ORIGINAL ARTICLE
ARTICOLO ORIGINALE

The activity of STI571 in human osteosarcoma cells

Attività di STI571 su linee cellulari di osteosarcoma umano

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Summary

Aims. We investigated the potential activity of STI571 on signal transduction pathways and its pro-apoptotic effect in osteosarcoma cell lines expressing PDGF and its receptor.
Materials and Methods. SCF-receptor and PDGF-receptor expression was evaluated in five human osteosarcoma cell lines by cytofluorimetry and immunohistochemistry. The activation status of these receptors was analysed by immunoprecipitation studies in basal conditions, after stimulation with PDGF, and after stimulation with PDGF and inhibition with STI571, simultaneously. We detected the presence of PDGF in culture media using an Enzyme-Linked ImmunoSorbent Assay. We then performed apoptosis analysis by Terminal desoxy-Uridine Nucleotide Extending Labelling (TUNEL) assay with a scalar concentration of STI571 at 24 and 48 hours.
Results. Four cell lines were positive for PDGF-Rβ, one for PDGF-Rα and one slightly positive for both receptors. We observed low levels of c-Kit expression. Detectable levels of PDGF were found in all the supernatants analysed. Immunoprecipitation studies showed a receptor constitutive phosphorilation in basal conditions, amplification of this status with exogenous PDGF and a dose-dependent inhibition by STI571.
Discussion. STI571 might interfere with osteosarcoma proliferative signals *in vitro* by selective inhibition of PDGF-R, suggesting a potential use of this drug in association with traditional antineoplastic agents.

Key words

Osteosarcoma • Tyrosine kinase receptor • Autocrine loop • PDGF • STI571

Riassunto

Obiettivi. *Abbiamo valutato i potenziali effetti pro-apoptotici di STI571 su linee cellulari di osteosarcoma umano esprimenti il PDGF ed il suo recettore.*
Materiali e metodi. *Attraverso indagine citofluorimetrica ed immunocitochimica abbiamo analizzato l'espressione dei recettori per il PDGF e lo SCF su 5 linee cellulari di osteosarcoma. Lo stato di attivazione di tali recettori è stato valutato attraverso studi di immunoprecipitazione in condizioni basali, dopo stimolazione con PDGF e dopo contemporanea stimolazione con PDGF ed inibizione con STI571. Attraverso test immunoenzimatici abbiamo ricercato la presenza del PDGF nel surnatante delle linee cellulari. Infine, abbiamo valutato l'apoptosi indotta da concentrazioni scalari di STI571 dopo 24 e 48 ore.*
Risultati. *Quattro linee cellulari esprimevano il PDGF-Rβ, una esprimeva il PDGF-Rα ed un'altra esprimeva debolmente entrambi i recettori; abbiamo osservato bassi livelli di espressione del c-kit. Inoltre, nei surnatanti delle linee cellulari era presente il PDGF. Gli studi di immunoprecipitazione hanno infine evidenziato una costitutiva fosforilazione del PDGF-R in condizioni basali, un'amplificazione di tale stato dopo stimolo esogeno con PDGF ed una inibizione dose-dipendente dopo aggiunta di STI571.*
Discussione. *STI571 può interferire in vitro con il segnale proliferativo dell'osteosarcoma attraverso inibizione selettiva del PDGF-R, suggerendone un possibile impiego in associazione alla chemioterapia tradizionale.*

Parole chiave

Osteosarcoma • Recettore tirosin-chinasi • Ansa autocrino • PDGF • STI571

Submitted: December 10, 2004
Accepted: January 16, 2005

Acknowledgements
This work was supported by AIRC – Milan, Italy, grant to E.M.

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Introduction

Osteosarcoma is the most common primary malignant bone tumour in children. Its prognosis remains poor despite recent advances, with a 5-year survival rate of 40-50% for patients with metastatic disease at diagnosis [1]. For

patients with osteosarcoma in metastatic relapse [2] overall survival rates are between 20 and 25% after metastasectomy and aggressive second line chemotherapy. These results in poor prognosis patients underline the need for additional therapies to preserve complete remission. Several studies [3 4] show that an aberrant activation of tyrosine kinase receptors such as Stem Cell Factor receptor (c-kit) or Platelet Derived Growth Factor-Receptor (PDGF-R) protect tumour cells against apoptosis and enhance their invasive potential, suggesting an autocrine and paracrine mechanism between receptors and their ligands.

Platelet Derived Growth Factor (PDGF) is a major mitogen for fibroblasts, smooth muscle cells and other cells of mesenchymal origin; it plays a central role in regulating cell proliferation, chemotaxis and survival in normal cells as well as in various disease states such as cancer, arteriosclerosis and fibrotic disease. The PDGF/PDGF-R system involves two receptors (PDGF-R α and PDGF-R β) and four ligands (PDGF-A, B, C and D). The binding of PDGF determines receptor dimerisation, structural changes and autophosphorylation and subsequent activation of an intracytoplasmic cascade that leads to multiple events such as cytoskeletal changes, gene transcription, cell motility, cell proliferation, cell differentiation, cell scattering and many others [5].

Coexpression of PDGF and its receptor has been observed in various human tumours including meningioma, astrocytic glioma and other brain tumours, melanoma, ovarian cancer, neuroendocrine tumours, pancreatic cancer, gastric cancer and prostate cancer [6 7]. Similar data about a Stem Cell Factor (SCF)-dependent autocrine loop in neuroblastoma cell lines and primary tumours were reported by our group [8 9]. Autocrine or paracrine stimulation of c-kit was also described in Ewing sarcoma – the second most common bone tumour in children and adolescents [10] – and in uveal melanoma [11]. Coexpression of c-kit and its ligand SCF mRNA in synovial sarcoma was described by Tamborini et al. [12], who also recently investigated the simultaneous expression of c-kit and PDGF-R β in this tumour, suggesting a mutually exclusive expression of the two receptors [13]. Selective inhibition of these growth factor-receptor signalling loops may represent a new treatment strategy for managing osteosarcoma as well as other types of cancer. Co-targeting more protein tyrosine kinases might also represent a new therapeutic approach, to obtain a synergetic effect in proliferation inhibition and apoptosis induction [14].

At present, tyrosine kinase inhibitors include multiple agents, some of these in early clinical development: Trastuzumab, which inhibits Her2/neu in breast cancer; Cetuximab, which inhibits Epidermal Growth Factor Receptor (EGFR) in colorectal cancer; ZD1839, which targets EGFR in non-small cell lung cancer; OSI-774, which inhibits EGFR in pancreatic cancer and many others [15].

STI571 or Imatinib, a 2-phenylaminopyrimidine derivative, is a well-known inhibitor of the proto-oncogene c-abl, the bcr-abl fusion protein – derived from the chromosomal translocation t(9;22) – and PDGF-R tyrosine kinases [16]. New approaches using this drug in GastroIntestinal Stromal Tumors (GISTs) are showing encouraging results [17]: moreover on February 1st 2001, Imatinib mesylate was approved by the United States Food and Drug Administration for the treatment of malignant metastatic and/or unresectable GISTs [18].

There is also a potential role for STI571 in other tumours bearing c-kit abnormalities, such as small cell lung cancer [19].

The therapeutic efficacy of STI571 on osteosarcoma is now the object of increasing attention. Recent studies have shown an over-expression of PDGF and its receptors [20] and the presence of c-kit on the surface of human osteosarcoma cells [21], thus predicting an efficacy of STI571 also in osteosarcoma. Therefore, our study focused on the anticancer activity of STI571 in human osteosarcoma cells *in vitro*.

In this study, we analysed the expression of PDGF and its receptors in osteosarcoma cell lines and investigated a potential interference of STI571 on its signal transduction pathways (modulation of the receptor activation status) and its possible pro-apoptotic effect.

Materials and methods

Cell lines

We analysed five human osteosarcoma cell lines: MG-63, HOS, SJSA-1, U-2 OS, Saos-2, obtained from the American Type Culture Collection. MG-63 and HOS cell lines were maintained in Eagle's Minimum Essential Medium (EMEM, EuroClone), while SJSA-1, U-2 OS and Saos-2 cell lines were maintained in Roswell Park Memorial Institute Medium (RPMI, Sigma), both supplemented with 10% Foetal Bovine Serum (FBS) and 0.5 mg/ml penicillin/streptomycin. The human chronic myelogenous leukaemia cell line K562, expressing c-kit, was used as a control.

Cytofluorimetric detection of surface c-kit and PDGF-R α and β chains

Five x 10^5 cells were incubated with anti-CD117 phycoerythrin (PE)-conjugated monoclonal antibody (MoAb) (Caltag) and anti α and β chain of PDGF-R MoAb (R&D System), at 4 °C for 30 minutes, then washed twice in Phosphate-Buffered Saline (PBS). Another incubation with Fluoresceine Isotyocianate (FITC)-conjugate Goat anti-Mouse secondary antibody and two further washes were performed to identify PDGF-R. All samples were analysed by flow cytometry (FACS Calibur, Becton Dickinson). PE and FITC conjugated isotypic antibodies were used as controls. To quantify the average number of receptors per cell, the Quantum (TM) 27 R-Phycoerythrin-conjugated kit (Dako) was used. This contains a set of calibrated standards, with four populations of microbeads displaying increasing and predetermined fluorescence intensity

(expressed in terms of number of Molecules of Equivalent Soluble Fluorochromes [MESF]) and one blank reference population. Using this method, the relative channel number obtained by flow cytometry analysis of a cell population was directly transformed into the number of MESF.

Immunohistochemistry for c-kit and PDGF-R α and β expression

To detect the presence of PDGF-R α and β chains on the osteosarcoma cell surface we used the commercial EnVision + System-Peroxidase kit (DAKO), according to the manufacturer's protocol.

Immunoprecipitation studies

The c-kit and/or PDGF-R positive cell lines were analysed for the phosphorylation of these receptors in different conditions: 1) basal condition; 2) stimulation with exogenous PDGF 10 ng/ml for 10 minutes; 3) stimulation with PDGF for 10 minutes plus inhibition with scalar doses of STI571 (1, 10 and 50 μM) for 90 minutes. Cells were then resuspended in serum-free medium and incubated for 24 hours at 37 °C 5% CO2. STI571 treated and untreated cells were rapidly pelleted and resuspended in lysis buffer (10 mmol/l trisHCl, pH 8.6; 1.5 mmol/l MgCl2; 0.14 mol/l NaCl; 1% NP40; 2 mmol/l phenylmethylsulfonylfluoride; 2 μg/ml leupeptin; 2 μg/ml aprotinin; 1 mg/ml pepsin). The lysates were incubated for a minimum of 30 minutes on ice, frozen, thawed, and centrifuged at 13,000 rpm at 4 °C for 20 minutes. Immunoprecipitation of c-kit and PDGF-R α and β chains were performed on the clarified supernatant with specific MoAb (R&D Systems) coupled to sepharose-protein (Sigma Chemical, St Louis, MO). After incubation for 16 hours at 4 °C, immunoprecipitates were washed, first with lysis buffer without NP-40, then with tris HCl 50 mmol/l, pH 6.5. After elution of samples from proteins with Sodium Dodecyl Sulphate (SDS) a sample buffer was carried out. The resulting proteins underwent a 7% SDS-PolyAcrylamide Gel Electrophoresis (PAGE). The proteins were electrophoretically transferred to nitro-cellulose filters, which had been incubated overnight at 4 °C with a 3% albumin bovine (Sigma) blocking solution in TBS-Tween buffer (20 mmol/L Tris HCl, pH 7.6; 137 mmol/L NaCl; 0.1% Tween 20). Antiserum anti-phosphotyrosine PY20 (Transduction Laboratories, Lexington, KY) was added to the solution and incubated for 2 hours at room temperature. The filters were then washed three times for 10 minutes with PBS-Tween buffer and incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated (Biorad) rabbit anti-mouse antibody. The filters were washed as above and visualised using Immun-Star Chemiluminescent Protein Detection System (Biorad). The mean of intensity of the bands visualised on the x-ray film was semiquantitatively evaluated with Quantity One Software (Biorad) and expressed as intensity (INT).

Detection of soluble SCF and PDGF production by tumour cell lines

To detect and quantify the presence of PDGF soluble forms -AA, -BB and -AB, we harvested the media of all cell lines in basal serum-free conditions and after 24 and 48 hours of incubation with STI571 at concentrations of 0.1, 1 and 10 μM. We used an ELISA method (Quantikine, R&D Systems) with a sensitivity of 4 pg/mL.

Evaluation of apoptosis by TUNEL assay analysis

The cells were cultured in serum-free conditions with and without STI571 at a scalar concentration (0.1, 1, 10 μM) for 24 and 48 hours. The cells were fixed by adding formaldehyde (1% in PBS) for 15 minutes on ice. After resuspension in PBS, the cells were stored at 4 °C in ethanol (70% in PBS). To perform the apoptosis analysis, the cells were rehydrated in PBS and then washed twice with PBS and incubated with a cacodylate buffer containing 5 U of TerminaldesossinucleotidylTransferase (TdT) (Boehringer) and 0.5 μmol/L of FITC-desoxyUridineTriPhosphate (Caltag) for 1 hour at 37 °C. The cells were rinsed in PBS and incubated with 0.4 μg/mL propidium iodide for 30 minutes before cytofluorimetric analysis.

Results

Cytofluorimetric detection of surface c-kit and PDGF-R α and β chains

Surface expression of PDGF receptor α and β chains and c-kit was tested in basal culture conditions in all five cell lines. Four cell lines (HOS, SJSA-1, Saos-2 and U-2 OS) were positive for PDGF-R β, though with a different expression as shown in Table I. The control K562 cell line also showed a low positivity for PDGF-R β and only the MG-63 cell line had a high positivity for PDGF-R α expression. The Saos-2 cell line was weakly positive for both PDGF-R β and c-kit. All the osteosarcoma cell lines analysed showed low levels of MESF values of c-kit expression, with the MESF value comparable with the blank control, except for a weak expression in Saos-2. Therefore, in our subsequent experiments we focused our attention on the study of PDGF and its receptors (Fig. 1).

Tab. I. MESF value of the cells positive for PDGF-R α and β and for c-kit in all the osteosarcoma cell lines analysed.

	PDGF-R α	PDGF-R β	c-KIT
HOS	0,00	72720,18	0,00
MG-63	116220,50	0,00	0,00
SAOS-2	0,00	784,90	120,80
SJSA-1	0,00	76872,80	0,00
U-2 OS	0,00	22625,57	0,00
K562	0,00	4561,37	114571,84

Fig. 1. Cytofluorimetric detection of PDGF-R β, in HOS cell line (A, B), Saos-2 cell line (C, D), SJSA-1 cell line (E, F), U-2 OS cell line (G, H); PDGF-R α expression in MG-63 cell line (I, L). Cytofluorimetric analysis of the cells stained with isotypic antibody (negative control) (left); histograms of the positive cells (right).

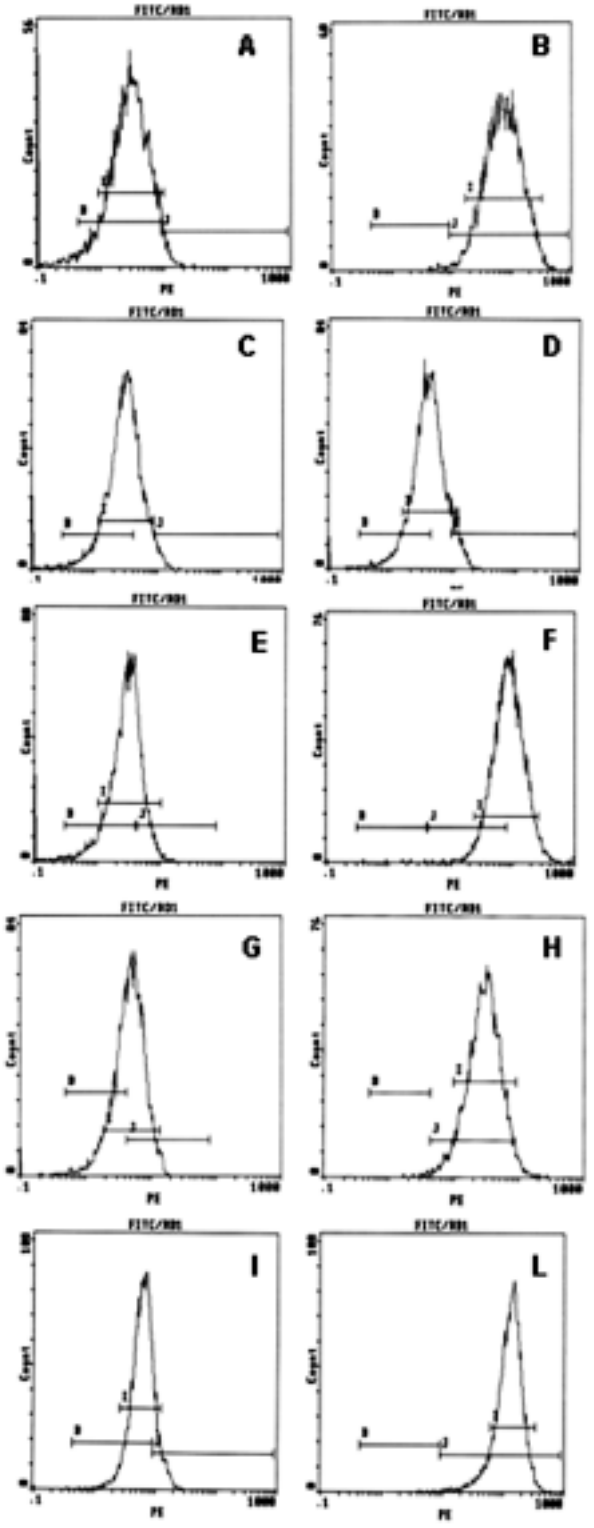
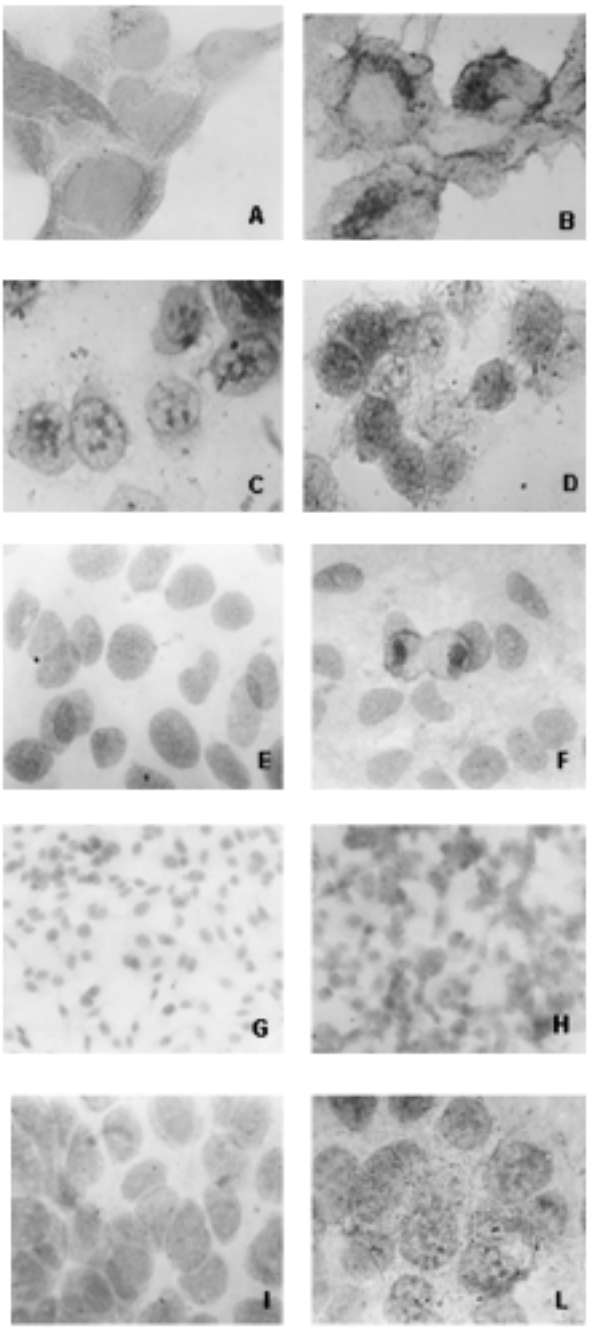


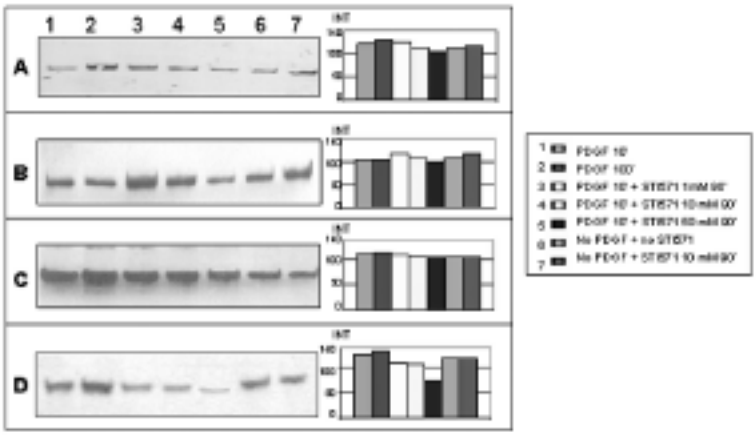
Fig. 2. Immunohistochemistry for PDGF-R α and β expression: in MG-63 cell line (A: negative control, B: PDGF-R α expression), SAOS-2 cell line (C: negative control, D: PDGF-R β expression), SJSA-1 cell line (E: negative control, F: PDGF-R β expression) (m.o. 100x), HOS cell line (G negative control, H PDGF-R β expression; m.o. 20x), U-2 OS cell line (I negative control, L PDGF-R β expression; m.o. 100x). PDGF-R expression is evident as a small brown granulation.



IMMUNOHISTOCHEMISTRY FOR C-KIT AND PDGF-R α AND β EXPRESSION

Immunohistochemistry analysis confirmed these results. PDGF-R expression was revealed by small brown granulation on the cell surface, for membrane

Fig. 3. Immunoprecipitation studies: modulation of the activation status of PDGF-R α chain in MG-63 (A) and of PDGF-R β chain in HOS (B), SJSA-1 (C) and U-2OS (D). Left: the illustration of Western Blotting analysis; right: the semi-quantitive analysis histograms of the mean intensity (INT) of the bands visualised on the x-ray film and analysed by Quantity One software (Biorad). In all cell lines the different experimental conditions (Lines 1-7), indicated in the legend on the right-hand, showed an activation status of the PDGF-R in basal condition (Line 6) that increases after adding exogenous PDGF according to exposure time (Lines 1 and 2) and decreases after stimulation with STI571 in a dose dependent manner (Lines 3, 4, 5). The condition without stimulation with exogenous PDGF, and only with STI571 10 μM (Line 7) did not show a decrease of the activation status compared to the basal condition.



receptor, or in the cytoplasm, for internalised forms (Fig. 2).

Immunoprecipitation studies

We tested the ability of PDGF to induce receptor tyrosine phosphorilation and the capacity of STI571 at concentrations of 1, 10 and 50 μM to inhibit the PDGF receptor kinase activity in the cell lines MG-63, HOS, SJSA-1 and U-2 OS. As shown in Figure 3, all the cell lines analysed showed a receptor constitutive phosphorilation in basal conditions (experimental condition 6) and this activation status increased after addition of exogenous PDGF according to exposure time (experimental conditions 1, 2). Inhibition of activation status of PDGF-R was dose-dependent, with the highest inhibition observed with 50 μM (experimental conditions 3, 4, 5). The activation status of PDGF-R was not down modulated after incubation with STI571 10 μM only. The modulation of the PDGF-R α and β chains on the cell lines analysed was similar in all the experimental conditions proposed. We found that PDGF activates its receptors and this activation is effectively inhibited by STI571 from 1 to 50 μM.

Detection of PDGF production by tumour cell lines

We studied the concentration of PDGF-AA, PDGF-AB and PDGF-BB in the culture media of HOS, U-2 OS, SJSA-1 and MG-63 cell lines without STI571, and in presence of rising concentrations (1, 10, 50 μM) of inhibitor after 24 and 48 hours.

We observed high levels of PDGF-AA isoform in the HOS cell line (range 974.5-1353.7 pg/ml at 24 hours and 1653.9-2043.1 pg/ml at 48 hours), in the U-2 OS cell line (range 2529.8-2861.4 pg/ml at 24 hours and 2727.5-3169.2 pg/ml at 48 hours) and in the SJSA-1 cell line (range 811.8-1278.6 pg/ml at 24 hours and 939.5-1638.9 pg/ml at 48 hours). No evidence of PDGF-AA was found in the MG-63 cell line. The PDGF-AB isoform was detectable in lower concentrations in the HOS cell line (range 345-687.5 pg/ml at 24 hours and 532.7-1616.1 pg/ml at 48 hours); for U-2 OS, PDGF levels were between 932.3 and 1630.4 pg/ml at 24 hours and between 1555.8 and 2037.7 pg/ml at 48 hours. PDGF-BB concentration was not significant in any of the cell lines analysed. The results are shown in Figure 4.

Evaluation of apoptosis

The effect of STI571 treatment on apoptosis was tested 24 and 48 hours after STI571 addition by the TUNEL assay method, using K562 as a drug-sensitive cell line. Only a small fraction of the cell lines analysed underwent apoptosis: two out of five cell lines showed apoptosis ranging from 23.45% in HOS to 24.07% in SJSA-1, as shown in Figure 5. After 48 hours, only one cell line (Saos-2) showed apoptosis (28.05%). These levels of apoptosis were observed at a STI571 concentration of 10 μM.

Discussion

The prognosis of patients with high grade osteosarcoma has greatly improved over the past 25 years, with overall survival rates rising from 15% to 70%. This improvement is attributed to: a) the effect of preoperative chemotherapy; b) the introduction of aggressive chemotherapy with different combinations of high dose methotrexate, doxorubicin, cisplatinum and ifos-

Fig. 4A. Concentration of PDGF-AA isoform in the supernatants of HOS, U-2 OS, SJSA-1 without (basal condition: ctrl) and with STI571 1, 10, 50 μM at 24 (a) and 48 hours (b).

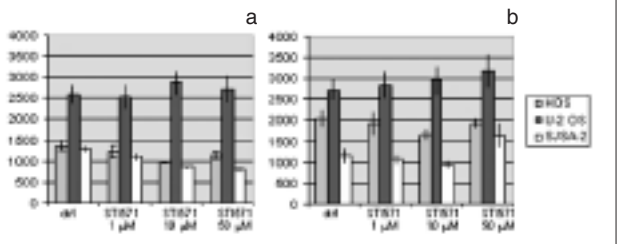
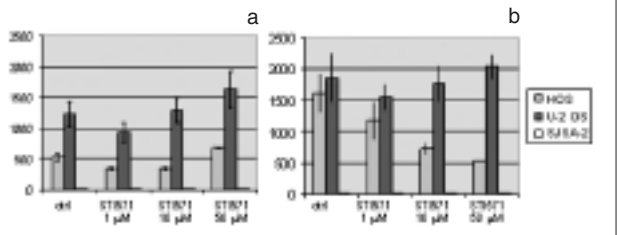
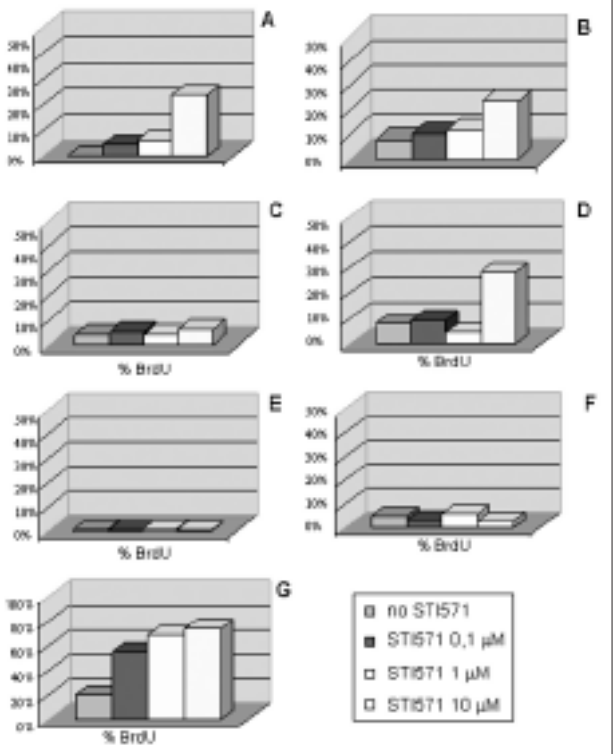


Fig. 4B. Concentration of PDGF-AB isoform in the supernatants of HOS, U-2 OS, SJSA-1 without (basal condition: ctrl) and with STI571 1, 10, 50 μM at 24 (a) and 48 hours (b).



famide; c) the identification of a dose-response relationship with methotrexate, doxorubicin, cisplatinum; d) the recognition of the histological response to preoperative chemotherapy as the main prognostic factor that might change the postoperative chemotherapy [22-24]. The prognosis of patients with osteosarcoma in metastatic relapse is very poor, with overall survival rates between 20%-25% after metastasectomy and aggressive second line chemotherapy [2]. These results in poor prognosis patients underline the need for additional therapies to maintain complete remission and to eliminate the minimal residual disease. The aim of our study was to evaluate the potential anticancer activity of STI571 in human osteosarcoma cells *in vitro*. Our results showed that all the cell lines analysed express, in different percentages, PDGF-R, while only a very low expression of c-kit was assessable on the Saos-2 cell line. Therefore, we primarily focused our attention on the role of PDGF and its receptor in anti-apoptotic pathways and on a potential effect of STI571 in this signalling. A constitutive phosphorilation of PDGF-R Western Blotting was revealed by western blotting analysis in basal conditions. Furthermore, ELISA studies suggest that this constitutive activation status might be sustained by the endogenous production of the PDGF ligand by osteosarcoma cells. This data demonstrated an autocrine/paracrine mechanism dependant on PDGF/PDGF-R pathway involved in the pathogenesis of osteosarcoma. The production of PDGF after stimulation with STI571 showed differing modulations in the cells lines, in terms of drug doses and stimulation times at 24 and 48 hours in the osteosarcoma cell lines

Fig. 5. Apoptosis study by TUNEL assay: in the histogram the apoptotic cells are identified as cells positive to the BrdU FITC antibody. The experimental conditions are given in the legend and were tested on the SJSA-1 (A) and HOS (B) cell line after 24 hours; on the Saos-2 cell line after 24 (C) and 48 (D) hours; on U-2 OS cell line after 24 (E) and 48 (F) hours. We used K562 as a drug-sensitive line (G).



analysed. For example, in the U-2 OS cell line, the PDGF-AB concentration after 24 and 48 hours of pharmacological treatment increases with higher drug concentrations (Fig. 4B: a-b); the same trend is slightly evident for PDGF-AA isoform after 48 hours (Fig. 4A: b). However, PDGF-AB production by the HOS cell line after 48 hours of Imatinib treatment showed a decreasing trend, probably due to the different biological and proliferative responses of cell lines to the drug. (Fig. 4B: b). Nevertheless, immunoprecipitation studies showed a slight dose-dependent inhibition of PDGF-R after the addition of increasing concentrations of STI571, suggesting a short-term interference of this drug in intracellular signalling processes. We then tried to verify whether this signal interference by STI571 determines programmed cell death or apoptosis. Using the TUNEL assay method, we measured the number of apoptotic cells 24 and 48 hours from the addition of scalar concentrations of STI571. We observed the highest levels of apoptosis in SJSA-1, HOS (24.07% and 23.45% after 24 hours respectively) and in the Saos-2 cell line (28.05% after 48 hours). Preliminary data seem to suggest that the inhibition of the proliferative message arising from tyrosine-kinase receptors such as PDGF-R might be considered as a molecular target ap-

proach in osteosarcoma therapy. Nevertheless, apoptosis data suggest a possible involvement of numerous signalling pathways insensitive to STI571 inhibition. A more complete approach might be the association of different tyrosine-kinase inhibitors that would interfere at multiple levels with the neoplastic signal.

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