In vitro antiproliferative and antimigratory activity of dasatinib in neuroblastoma and Ewing sarcoma cell lines

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Abstract. Neuroblastoma (NB) and Ewing sarcoma (ES) are neuroectodermal tumors typical of pediatric age that, despite aggressive treatment, still present a poor prognosis when in advanced stages. Studies indicate that c-KIT and platelet-derived growth factor receptor (PDGFR) play a substantial role in the proliferation and survival of NB and ES cells. Dasatinib, an oral multi-targeted inhibitor of several kinases including BCR-ABL and SRC-family kinases, is also active against c-KIT and PDGFR. Here, we evaluated the effect of dasatinib on the NB cell lines SJ-N-KP, SK-N-BE, AF8 and IMR5, and on the ES lines PDE02, TC106 and 6647. Proliferation and viability assays showed that dasatinib exerts an antiproliferative activity with a peak effect occurring at 24 h. After a 24-h exposure to dasatinib at 100 nM, proliferation was inhibited by 29.4±5.7% in SJ-N-KP, 41.3±11.7% in IMR5, 35.3±7.6% in PDE02 and 14±10.6% in 6647. Dasatinib did not induce apoptosis in NB and ES cell lines. A possible antimigratory activity of dasatinib was evaluated by scratch test. Dasatinib at 100 nM inhibited the migration of NB and ES cell lines by a mean of 30.2 and 25.3%, respectively. This activity suggests a possible role of dasatinib in inhibiting metastasis and appears of particular interest, given the association between metastatic disease and poor prognosis in these tumors. In conclusion, the cytostatic and antimigratory activity of dasatinib in NB and ES cell lines and the lack of pro-apoptotic activity suggests a possible use for this compound in the treatment of these tumors as a combination with other cytotoxic therapy.

Introduction

Neuroblastoma (NB) and Ewing sarcoma (ES) are neuroectodermal tumors that most commonly affect pediatric age (1). Despite advances in treatment, the prognosis of these patients remains poor. Twenty to 40% of patients with localized ES eventually die from the disease (2) and the overall survival rates for patients with NB were reported at 68.5% (3). In both neuroblastoma and Ewing sarcoma, metastatic disease is predictive of a particularly poor treatment outcome. The probability of long-term survival for patients with either metastatic neuroblastoma or Ewing sarcoma is ~25%, highlighting the need for new therapeutic approaches (3,4).

Numerous studies have suggested that the tyrosine kinase platelet-derived growth factor receptor (PDGFR) and c-KIT are involved in the pathogenesis of Ewing sarcoma and neuroblastoma. In neuroblastoma cells, an autocrine stem cell factor (SCF)/c-KIT loop was shown to protect against apoptosis (5). Autocrine stimulation of KIT receptor has also been reported in Ewing sarcoma (6-8), implicated in tumor growth and metastasis (7) and suggested to prevent the initiation of apoptosis (8). Moreover, the simultaneous expression of c-KIT and SCF in neuroblastoma tumors was shown to be associated with a more aggressive phenotype (9,10).

PDGFR is also expressed in Ewing sarcoma and neuroblastoma tumor cell lines (11,12) and has been implicated in disease pathogenesis (12,13). Zwerner and May showed that a platelet-derived growth factor (PDGF)-C/PDGFR autocrine loop may be involved in the neoplastic transformation of Ewing sarcoma cells (13). Furthermore, the inhibition of PDGFR signaling with a PDGFR-specific kinase inhibitor blocked growth and chemotaxis of TC-32 cells *in vitro* (12). Similarly, neuroblastoma cells were found to express PDGFR-α and -ß (11) and the inhibition of ligand stimulated-phosphorylation of both PDGFR and c-KIT correlated with the growth inhibition of neuroblastoma cells *in vitro*.

The involvement of PDGFR and c-KIT in Ewing sarcoma and neuroblastoma raises the possibility that these signaling molecules could be exploited as potential therapeutic targets.

Dasatinib (BMS-354825, SPRYCEL, Bristol-Myers Squibb, NY, USA), is an oral multi-targeted inhibitor of several kinases including BCR-ABL and SRC-family kinases (14). Additionally, dasatinib inhibits c-KIT and PDGFR, and has demonstrated activity against PDGFR and c-KIT-mediated cellular events (14). Chen *et al* showed that dasatinib

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inhibited PDGF-stimulated migration and proliferation of vascular smooth muscle cells at concentrations in the low nanomolar range (15). In another study, dasatinib was found to potently inhibit both wild-type and mutant forms of KIT (including imatinib-resistant forms) in mast and leukemic cell lines (16).

On the basis of this activity, coupled with the known role of PDGFR and c-KIT in the biology of neuroblastoma and Ewing sarcoma, we investigated the effects of dasatinib on the proliferation, viability and migration of neuroblastoma and Ewing sarcoma cell lines.

Materials and methods

Dasatinib preparation. All compounds were synthesized by the medicinal chemistry group at Bristol-Myers Squibb Pharmaceutical Research Institute. Compounds were solubilized in 100% DMSO at 10 mM as stock solution for all experiments.

Cell cultures. Four neuroblastoma cell lines (SJ-N-KP, SK-N-BE, AF8 and IMR5) and three Ewing sarcoma cell lines (PDE02, TC106 and 6647) were used in this study (5,8,17). Cell lines were maintained in monolayer cultures in RPMI-1640 medium + 10% fetal calf serum (FCS).

Cell proliferation assays. To assess proliferation, cells were cultured in the presence or absence of dasatinib at 10, 50, 100, 250, 500, 1,000, 2,000, 5,000 and 10,000 nM for 24, 48, or 72 h. Following exposure to dasatinib or DMSO (control), cells were harvested with trypsin and stained with trypan blue. Cellular proliferation was determined by quantifying viable cells manually using a hemocytometer. Viability was based on trypan blue exclusion. Inhibiting concentrations of 50% (IC_{50}) were calculated by the SPSS 11.5 program. Experiments were performed in triplicate.

Apoptosis assays. Following 24- and 48-h exposure to dasatinib or DMSO (control), cells were harvested with trypsin. Apoptosis was evaluated by flow cytometry, using annexin V-FITC conjugates (Apoptosis Detection Kit, R&D Systems), according to the manufacturer's instructions, with an EPICS XL2 flow cytometer. Experiments were performed in triplicate.

Cell cycle analysis. Cell cycle was evaluated using the Coulter Prep Reagents kit (Beckman Coulter, CA, USA). Cells were processed according to the protocol following a 24- or 48-h exposure to dasatinib or DMSO (control) and analyzed by flow cytometry. Experiments were performed in triplicate.

Cell migration assays. Cell migration was evaluated using the scratch test as previously described (18). In brief, cells were grown to confluence on tissue culture dishes and a single scrape was made in the confluent monolayer using a sterile pipette tip. After washing the cells in PBS, a complete medium containing dasatinib (100 nM) or DMSO (control) was added. Cells were then incubated for an additional 48 h. The number of cells that migrated beyond the borders of the scrape were then quantified (18).

Results

Proliferation assays performed on neuroblastoma (SJ-N-KP and IMR5) and Ewing sarcoma (PDE02 and 6647) cell lines following a 24-, 48- or 72-h exposure to dasatinib, demonstrated a concentration-dependent antiproliferative effect of dasatinib in both NB and ES cell lines with a peak effect occurring at 24 h (Fig. 1). IC_{50} values calculated from the cell proliferation assays showed that the comparative sensitivity to dasatinib was 6647 < SJ-N-KP < IMR5 < PDE02. IC_{50} at 24 and 48 h were respectively 6,112 and 4,970 nM for SJ-N-KP, 1,379 and 31,513 nM for IMR5, 433 and 661 nM for PDE02, 19,897 and 21,142 nM for 6647 cell lines.

To evaluate a possible pro-apoptotic effect of dasatinib on neuroblastoma and Ewing sarcoma lines annexin-V/PI staining was quantified in SJ-N-KP and PDE02 cells treated with various concentrations of dasatinib (50, 100, 250, 500, 1,000 and 2,000 nM). The 6647 and IMR5 lines were evaluated for apoptosis only after exposure to dasatinib at 100 nM. Dasatinib did not induce apoptosis in neuroblastoma and Ewing sarcoma cells (Table I).

Cell cycle effects of dasatinib were analyzed in neuroblastoma cells (SJ-N-KP and IMR5) and in Ewing sarcoma cells (6647 and PDE02) after 24-, 48-, 72-h exposure to dasatinib at 100 nM. Exposure to dasatinib for 48 h induced a mean S-phase reduction of 28.1% and a 34.8% increase in G0/G1 in 6647 and IMR5 cells (Table II, Fig. 2). No significant effect on cell cycle was observed in SJ-N-KP and PDE02 cells (data not shown).

The scratch test demonstrated an inhibitory effect of dasatinib on cell migration in all of the NB and ES lines, ranging from a mean of 12 to 42% (Table III, Figs. 3 and 4).

Discussion

The expression of c-KIT and PDGFR in Ewing sarcoma and neuroblastoma tumor cells suggested that inhibiting these signaling molecules may be an effective treatment strategy. Evidence of a c-KIT/SCF autocrine loop was previously demonstrated in each of the cell lines used in this study (5,8).

The known activity of dasatinib against c-KIT and PDGFR provided the basis for testing effects of this agent. Our results show that dasatinib induces substantial anti-proliferative and antimigratory effects on both Ewing sarcoma and neuroblastoma cells *in vitro*.

A dose-dependent antiproliferative effect was observed in all of the cell lines tested and occurred rapidly with the peak effect at 24 h. It should be noted that the IC_{50} values calculated for the Ewing sarcoma and neuroblastoma cell lines used in this study were substantially higher than those reported for other cell types (14). However, after a 24-h exposure to the relatively low concentration of 100 nM, proliferation was inhibited by 29.4±5.7% in SJ-N-KP, 41.3±11.7% in IMR5, 35.3±7.6% in PDE02 and 14±10.6% in 6647 cell lines. Dasatinib at 100 nM also induced a reduction of the S phase and an increase in the G0/G1 phase of the cell cycle in 6647 (Ewing sarcoma) and IMR5 (neuroblastoma) lines.

The effects of dasatinib on Ewing sarcoma and neuroblastoma cells appears to be cytostatic, whereas results

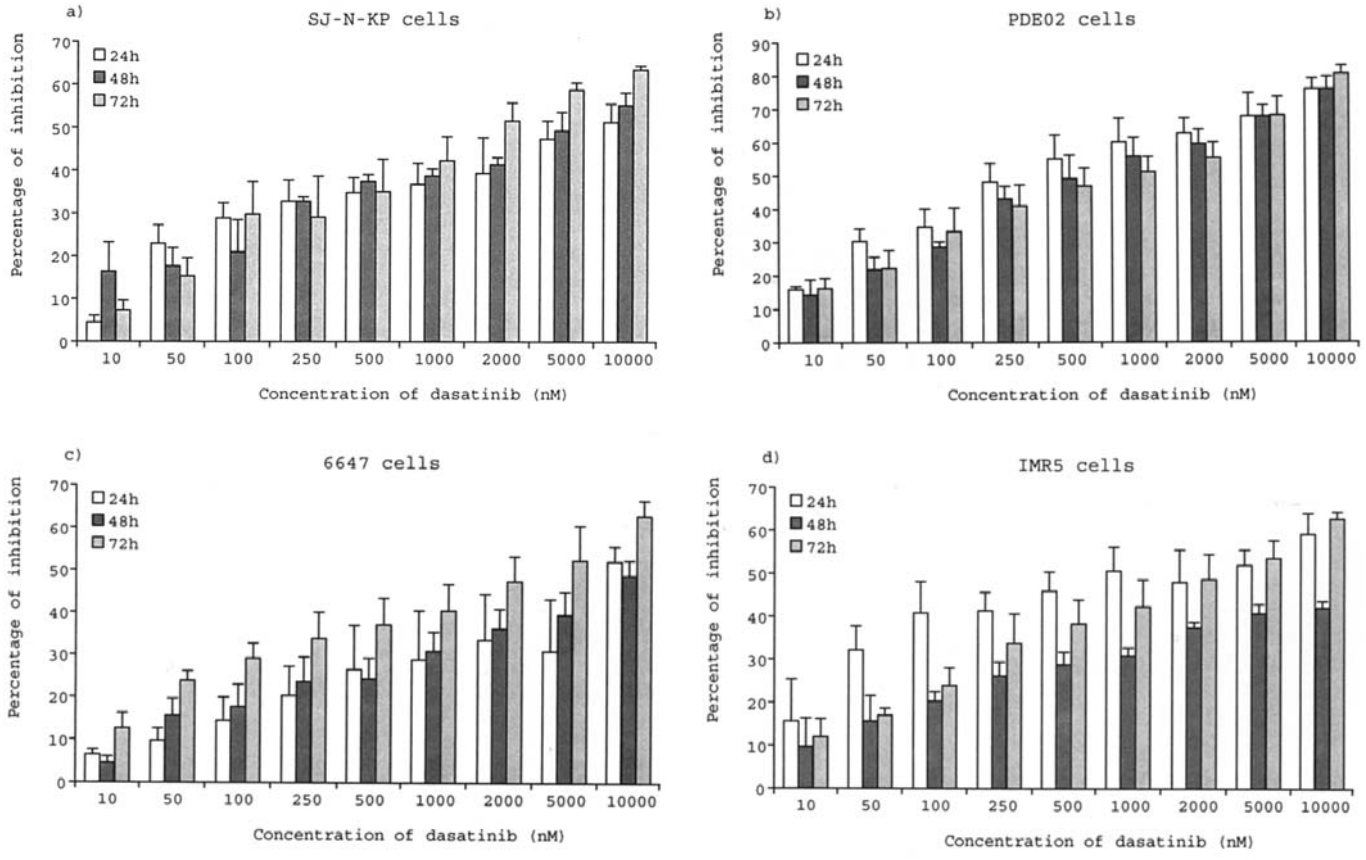

Figure 1. Dasatinib inhibits proliferation of neuroblastoma (SJ-N-KP and SK-N-BE) and Ewing sarcoma (PDE02 and 6647) cells. Percentage of proliferation inhibition at various concentrations of dasatinib after 24, 48 or 72 h. The values represent the mean of triplicate samples.

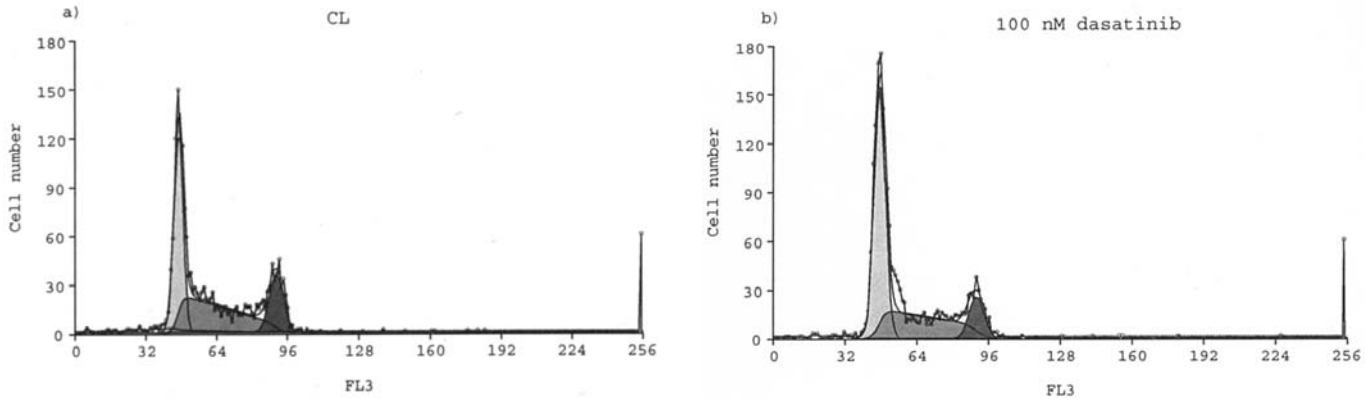

Figure 2. A representative flow cytometric evaluation of cell cycle in the IMR5 cell line. An increase in G0/G1 phase and a decrease in the S phase after a 48 h-exposure to dasatinib at 100 nM. CL, control.

from apoptosis assays showed that dasatinib did not induce cell death in any of the cell lines tested.

Dasatinib treatment inhibited the migration of neuroblastoma and Ewing sarcoma cell lines by a mean of 30.2 and 25.3%, respectively. This effect occurred at 100 nM, a concentration well below the calculated IC_{50} for proliferation. The dasatinib-mediated inhibition of cell migration likely occurs through its effects on c-KIT and PDGFR. SCF plays a central role in the migration of neural crest-derived cells (19). SCF has been shown to induce migration of Ewing sarcoma cells (7). Scotlandi *et al* reported that imatinib blocked SCF-mediated migration of Ewing sarcoma cells expressing high levels of c-KIT (20). Similarly, Uren *et al* showed that the Ewing sarcoma cell line TC-32 migrated towards PDGF-BB and that this migration was inhibited when PDGFR-ß activation was blocked with AG1295 (12). Other reports have demonstrated that the antimetastatic effects of dasatinib in a variety of tumor models were linked to its potent activity against SRC (18,21-23). Furthermore, SRC has been implicated in PDGF-dependent chemotaxis (24). The involvement of SRC in PDGF-mediated and other forms of cellular migration suggests the possibility that, in addition to

Table I. Dasatinib does not induce apoptosis in the SJ-N-KP or PDE02 cell lines.

A, Neuroblastoma (SJ-N-KP)

SJ-N-KP	Apoptosis (%)			
	Annexin V$^+$	PI$^+$/Annexin V$^+$	PI$^+$	Alive
CL (24 h)	2.03	3.68	2.03	92.25
50 nM	1.45	2.55	1.65	94.40
100 nM	0.78	2.30	1.53	95.45
250 nM	1.25	2.93	1.18	94.65
500 nM	1.75	3.05	0.95	94.25
1,000 nM	2.18	3.25	0.80	93.75
2,000 nM	2.10	3.05	1.00	93.80
CL (48 h)	2.10	3.05	3.45	91.4
50 nM	1.75	2.15	5.75	90.4
100 nM	4.25	3.30	6.55	85.9
250 nM	0.20	3.45	7.20	89.1
500 nM	2.35	3.95	5.05	88.6
1,000 nM	2.85	3.20	5.50	88.4
2,000 nM	0.35	0.95	5.25	93.4

B, Ewing sarcoma (PDE02)

PDE02	(%)			
	Annexin V$^+$	PI$^+$/Annexin V$^+$	PI$^+$	Alive
CL (24 h)	5.20	2.15	1.95	90.75
50 nM	3.63	2.18	1.45	92.70
100 nM	2.48	1.70	1.68	94.10
250 nM	3.35	1.35	1.50	93.75
500 nM	3.98	2.78	2.13	91.20
1,000 nM	3.55	1.85	1.38	93.30
2,000 nM	1.93	1.13	3.30	93.65
CL (48 h)	0.95	3.45	5.10	90.5
50 nM	0.75	1.65	2.20	95.4
100 nM	0.35	0.85	4.60	94.2
250 nM	0.25	0.45	3.00	96.3
500 nM	0.65	0.90	2.10	96.4
1,000 nM	1.80	2.95	2.20	93.0
2,000 nM	0.90	3.90	2.80	92.4

its direct effects on c-KIT and PDGFR, the anti-SRC activity of dasatinib may play a role in inhibiting neuroblastoma and Ewing sarcoma cell migration.

The inhibitory effect of dasatinib on neuroblastoma and Ewing sarcoma cell migration is of particular interest. While further studies are necessary to fully characterize the anti-metastatic activity of dasatinib, the results presented here suggest that dasatinib may prevent metastasis of certain neuroblastoma and Ewing sarcoma tumors *in vivo*. Given the association between metastatic disease and poor prognosis in these tumors (3,4,25), the inhibition of metastasis could have a significant impact on the overall treatment outcome of patients with these diseases.

Another c-KIT/ PDGFR inhibitor, imatinib, has also shown activity against neuroblastoma and Ewing sarcoma cells. In preclinical studies, imatinib inhibited the growth of both Ewing sarcoma and neuroblastoma cells (6,9,20,26-28). Several of these reports have linked the activity of imatinib in

Table II. Cell cycle modifications in IMR5 and 6647 cell lines after exposure to dasatinib at 100 nM for 24, 48 and 72 h.

	G0/G1 CL	G0/G1 dasatinib	S CL	S dasatinib
IMR5 24 h	49±3.2%	39.7±3.7	30.8±3.8	39±3.3
IMR5 48 h	41.1±4.6	52.4±5.5	39.2±2.2	29.6±2.1
IMR5 72 h	53.2±2.5	68.2±1.6	28.9±2.7	20.9±1.5
6647 24 h	42.1±17	38.5±3.6	38.7±11.9	44.8±3.4
6647 48 h	41.8±0.7	59.4±3.8	40.9±1.5	27.9±4.0
6647 72 h	57.0±4.9	73.2±1.6	26.2±2.5	17.9±1.9

CL, control.

Table III. Percentage of inhibition of migration in neuroblastoma and Ewing sarcoma cell lines evaluated by scratch test after exposure to dasatinib at 100 nM for 48 h.

	48 h
NB	
SJ-N-KP	33±9.6%
IMR5	20±2.1%
SK-N-BE	30±1.4%
AF8	38±11.3%
ES	
PDE02	42±7.8%
TC106	22±8.5%
6647	12±2.1%

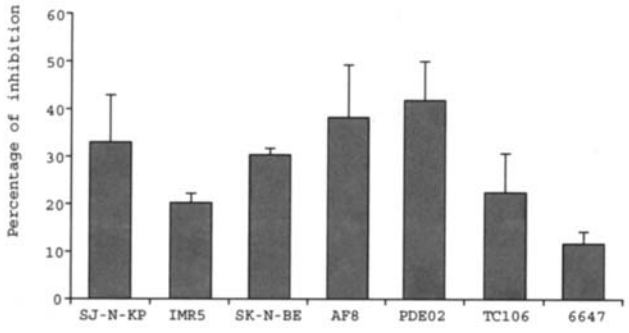

Figure 3. A scratch test in neuroblastoma and Ewing sarcoma lines after exposure to dasatinib at 100 nM for 48 h. Bars show the percentage of inhibition of cell migration through the scrape.

Ewing sarcoma cells to c-KIT inhibition (6,20,26,27). However, other studies showed that imatinib inhibited the growth of cells not expressing c-KIT (9) and that SCF stimulation persisted during imatinib treatment (28), suggesting that the activity of this agent is at least partially independent of its effects on c-KIT. Beppu *et al* showed that the activity of imatinib in neuroblastoma cells coincided with its inhibition of ligand-stimulated activity of both c-KIT and PDGFR (26). Furthermore, treatment with another PDGFR-specific inhibitor (AG1295) delayed the formation of Ewing sarcoma family tumors in animal models (12). These latter reports suggest that both c-KIT and PDGF are potential targets for the treatment of neuroectodermal-derived tumor cells.

While imatinib has shown preclinical efficacy against both Ewing sarcoma and neuroblastoma cells growing *in vitro* and

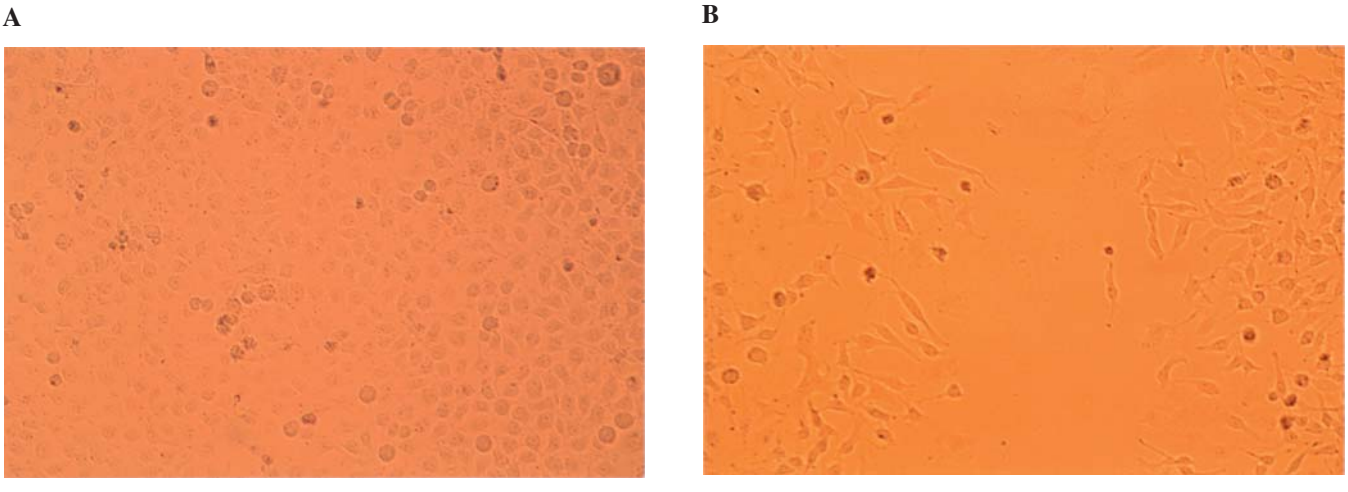

Figure 4. A Scratch test in the SJ-N-KP line. (A) In the control, the scrape is entirely filled by migrating cells. (B) Substantial inhibition of cell migration through the scrape.

in vivo (6,9,20,26-28), clinical results in this indication have been disappointing. In a phase II study in pediatric patients with relapsed or refractory solid tumors, single agent imatinib had only minimal efficacy, with a partial response in only 1/24 patients with Ewing sarcoma and no responses among 10 patients with neuroblastoma (29).

The greater activity of dasatinib against PDGFR and c-KIT compared with that of imatinib may allow for improved efficacy in neuroblastoma and Ewing sarcoma tumors expressing these targets (14-16). Moreover, the potent effects of dasatinib on SRC may improve the treatment response of dasatinib in Ewing sarcoma cells.

Recently reported results from an ongoing clinical trial showed that dasatinib had a favorable safety profile in pediatric patients with relapsed or refractory leukaemia (30), suggesting that this agent could be used safely for treating young children and adolescents with neuroblastoma or Ewing sarcoma.

In conclusion, the antiproliferative and antimigratory activity of dasatinib on neuroblastoma and Ewing sarcoma cell lines may be clinically beneficial in the treatment of these tumors. However, the lack of apoptotic response suggests that dasatinib will likely yield the greatest clinical effect when combined with another cytotoxic therapy. Animal studies are warranted.

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