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SHORT COMMUNICATION

**Marc Hotfilder · Claudia Lanvers · Heribert Jürgens
Joachim Boos · Josef Vormoor**

c-KIT-expressing Ewing tumour cells are insensitive to imatinib mesylate (STI571)

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Abstract *Purpose*: In order to determine whether Ewing tumour patients may be potential candidates for imatinib mesylate therapy, we analysed the expression of the currently known imatinib mesylate-sensitive tyrosine kinases and tested sensitivity to imatinib mesylate in a panel of eight Ewing tumour cell lines in vitro. *Methods*: Expression of the different tyrosine kinases was assessed by flow cytometry and RT-PCR. Sensitivity to imatinib mesylate was analysed using a standard MTT proliferation assay. *Results*: Flow cytometric and RT-PCR analyses in a panel of eight Ewing tumour cell lines demonstrated expression of several imatinib mesylate-sensitive tyrosine kinases, including c-KIT, platelet-derived growth factor receptor, c-ABL and c-ARG. However, in the MTT proliferation assay, all eight Ewing tumour cell lines were found to be resistant to imatinib mesylate at concentrations ranging from 0.1 to 10 μM. *Conclusions*: Despite the expression of imatinib mesylate-sensitive tyrosine kinases, Ewing tumour cells proved resistant to imatinib mesylate in vitro. This observation has implications for the selection of patients for experimental therapy with imatinib mesylate.

Keywords Ewing tumour · Sarcoma · c-kit · Imatinib mesylate (STI571) · Signal transduction inhibitor

Introduction

Deregulation of protein-tyrosine kinase signalling is one of the principal mechanisms involved in abnormal cell growth and survival of malignant cells. Protein-tyrosine kinase inhibitors that disrupt these autonomous signalling loops are currently being developed for clinical use. One of these is the signal transduction inhibitor imatinib mesylate (STI571, Glivec; Novartis, Basel, Switzerland). Imatinib mesylate was initially designed as an inhibitor of platelet-derived growth factor receptor (PDGF-R). However, it has also been shown to inhibit other tyrosine kinases including the receptor for stem cell factor (SCF) c-KIT, the non-receptor-type tyrosine kinases c-ABL and c-ARG and related fusion oncogenes, such as BCR/ABL. Imatinib mesylate has been used successfully in patients with constitutive activation of one of these tyrosine kinases, e.g. in BCR/ABL-positive chronic myelogenous leukaemia (CML) [7] and in gastrointestinal stromal tumours that harbour activating mutations of c-KIT [5].

Ewing tumours are the second most common primary bone tumour in childhood and adolescence with an incidence of approximately 0.6 per million population. With combined intensive chemotherapy, surgery and radiotherapy approximately 50% of patients with localized disease can be cured. However, patients with primary metastatic disease or relapse have a dismal prognosis [8]. New treatment options are therefore needed for these patients. In order to determine whether Ewing tumour patients may be potential candidates for imatinib mesylate therapy, we analysed the expression of the currently known imatinib mesylate-sensitive tyrosine kinases and tested sensitivity to imatinib mesylate in a panel of Ewing tumour cell lines in vitro.

Materials and methods

Cell lines

The panel of Ewing tumour cell lines comprised RM-82, WE-68, VH-64, STA-ET-1 and STA-ET-2.1 (kindly provided by F. van Valen, Department of Orthopedics, Münster, Germany), TC-71 (kindly provided by T.J. Triche, Children's Hospital, Los Angeles, Calif.), and CADO-ES1 and RD-ES (purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

M.H. and C.L. contributed equally to this work.

M. Hotfilder · C. Lanvers · H. Jürgens · J. Boos · J. Vormoor (✉)
University Children's Hospital Muenster,
Department of Pediatric Hematology and Oncology,
University of Muenster, Albert-Schweitzer-Str. 33,
48129 Muenster, Germany
E-mail: vormoor@uni-muenster.de
Tel.: +49-251-8347742
Fax: +49-251-8347828

Flow cytometry

Flow cytometric analysis of CD117 expression (antibody: clone 95C3; Beckman Coulter, Krefeld, Germany) of single cell suspensions of the different Ewing tumour cell lines was performed according to our standard protocol [9].

RT-PCR assay

Total RNA (2 μg, isolated with TriReagent; Biozol, Eching, Germany) was reverse transcribed utilizing a Superscript II Reverse Transcriptase Kit (Life Technologies, Karlsruhe, Germany). The resulting cDNA was diluted (1:5–1:20) and subjected to PCRs for GAPDH (control), c-ARG (sense: GCACAAGATGCCACAGAAAA, antisense: GGTTCCTCTGTGCAAAGCTC; 3 min at 94°C followed by 35 cycles of 60 s at 94°C, 60 s at 60°C, 60 s at 72°C), c-ABL (see reference 3), and PDGF-Rα and β (see reference 1). The final reaction mix contained 2.5 U Taq polymerase (Life Technologies), 0.2 μ*M* of each primer, 10 m*M* Tris/HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1 mg/ml bovine serum albumin, 0.05% Triton X-100 and 200 μ*M* of each dNTP. The amplification products were separated by electrophoresis on a 0.8% agarose gel.

MTT assay

The responses to increasing concentrations of imatinib mesylate (Glivec kindly provided by Novartis) were evaluated in a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) proliferation assay [4].

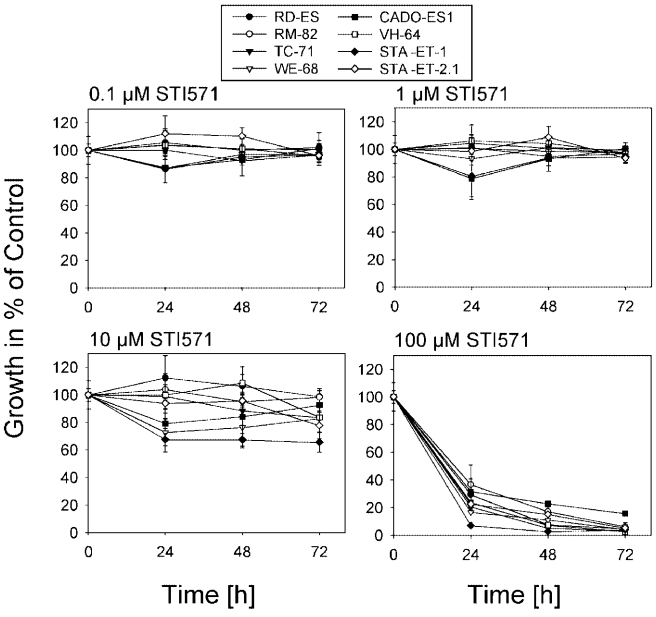

Fig. 1. Inhibition of Ewing tumour proliferation by imatinib mesylate (STI571). Only at a concentration of 100 μ*M* imatinib mesylate was a significant irreversible inhibition of all eight tested Ewing tumour cell lines detected, while lower concentrations had no or only marginal effects on cell proliferation in MTT tests

Results

In order to determine whether Ewing tumour patients may be potential candidates for imatinib mesylate therapy, the expression of the currently known imatinib mesylate-sensitive tyrosine kinases c-KIT, PDGF-Rα and β, c-ABL and c-ARG was analysed in a panel of eight different Ewing tumour cell lines. All eight Ewing tumour cell lines were positive for CD117 (c-KIT) expression by flow cytometry (Table 1). In addition, by RT-PCR analysis, two out of six cell lines expressed PDGF-Rα, five out of six cell lines expressed PDGF-Rβ and all six cell lines had detectable mRNA for c-ABL and c-ARG (Table 1).

The responsiveness to imatinib mesylate was investigated in the same panel of cell lines. Neither standard MTT proliferation assays (Fig. 1) nor clonogenic assays (data not shown) showed cytotoxicity of imatinib mesylate in any of the eight Ewing tumour cell lines at concentrations ranging from 0.1 to 10 μ*M*. At 10 μ*M* a minor transitory growth retardation occurred during the first 24 h in three of the cell lines (Fig. 1). Only at very high concentrations (50–100 μ*M*) was it possible to detect an irreversible cytotoxic effect of imatinib mesylate in all Ewing tumour cell lines. Thus, despite expression of c-KIT and other imatinib mesylate-sensitive tyrosine kinases, Ewing tumour cells proved to be resistant to imatinib mesylate in vitro.

Discussion

The homogeneous expression of c-KIT (and to a lesser extent other imatinib mesylate-sensitive tyrosine kinases, see Table 1) in all Ewing tumour cell lines suggested that Ewing tumours may be good targets for treatment with imatinib mesylate, particularly as c-KIT-positive gastrointestinal stromal tumours [5] and small-cell lung

Table 1. Expression of imatinib mesylate-sensitive tyrosine kinases in a panel of Ewing tumour cell lines (+ detectable expression, – no expression, *nd* analysis not performed)

Cell line	Flow cytometric analysis for c-KIT	RT-PCR analysis for			
		PGDF-Rα	PDGF-Rβ	c-ABL	c-ARG
CADO-ES1	+	+	+	+	+
RD-ES	+	+	+	+	+
RM-82	+	–	+	+	+
TC-71	+	–	+	+	+
VH-64	+	–	+	+	+
WE-68	+	–	–	+	+
STA-ET1	+	nd	nd	nd	nd
STA-ET-2.1	+	nd	nd	nd	nd

cancer cell lines [10] have been shown to be responsive to imatinib mesylate. A central role of c-KIT and its ligand SCF in survival and metastasis of Ewing tumours has been indicated [6]. SCF was shown to induce downregulation of c-KIT on the cell surface in all six cell lines tested in that study indicating functionality of c-KIT. SCF was also shown to be a chemoattractant in three of the six cell lines. However, only in one cell line was SCF found to stimulate proliferation and reduce apoptosis [6]. Thus, the exact role of SCF and its receptor in the biology of Ewing tumours needs further investigation.

In contrast to the expression pattern of imatinib mesylate-sensitive tyrosine kinases, no cytotoxicity was observed in any of the eight Ewing tumour cell lines at concentrations of imatinib mesylate ranging from 0.1 to 10 μM. This corresponds to plasma concentrations that show an inhibitory activity in CML in vitro [7] and that are achieved after once-daily dosing of 600 mg imatinib mesylate orally [7].

In conclusion, imatinib mesylate may only be effective in tumours that have constitutively activated imatinib mesylate-sensitive tyrosine kinases, e.g. due to receptor mutations, fusion oncogenes or otherwise upregulated autocrine loops. The resulting abnormal signalling cascades can be disrupted by imatinib mesylate. However, as shown in our experiments, expression of imatinib mesylate-sensitive tyrosine kinases, including c-KIT, PDGF-R, c-ABL and c-ARG, alone is not sufficient to render malignant cells sensitive to imatinib mesylate – at least not to clinically achievable imatinib mesylate concentrations.

There has been some concern that imatinib mesylate may have tumour-promoting effects in certain malignancies as it enhances baseline and hepatocyte growth factor-induced migration of thyroid carcinoma cells [2]. However, no tumour-promoting activity of imatinib mesylate in Ewing tumours was detected. The effects of imatinib mesylate on Ewing tumour migration are currently under investigation.

Imatinib mesylate is the first example of a novel class of signal transduction inhibitors that provide a new insight into the biology of clinical cancers. It is an important therapeutic tool providing its use is based on valid preclinical and/or clinical evidence.

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