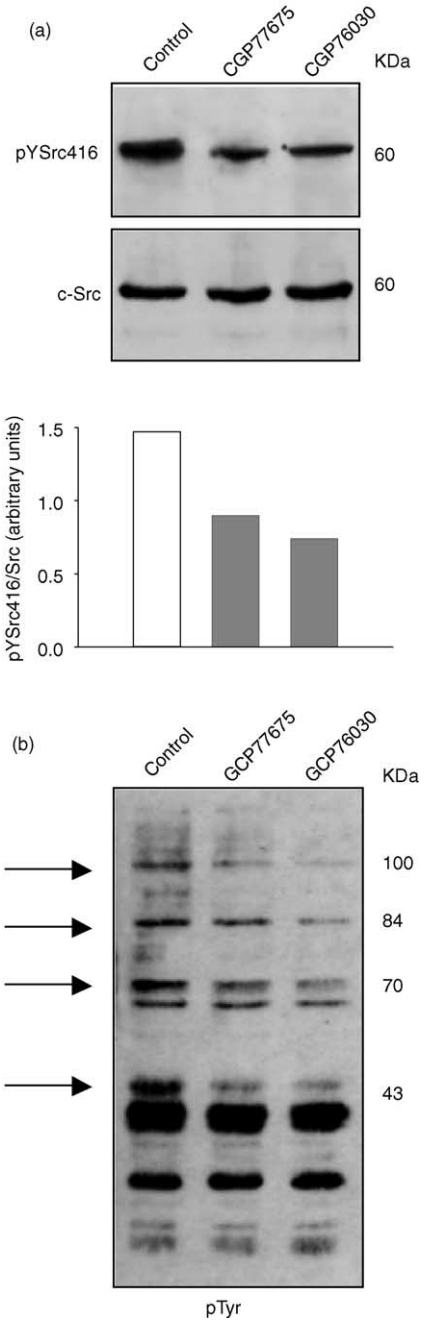

Fig. 1. Western blot analysis of c-Src activity: (a) filter probed with anti-pYSrc416 antibody, stripped and re-probed with anti-Src antibody; (b) filter probed with anti-pTyr antibody. Arrows: bands of reduced intensity. The experiment was repeated three times with similar results.

2.10. Densitometric analysis

Band areas were analysed by scanning densitometry, using the Molecular Analyst software for the Bio-Rad Laboratories (Hercules, CA, USA) model 670 scanning densitometer. Band areas of pY-Src416 were normalised against those of total c-Src as an internal control. MMP and PA activities and TIMP expression were normalised against total protein amounts, measured by the Bradford method.

2.11. Statistics

Data are expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Statistical analysis was performed by the unpaired Student's *t*-test. A P value <0.05 was conventionally considered as statistically significant.

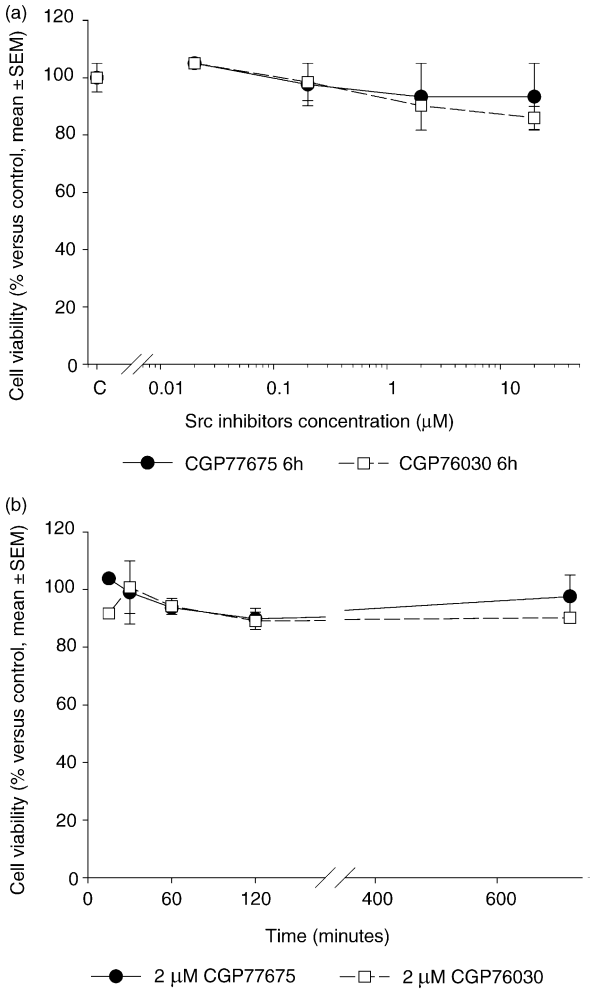

Fig. 2. Cell viability. PC3 cells were treated with test compounds at the concentrations (a) and times (b) indicated on *abscissae*. Cells were then released by trypsinisation and subjected to the trypan blue exclusion test. Mean ± Standard Error of the Mean (SEM) of three independent experiments. Differences were non significant.

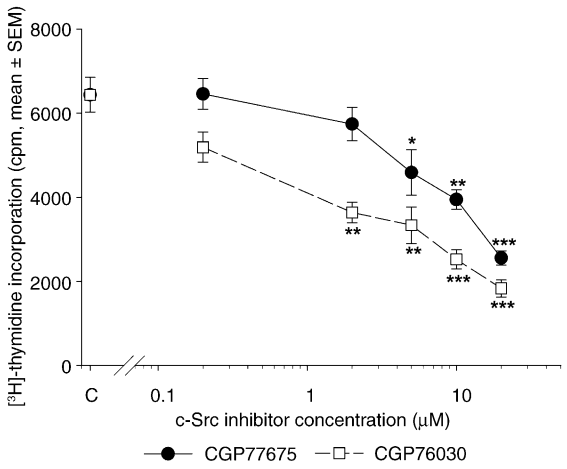

Fig. 3. Proliferation assay. PC3 cells were serum-starved and exposed to 20% FBS (v/v) as a source of mitogens in the presence of [^{3}H]-thymidine and of the test compounds or dimethyl suphoxide (DMSO) alone. *$P<0.05$, **$P<0.005$, ***$P<0.0005$.

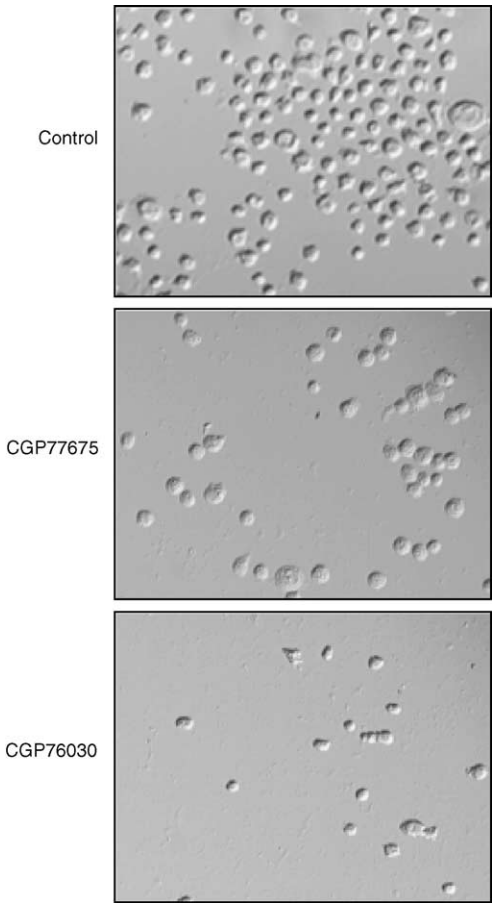

Fig. 4. Adhesion assay. PC3 cells were trypsinised, treated with 2 μM of c-Src inhibitors for 30 min and re-plated in wells. Two hours later, morphological observation was performed using the Nomarski microscopy. Original magnification 20×.

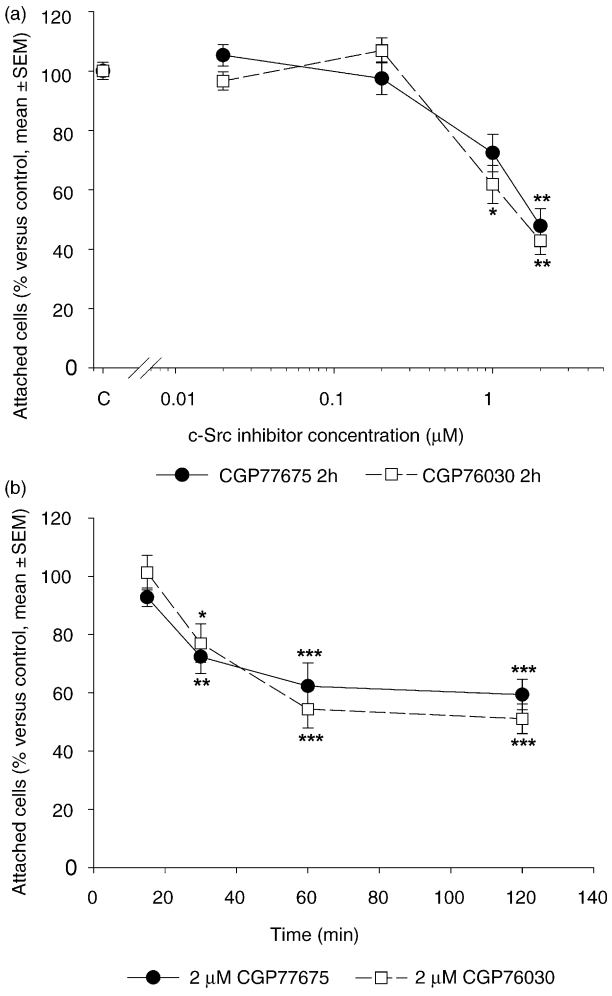

Fig. 5. Adhesion to substrate. (a) Cells were treated with dimethyl suphoxide (DMSO) (control) or test compounds, at the concentrations indicated on *abscissa*, for 120 min. (b) Cells were treated with DMSO or 2 μM test compound, and allowed to adhere for the times indicated on *abscissa*. Mean±Standard Error of the Mean (SEM) of three independent experiments. *$P<0.05$, **$P<0.005$, ***$P<0.0005$.

3. Results

3.1. Inhibition of c-Src activity

In this study, we employed two substituted 5,7-diphenyl-pyrrolo[2,3-*d*]pyrimidines, denominated CGP77675 and CGP76030, which proved to be active at inhibiting the phosphorylation of c-Src peptide substrates and c-SrcY416 auto-phosphorylation. They exhibited a high selectivity towards other tyrosine kinases from receptor and non-receptor families, including other members of the c-Src group [9–11]. As shown in Fig. 1a, decreases of approximately 40 and 50% of pYSrc416 compared with the control were observed in the PC3 cells treated with 2 μM CGP77675 and CGP76030, respectively. Total c-Src expression levels were unaffected. An overall decrease of tyrosine phosphorylation of several proteins, believed to be c-Src substrates (Fig. 1b, arrows) was also detected.

The CGP76030 appeared slightly more efficacious than CGP77675.

3.2. PC3 cell viability and apoptosis

The trypan blue exclusion test demonstrated that our inhibitors did not reduce PC3 cell viability at the indicated concentrations (Fig. 2a) and times (Fig. 2b). Bisbenzimide staining showed no increase of condensed chromatin or apoptotic bodies in c-Src inhibitor- versus vehicle-treated cultures (Mean% ± SEM, Control: 2.8 ± 1.44; CGP77675: 5.5 ± 1.23; CGP76030: 4.3 ± 2.2; $n = 3$, non-significant (N.S.)).

3.3. PC3 cell proliferation

Serum-starved cells were exposed to increasing doses of the test compounds and 20% FBS (v/v). c-Src inhibitors decreased [^{3}H]-thymidine incorporation in a concentration-dependent manner (Fig. 3), with CGP77675 again being less effective ($IC_{50} = 7$ μM) than CGP76030 ($IC_{50} = 1$ μM).

3.4. Adhesion to the extracellular matrix

A reduced adhesion of PC3 cells treated with either CGP77675 or CGP76030 was observed 2 h after plating (Fig. 4). The effect was concentration- and time-dependent, with a similar efficacy being observed for the two compounds (Fig. 5a and b). IC_{50} were 1 μM for CGP77675 and 0.7 μM for CGP76030.

3.5. Cell spreading

PC3 cells were allowed to adhere for 2 h (Fig. 6a, upper panels, C_0) on Matrigel-coated coverslips and treated for 6 h with 2 μM of the test compounds (Fig. 6a, lower panels, T_6) or vehicle alone (Fig. 6a, upper panels, C_6). Nomarski microscopy showed that vehicle-treated cells (C_6) were fully spread and had acquired a motile phenotype compared with cells at time 0 (C_0). In contrast, in the c-Src inhibitor-treated cultures, spreading was nearly abolished (Fig. 6a, compare T_6 with C_6). Quantitative analysis showed that, in these experimental conditions, the number of cells attached to the substrate

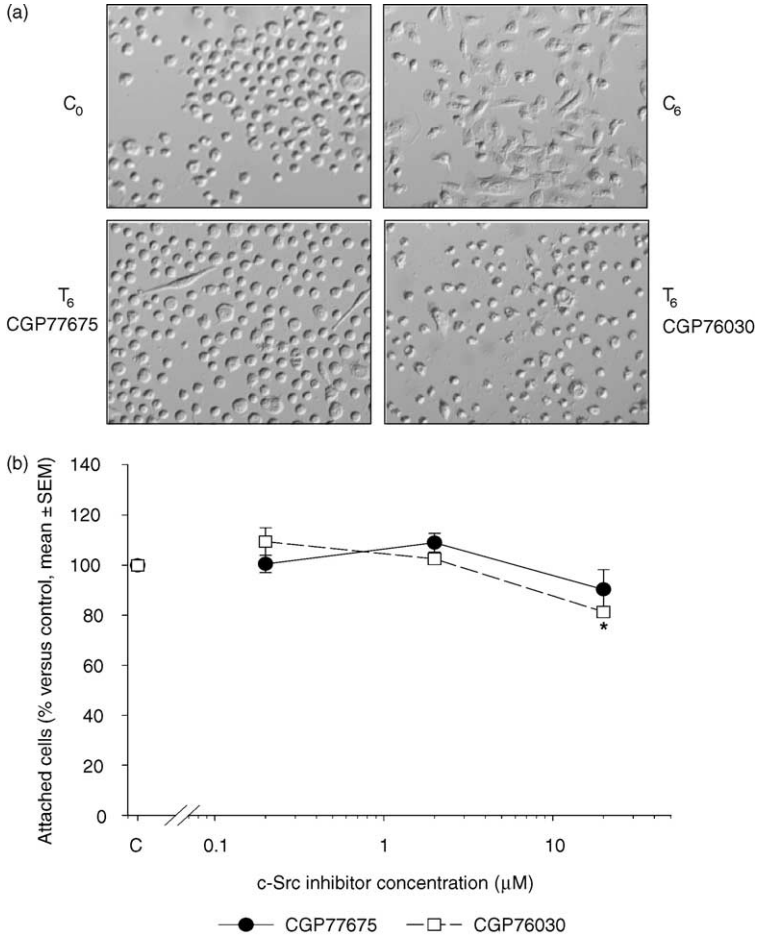

Fig. 6. Cell spreading. (a) Nomarski microscopy of cells after 2 h from plating (C_0), or plus 6 h of treatment with vehicle (C_6) or with test compounds (T_6). Original magnification 20×. (b) Quantitative analysis. Mean ± Standard Error of the Mean (SEM) of three independent experiments. *$P < 0.05$.

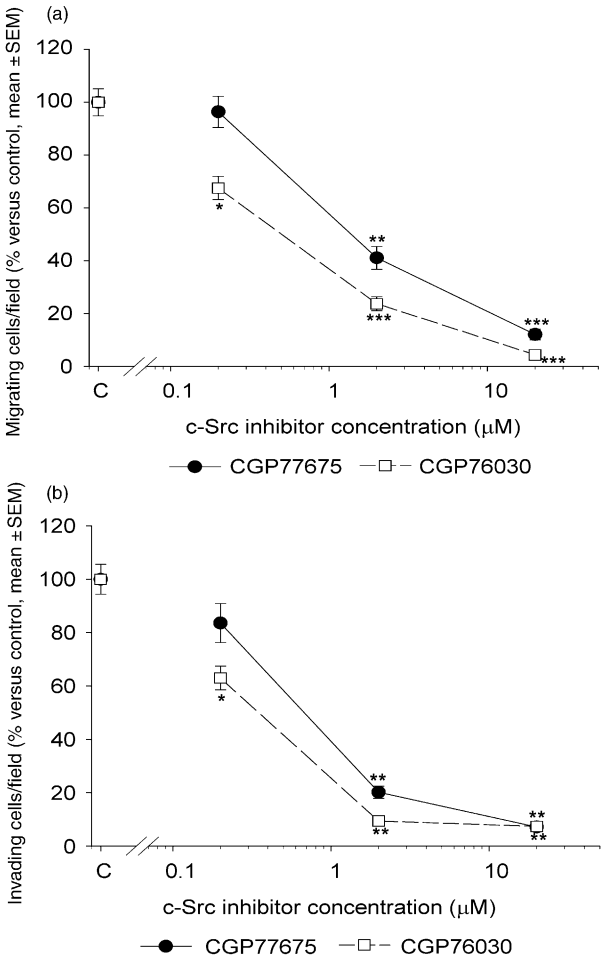

Fig. 7. Migration and invasion assays. PC3 cells were cultured in the upper compartments of Boyden chambers, on porous membranes coated with (a) gelatine or (b) Matrigel substrates, and treated as indicated in Fig. 6. The lower compartment contained NIH3T3-conditioned media as a source of chemoattractants. Number of cells migrating to the lower side of the membranes was counted to estimate the migration (a) or invasive (b) abilities. Mean ± Standard Error of the Mean (SEM) of three independent experiments. $^{*}P<0.001$, $^{**}P<0.0001$, $^{***}P<0.00001$.

was only modestly affected at the highest inhibitor concentration tested (15–19% reduction at 20 μM, Fig. 6b).

3.6. Migration and invasion

In the migration experiments, cells were allowed to move through gelatine-coated polycarbonate filters towards the chemo-attractant-conditioned medium from NIH3T3 cells. The graph in Fig. 7a shows a concentration-dependent decrease of migrating cells/field in response to both inhibitors. At 20 μM, migration was nearly abolished, with 88% inhibition with CGP77675 and 96% with CGP76030 versus the control. Invasion through a Matrigel barrier was also markedly impaired by the test compounds (Fig. 7b). In both assays, CGP76030 was slightly more efficacious than CGP77675. (IC_{50} migration: CGP77675, 1 μM; CGP76030, 0.48 μM. IC_{50} invasion: CGP77675, 0.6 μM; CGP76030, 0.3 μM).

3.7. Matrix degrading enzymes activities and TIMP expression

Fig. 8 demonstrates that conditioned media from control PC3 cells showed high urokinase-type plasminogen activator (uPA) activity, with no modulation by the c-Src inhibitors (top panels). In contrast, the metalloproteinase 9 (MMP-9) was concentration-dependently decreased by the two inhibitors (second from the top panels). MMP-2 was barely active under our conditions and was not modulated in a c-Src-dependent manner (data not shown). Consistent with the inhibition of MMP-9, TIMP-1 and 2 expression levels, known to contribute to the targeting and regulation of metalloproteinases, were also reduced by the c-Src inhibitors in the PC3 cells (Fig. 8, lower two rows of panels). However, both MMP-9 and TIMPs were significantly inhibited only at concentrations of both compounds that were >2 μM.

4. Discussion

Numerous studies have provided compelling evidence that c-Src plays a central role in the development of several tumours, where it is often overexpressed and deregulated [4]. Development of small-molecular weight inhibitors of tyrosine kinases has proved to be useful for the treatment of cancers. Disappointingly, however, these compounds often display a poor selectivity among the various classes of tyrosine kinases, thus significantly restricting their therapeutic applications. 5,7-Diphenylpyrrolopyrimidines have been identified as potent inhibitors of the c-Src tyrosine kinase, with a high specificity not only relative to other receptor- or non-receptor tyrosine kinases, but also relative to the other members of the c-Src family itself. We have previously shown that these inhibitors exhibit at least a 10-fold greater specificity for c-Src versus c-Lck [9], whereas their efficacy against c-Yes is similar to that against c-Src, at least in enzymatic assays [11]. Both c-Lck and c-Yes are expressed in prostate cancers [13]. However, their relevance in the malignant phenotype remains to be elucidated. Nevertheless, the remarkable *in vitro* activity of CGP77675 and CGP76030 has been demonstrated in our cellular model of human prostate carcinoma, providing evidence of their effectiveness at inhibiting the autophosphorylation of c-Src Y416 and tyrosine phosphorylation of c-Src substrates, as well as various functions closely related to the malignant phenotype of this cell line. The observed effects were not due to toxicity or to apoptosis, as confirmed by an analysis of both cell viability and nuclear damage.

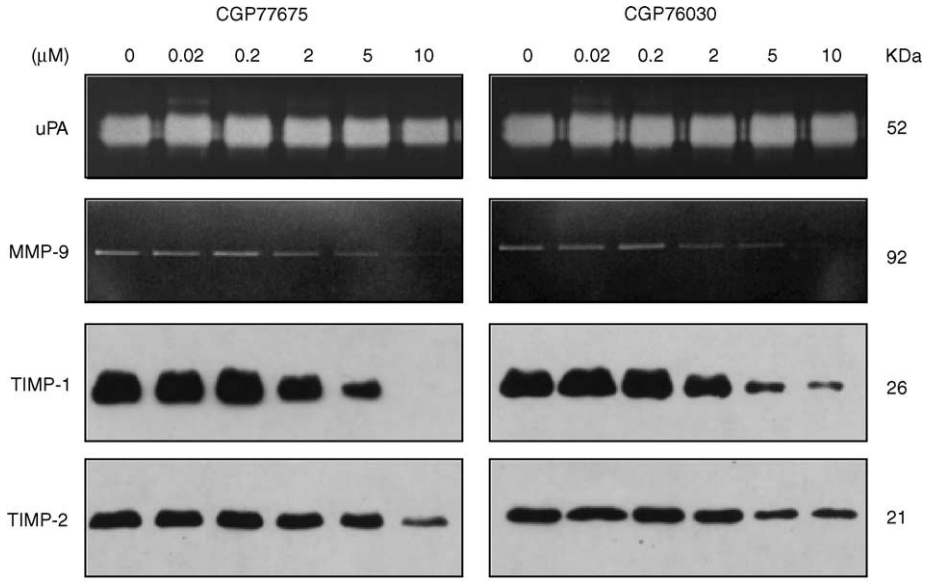

Fig. 8. Matrix degrading enzymes and Tissue Inhibitors of Metalloproteinases (TIMPs). Conditioned media from PC3 cells treated with vehicle (dimethyl sulphoxide (DMSO)) or with the indicated concentrations of c-Src inhibitors were tested by zymography for uPA and MMP-9 activities, and by western blot analysis for TIMP-1 and TIMP-2 expression levels. The experiment was repeated three times with similar results.

Proliferation is under the control of numerous extracellular factors and intracellular signals, and c-Src is known to play a pivotal role in the control of various steps of the cell cycle. For instance, c-Src is downstream of a number of growth factor receptors, which lead to the activation of the Ras mitogen-activated protein kinase (MAPK) pathway stimulating cell proliferation [14]. Thymidine incorporation was concentration-dependently decreased in PC3 cells treated with our compounds, thus indicating an inhibitory effect on one of the most relevant events in carcinogenesis. However, the calculated IC_{50} for both compounds was above 2 μM, where additional tyrosine kinase activities could be blocked and contribute to the inhibition of proliferation. Remarkably, our inhibitors proved to be effective against another essential cell activity, adhesion to the substrate, which is believed to determine controlled cell growth and elicit physiological signal transduction pathways within the cells [15]. Adhesion to the extracellular matrix is a mandatory requirement of most cell types of our body for proliferation, regulation of gene expression [16,17] and cell survival [18]. Migration is another event requiring integrin signals, as it depends on the ability to form dynamic adhesions that allow the cell to move through the extracellular boundaries. c-Src is involved in all of these events as a tyrosine kinase enzyme, as well as an adapter protein [19], and enhancement of the FAK/c-Src signal transduction pathway correlates with an increased migratory capacity of prostate cancer cells [20]. We found that both the adhesion and spreading onto a substrate, as well as the ability of cells to migrate towards a chemotactic stimulus were potently inhibited by our compounds.

c-Src plays a central role in carcinoma cell motility and its dominant-negative mutant forms markedly reduce the motility of tumour cells expressing high levels of c-Src [21]. Our c-Src inhibitors potently inhibited the ability of PC3 cells to enter a reconstituted basal lamina (Matrigel) in response to chemoattractants. The passage through the Matrigel barrier may require increased motility, as well as degradation of its proteinaceous constituents. A number of matrix degrading enzymes are known to serve this purpose. Our assays have shown no effect on uPA and MMP-2 and a reduction of MMP-9 activity at c-Src inhibitor concentrations >2 μM. MMP-9 is a matrix degrading enzyme regulated in a c-Src-dependent manner at the transcriptional level through a GT box located downstream of the AP-1 site of its promoter [22]. Therefore, our results would be consistent with this property of c-Src. TIMP-1 and -2 were also inhibited in our study, which may also have influenced invasion. TIMP-1 is known to be regulated in a v-Src-dependent manner in transformed cells [23]. However, the high IC_{50} calculated for the migration and invasion assays would predict that the major role of c-Src is with regard to the motility capacity rather than the matrix degradation ability of PC3 cells.

PC3 cells are derived from a human prostate cancer bone metastasis. c-Src has been demonstrated to have a central role in bone cell homeostasis [24,25]. Bone metastases are common events in prostate carcinoma progression [26,27] and are responsible for much of the morbidity of the disease, including pain, pathological fractures, hypercalcaemia and the nerve compression syndrome [28]. In recent years, the important role of c-Src in osteoclast activity has also been clearly demon-

strated. In fact, the *c-src* gene has been shown to be critical for the bone resorbing activity, in that loss of function mutation in mice impairs osteoclast function [24]. Although the predominant pattern of prostate cancer metastases to the bone is osteoblastic, careful and extensive histological examination of metastatic bone lesions often reveals the presence of osteoclastic bone destruction together with osteosclerosis [29]. Increased biochemical indices of bone resorption have consistently been reported in patients with prostate carcinoma and bone metastases [30] and osteolytic lesions are responsible for some of the morbidity observed in these patients. Our previous *in vivo* data demonstrated that c-Src tyrosine kinase blockade prevented bone loss in ovariectomised rats [9]. Based on this knowledge, an efficacious treatment of prostate cancer bone metastases should be targeted to tumour cells and bone cells. Given their ability to inhibit prostate cancer cell growth, adhesion, motility and invasion, as well as bone resorption, we believe that our class of c-Src inhibitors could be further developed to increase their potency and selectivity, and tested as alternative therapeutics against the development not only of primary prostate cancers, but also of their skeletal lesions [31].

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