LABORATORY STUDY

The effect of imatinib mesylate on the proliferation, invasive ability, and radiosensitivity of retinoblastoma cell lines

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Abstract

Purpose Our aim was to evaluate the potential effect of imatinib mesylate (IM), a small molecule that specifically inhibits the tyrosine quinase receptors, on the proliferation and invasive abilities of two human retinoblastoma (Rb) cell lines. Furthermore, the ability of IM to radiosensitize Rb cells was evaluated. The potential targets of IM (C-kit, PDGRF-α and -β, and c-Abl) were also investigated in these cell lines.

Methods Two human Rb cell lines (WERI-RB-1 and Y79) were cultured under normal growth conditions. An MTT-based proliferation assay and a Matrigel invasion assay were performed with and without exposure to 10 μM of IM. The cells were also irradiated with graded dosages of 0, 2, 4, 6, 8, and 10 Gy with and without IM and their proliferations rates were analyzed. Western blot and immunocytochemical analysis of cytospins were performed to evaluate the expression of C-kit, PDGRF-α and -β, and c-Abl.

Results When IM was added to both cell lines a statistically significant ($P < 0.05$) reduction in proliferation and invasive ability were observed. Exposure to IM also significantly increased the radiosensitivity of both Rb cell lines. The c-Abl expression was strongly positive, PDGRF-α and -β expression were also positive but the C-kit expression was negative in both cell lines.

Conclusions These results indicate that Gleevec may be useful as an adjuvant treatment in Rb patients, specially those considered for radiation therapy.

Introduction

Retinoblastoma (Rb) is the most common primary malignant intra-ocular tumor in children and overall, the most common retinal tumor. It represents approximately 4% of all pediatric malignancies.[1] Early diagnosis can greatly improve both treatment efficacy and survival rates, although some patients still succumb because of metastatic disease. The current therapies for Rb include intravenous chemoreduction, thermotherapy, cryotherapy, laser photocoagulation, plaque radiotherapy, external beam radiotherapy, enucleation, orbital exenteration, and systemic chemotherapy for metastatic disease.[1]

Rb is generally a radiosensitive tumor. In the treatment of Rb, radiation is delivered in the form of brachytherapy (plaque radiotherapy) or using an external beam (EBR) delivering irradiation to the whole eye; the latter being used only as a last choice of treatment before enucleation in more advanced tumors. Earlier stages of the disease expectedly respond better, with success rates as high as 88%. When irradiation is used as salvage therapy in stages IV and V, half of the treated eyes still retain useful vision.[2] Unfortunately, the use of external beam radiotherapy increases the incidence of orbital hypoplasia, cataract, and second cancers

in the field of irradiation, specially in patients younger than 12 months of age.[3] In the past 10 years, there has been an effort by many centers to avoid EBR by the use of chemotherapy to decrease the tumor dimensions (chemoreduction). Focal treatment methods are then used to consolidate the tumor control.[4]

Despite systemic chemotherapy with a variety of chemotherapeutic agents, many tumors persist unaffected.[5,6] The presence of multiple side effects along with the multidrug resistance often observed has fostered the development of a new class of citotoxic agents. These agents target individual genes that have altered expression in tumors cells.[7]

Imatinib mesylate (IM, Gleevec, Novartis Pharma AG Basel, Switzerland), formerly referred to as STI571, is a therapeutic compound that specifically inhibits the tyrosine kinase receptors: c-Abl, C-kit, and platelet-derived growth factor (PDGF-α and -β) receptor. It is known that protein tyrosine kinases (PTKs) have an important role in cellular mechanisms, such as differentiation, proliferation, and regulatory mechanisms, as well as in signal transduction.[8,9] The expression of these targets and the use of IM as a new therapeutic option have been studied in a wide variety of human malignancies.[9–12] Our group has already demonstrated that the majority of Rb tumors express one of the targets of IM: C-kit.[13] The use of this drug is already approved by the FDA for the treatment of chronic myelogenous leukemia (CML) and gastrointestinal tumors (GIST).[8]

The purpose of this study was to examine the potential effect of Gleevec, on the proliferation and invasive abilities of two human Rb cell lines, as well as the ability to increase their radiosensitivity. To assess the expression of the potential targets of Gleevec, we performed western blot and immunocytochemistry on cytospin of both Rb cell lines.

Materials and methods

Cell culture

The Rb cell lines were purchased from ATCC (Manassas, VA, USA). Two non-adherent human Rb cell lines Y79 (ATCC catalog number HTB-18) and WERI-RB-1 (ATCC catalog number HTB-169) were incubated at 37 °C in a humidified 5% CO_2-enriched atmosphere. The cells were cultured in RPMI-1640 medium (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum, 1% fungizone, and 1% penicillin–streptomycin purchased from Invitrogen. Cells were cultured in 25 cm² flasks (Fisher, Whitby, Ontario, Canada) and observed twice weekly at every media change for normal growth by phase contrast microscopy.

Cells were centrifuged at 120 g for 10 min to form a pellet, and were then re-suspended in 1 ml of medium and counted using the Trypan blue dye exclusion test.

In vitro proliferation assay

The MTT-based assay kit (TOX-1, Sigma-Aldrich, St Louis, MO, USA) was performed in accordance with the manufacturer's recommendations. Briefly, the two human Rb cell lines were seeded into wells at a concentration of 10 000 cells per well, six wells per cell line. A row of six wells exposed only to RPMI-1640 medium was used as a control. Twenty-four hours following seeding, IM was added to the experimental wells at a concentration of 10 μM. The concentration of IM was chosen based on the blood levels reported in previous studies with the maximum tolerated dose of IM on clinical trials.[14] Cells were allowed to incubate for 48 h following cell seeding. After this 48-h period, 10% MTT was added to each well, then the cultures were reincubated for a further 3 h. Once removed from the incubator, the resulting formazan crystals were dissolved using MTT Solubilization Solution (M-8910), and finally cell number was determined spectrophotometrically by subtracting absorbance at a wavelength of 690 nm from the result at 570 nm. Each experiment was done in triplicate.

In vitro invasion assay

A modified Boyden chamber consisting of a polyethelene teraphthalate membrane (PET) with 8-μm diameter pores, precoated with Matrigel, an artificial basement membrane, (Beckton Dickenson Labware, Bedford, MA, USA) was used as previously described,[13] to assay for invasive ability. PET membrane without Matrigel was used as a control.

Briefly, 1.25×10^5 cells were added to the upper chamber in RPMI-1640 medium with 0.1% FBS. RPMI-1640 medium with 10% FBS was added to the lower chamber as a chemoattractant to obtain the baseline invasive ability of the cell lines. The effect of IM on invasion was assayed by adding 10 μM of IM to the RPMI-1640 medium supplemented with 0.1% FBS in the upper chamber. The chambers were then incubated at 37 °C in a 5% CO_2- enriched atmosphere for 48 h to allow for cellular invasion through the Matrigel.

Non-invading cells were removed from the upper chamber by gently wiping the surface of the membrane with a moist cotton swab. The membranes were then stained using Diff-Quick staining set (Siemens Healthcare Diagnostics, Tarrytown, NY, USA), which stains cell nuclei purple and cytoplasm pink. Stained cells were counted microscopically in 20 random high-powered fields (40 ×).

Only cells whose nuclei had completely invaded through the membrane were counted. Each experimental condition, including control, was performed in triplicate and the average number of invading cells was then calculated for all experimental conditions.

Percent invasion was determined for each cell line under each experimental condition using the following formula: % invasion = (mean number of treated cells invading through the Matrigel/mean number of untreated cells migrating through the Matrigel) multiplied by a 100. The cell lines were then ranked according to their invasive ability.

Irradiation

Before irradiation, the cells were incubated with IM as a concentration of $10 \,\mu$M. Cells were seeded at a concentration of 500 000 cells per ml in micro-Petri dishes and allowed to incubate overnight. The following day the Petri dishes were exposed to graded doses of gamma irradiation (^{137} Cs source/gamma cell 1000) 0, 2, 4, 6, 8, and 10 Gy. One set of dishes per cell line with and without IM was kept as controls without exposure to irradiation.

After irradiation, RB cells were removed from all Petri dishes containing 500 000 cells per plate and spun down for 5 min at 120 g. Cells were then diluted to a concentration of 50 000 cells/ml in 5% FBS RPMI solution. These dilutions were then seeded in a 96-well plate format at a concentration of 10 000 cells per well and left to incubate 37 °C for 48 h. The MTT proliferation assay was done as described above in triplicate per exposure condition and controls.

Immunocytochemistry

Cells from the two human Rb cell lines were diluted to a concentration of 200 000 cells in 300 μM. Cytospins were prepared using a Cytospin3 machine (Shandon, Pittsburgh, PA, USA). All slides were then immunostained with a polyclonal primary antibody anti-human C-kit (A4502, DakoCytomation, Burlington, ON, Canada); PDGFR-β (sc-339, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); PDGFR-α (sc-338, Santa Cruz Biotechnology, Inc.), and c-Abl (sc-887, Santa Cruz Biotechnology, Inc.) using the Ventana automated immunostaining machine (Ventana Medical Systems Inc., Tucson, AZ, USA) programmed to stain wet loaded slides. The slides were incubated at a dilution of 1 : 30 (C-kit), 1 : 100 (PDGFR-β), 1 : 200 (PDGFR-α), and 1 : 200 (c-Abl) for 30 min at 37 °C, followed by the application of biotinylated secondary antibody (8 min, 37 °C), then an avidin/streptavidin enzyme conjugate complex (8 min, 37 °C). Finally, the antibody was detected by Fast Red chromogenic substrate and counterstained with hematoxylin.

Western blot

Protein samples from the cell lines were prepared using 100 μl of 1 $\times$ electrophoresis sample buffer (62.5 mM TRIS pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 50 mM DTT) per million cells, which was then boiled for 5 min. The same number of cells per sample was used. Twenty microliter of total protein was separated on 7.5% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Amersham Bioscience, Piscataway, NJ, USA). The membrane was blotted for specific antibody according to ProteoQwest Colorimetric Western Blot Kit (Sigma-Aldrich, Oakville, ON, Canada). The primary polyclonal antibody against C-kit (A4502, DakoCytomation), PDGFR-β (sc-339, Santa Cruz Biotechnology, Inc.), PDGFR-α (sc-338, Santa Cruz Biotechnology, Inc.), and c-Abl (sc-887, Santa Cruz Biotechnology, Inc.) were used at a dilution 1 : 1000 overnight at 4 °C. The secondary, goat anti-rabbit peroxidase-conjugated antibody (Sigma-Aldrich, Oakville) was used to visualize the proteins on the membrane diluted 1 : 10 000. TMB substrate was used to visualize the bands. A broad range molecular weight marker (Bio-Rad, Mississauga, ON, Canada) was used and the positive controls were used according to specificity of the date sheet of the antibody. We used P815 lysed cell line to C-kit, 3T3/NIH cell lysate to PDGFR-α, THP-1 cell lysate to PDGFR-β, and K562 cell lysate to C-Abl.

Statistical analysis

The Student's t-test was used to compare differences on the proliferation and invasion assays. It was also used to compare results after different doses of radiation. A value of $P < 0.05$ was considered statistically significant.

Results

Proliferation

The Y-79 cell line showed a higher proliferation rate than the WERI-RB-1 Rb cell line with an average absorption of 0.172 ± 0.006 compared with 0.048 ± 0.002. IM caused a statistically significant reduction in the proliferation rates in both of them ($P < 0.001$), although the Y-79 cell line revealed a more significant decrease (Figure 1).

Invasion

Statistically significant reduction in the invasion rates of the treated group was found for both cell lines. The invasion rate of the treated cells was 27.27% of the control for Y-79 ($P = 0.014$) and 40.74% for WERI-RB-1 ($P = 0.029$; Figure 2).
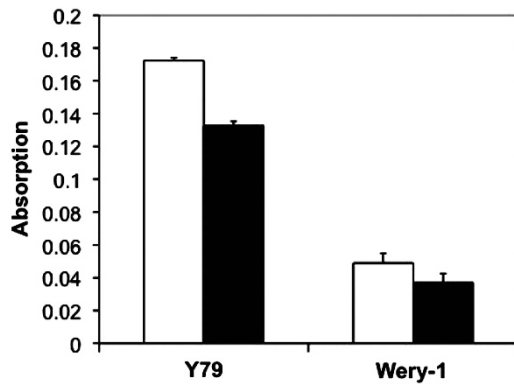
Figure 1 The graph shows the proliferation assay results comparing a control (white) and the cells treated with IM $10\,\mu$M (black). A statistical difference was seen for the two cell lines ($P < 0.001$).

Irradiation

The effect of radiation was significantly more pronounced in the Rb cell lines treated with IM. The difference in the proliferation rates between treated and non-treated cell lines was statistically significant in both cell lines at all radiation doses ($P < 0.05$; Figure 3).

Expression of the tyrosine kinase receptors

The two cell lines were strongly positive for c-Abl, positive for PDGFR-α and -β but negative for C-kit on immunocytochemistry (Figure 4) and western blot (Figure 5).

Discussion

Developing drugs to specifically inhibit oncogenes has been a major goal of cancer research. Identifying the appropriate intracellular targets is critical to this process. Some of the activated oncogenes implicated in the pathogenesis and progression of malignancy are tyrosine kinases.[14] PTK are enzymes that transfer phosphate from adenosine triphosphate to specific amino acids on substrate proteins. The phosphorylation of these proteins leads to the activation of signal-transduction pathways, which have a critical role in a variety of biologic processes, including cell growth, differentiation, and death. Several PTKs are deregulated and overexpressed in human cancers and are thus attractive targets for selective pharmacological inhibitors.[8]

Bcr–Abl, the causative molecular abnormality in CML is a prototypic oncogenic kinase. The FDA approved the tyrosine kinase inhibitor IM, in 2001, for the treatment of CML. It was the first time that this compound had the approval for the treatment of a human malignancy. The success of IM in CML led rapidly to studies in other
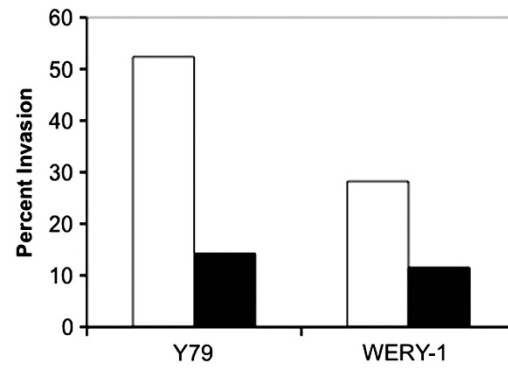

Figure 2 The graph shows the Matrigel invasion assay results for each Rb cell line before (white) and after (black) treatment with IM $10\,\mu$M. The data were presented and graphed as percentages calculated by normalizing values obtained for the untreated cells as 100%. A statistically significant reduction was seen for both cell lines.
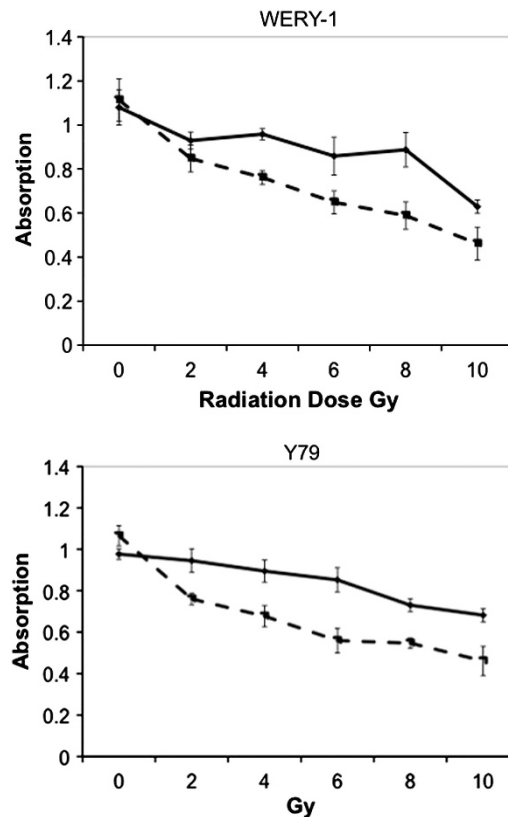

Figure 3 Graphical representation of the effect of IM and radiation on cell lines. The absorbance level is seen in the y axis while the doses of radiation are shown in the x axis. The difference in the proliferation rates between treated (solid line) and non-treated (dashed line) cell lines was statistically significant in both cell lines at all radiation doses.

cancers associated with activation of two other tyrosine kinases known to be sensitive to IM, C-kit the target of IM in the GIST, and PDGFR the target of this compound
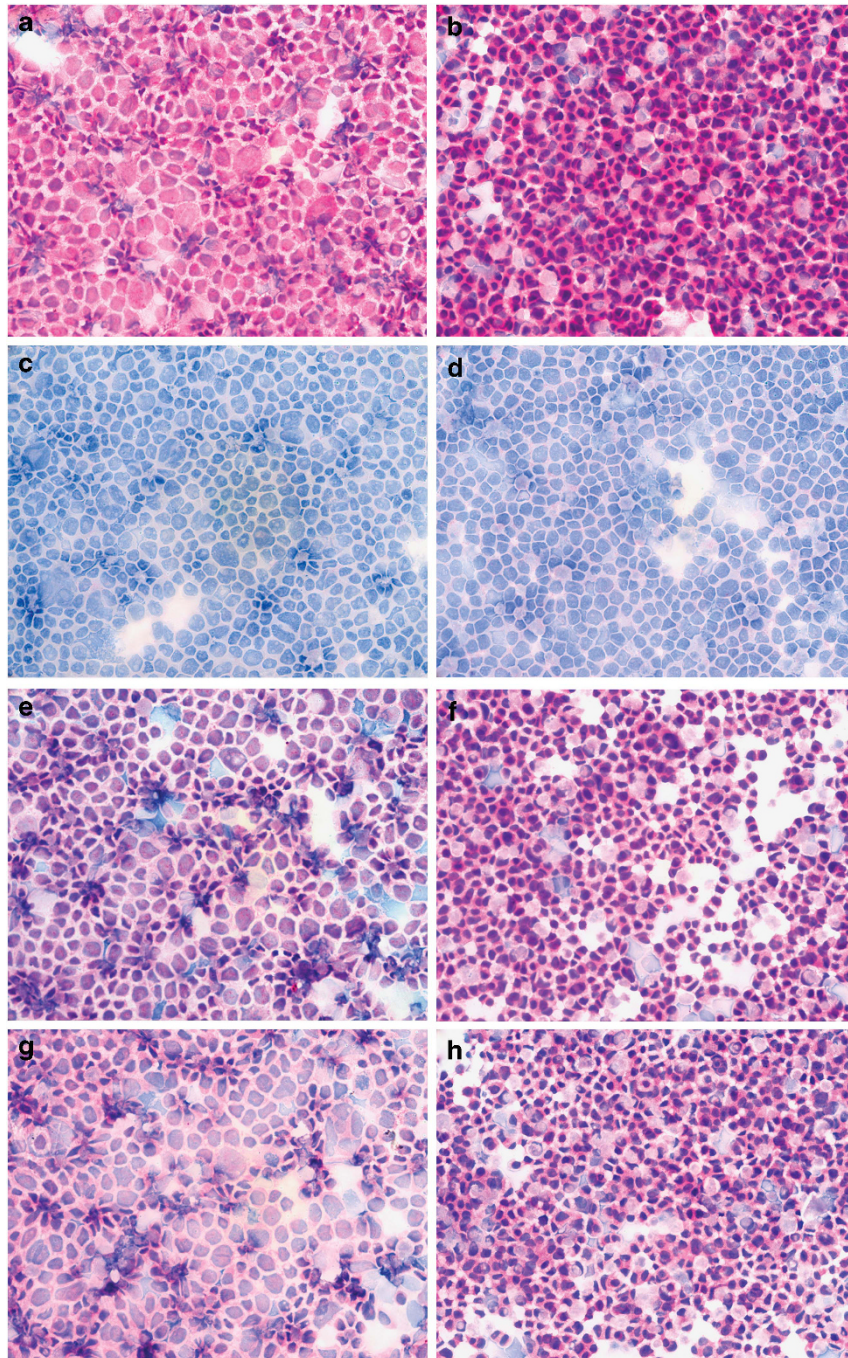
Figure 4 Immunocytochemistry of Rb cell lines. C-kit was negative for both cell lines whereas all other markers were positive. (a) Y-79 (Bcr–Abl, ×400). (b) WERI-RB-1 (Bcr–Abl, ×400). (c) Y-79 (C-kit, ×400). (d) WERI-RB-1 (C-kit, ×400). (e) Y-79 (PDGFR-α, ×400). (f) WERI-RB-1 (PDGFR-α, ×400). (g) Y-79 (PDGFR-β, ×400). (h) WERI-RB-1 (PDGFR-β, ×400).

in some solid tumors like glioma, osteosarcoma, and neuroblastoma.[14–17]

In an effort to identify additional agents for the treatment of Rb, we performed pre-clinical studies of IM and its targets in two human Rb cell lines. In the first part of our study, we evaluated the anti-proliferative and anti-invasive effects of IM in Y79 and WERI-RB-1 human Rb

cell lines. Cell proliferation has been suggested by Kim *et al*[18] to be a significant histopathological parameter that can predict the clinical outcome of Rb. In our study, IM reduced the proliferation rate of both cell lines, specially Y79, which considered the most aggressive one. Our study also demonstrated that IM markedly reduced the invasive ability of both cell lines. The invasion assay is
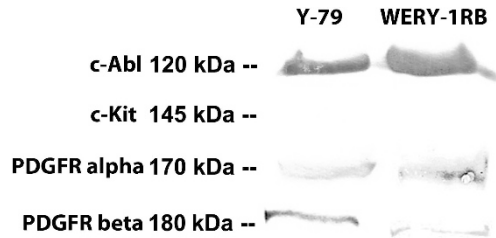
Figure 5 Western blot. None of the cell lines had detectable levels of c-kit. All other markers were positive.

important to show the ability of cells to invade an artificial basement membrane, reflecting the ability of cells to migrate through the extracellular matrix get access to the systemic circulation. The use of an artificial basement membrane provides us the opportunity to study if a drug can alter the invasive potential of cells by counting the number of cells that invade a matrigel layer. A drug that can inhibit or reduce the invasiveness ability of a cell, would not only decrease the chances of a malignant cell to escape the primary site of a tumor, but also decrease the ability of circulating malignant cells to extravasate and seed distant organs.

According to Chevez-Barrios *et al*[19], Rb cell lines used in this study (WERI-RB and Y79) have very distinctive behavior as observed in a murine animal model. When inoculated in these animals, the Y-79 cell line generated aggressive tumors with invasive and metastatic potential. On the other hand, the WERI-RB cell line gave rise to localized tumors that only invaded the anterior structures of the eye without any sign of extraocular spread or metastasis. In the study herein, the proliferation and invasive rate observed in these two cell lines supported that publication, confirming the more aggressive phenotype of the Y-79 cell line.

The concentration of IM used for our *in vitro* studies was 10 μM. This concentration is equivalent to the highest drug concentration that has been achieved in the blood of patients receiving 1000 mg per day of IM, the maximum tolerated dose reported by clinical trials.[20] At that dose and schedule, the plasma concentration of IM ranged from 6 to 10 μM. *In vitro* studies have been published investigating the effect of IM in other types of tumor cell lines. McGary *et al*[15] investigated the potential of IM as a therapy for osteosarcoma, the most common primary malignant bone tumor in children. They demonstrated that IM could inhibit PDGF-mediated growth and led to apoptosis of osteosarcoma cells *in vitro* by selective inhibition of the PDGFR tyrosine kinase. Pereira *et al*[9] reported a significant decrease by IM in the proliferation and invasion rates of five different cell lines of another intra-ocular tumor, uveal melanoma. To the

best of our knowledge, this is the first report about the *in vitro* effects of IM on Rb cells.

In the second part of our study, we investigated the ability of IM to radiosensitize Rb cells. There is increasing amount evidence that IM may work as radiosensitizing agent. Studies have been done to investigate the potentiating effect of this drug on the cytotoxic effect of ionizing radiation. Holdhoff *et al*[21] demonstrated that IM could enhance significantly the cytotoxic effect of the radiation in a human glioblastoma cell line, although this effect was not seen in human breast and colon cancer cell lines. One of the proposed mechanisms for this effect on the glioblastoma cell line was the decrease in the levels of cellular tyrosine phosphorylation, and specifically the phosphorylation of PDGFR-β. Oertel *et al*[22] studied the combined effect of IM and radiation *in vitro* and *in vivo*. They showed that IM could enhance the tumor growth reduction induced by fractioned radiotherapy in glioblastoma and carcinoma models. In Bcr–abl-positive cell lines IM and irradiation was also reported to be significantly synergistic.[23]

Gerweck *et al*[24] demonstrated that the intrinsic radiosensitivity of tumor cells is a major determinant of tumor response to radiation, and not the tumor stroma radiosensitivity, like some other studies have suggested. Zhang *et al*[25] studied the effect of radiation on the Rb cell lines Y79 and WERI-RB-1, the same cell lines of our study. They confirmed what has been demonstrated clinically, that Rb cells are very sensitive to radiation. They showed that, for both Rb cell lines, radiation resulted in a time and dose-dependent induction of apoptosis associated with decreased viability. Apoptosis was indeed the predominant form of cell death observed morphologically in Rb cells following radiation.[25] Kondo *et al*[26] also demonstrated that radiation in human Rb cell linesY79 and WERI-RB-1-induced apoptosis. To the best of our knowledge, this is the first time that IM has been shown to increase the radiosensitivity of Rb cells lines. The combination of IM and radiation is a promising emerging therapy as it is possible that IM can selectively enhance the radiosensitivity of the tumor, thereby reducing the needed doses of radiation. This reduction in radiation would hopefully lead to a reduction in treatment-related complications and the rates of secondary tumor development.

The mechanism underlying the synergistic effect of IM and radiation remains unclear and is probably multifaceted. Some authors believe that IM's anti-neoplastic activity *in vivo* could be attributed in part to its anti-angiogenic effects, and the combination of the drug and radiation could further reduce microvessel density.[22] The inhibition of specific PTKs, specially C-kit and PDGFR-α and -β, can synergistically increase the radiation damage in neoplastic cells, as it is known that

both are related to the repair of radiation-induced DNA damage. As an example, signaling of C-kit by stem cell factor is known to reduce radiation-induced apoptosis and cytotoxicity, thus conferring radioprotection to malignant cells.[27]

In the final part of our study, we investigated the presence of the targets of IM in both Rb cell lines by western blot and immunocytochemistry. The only target that was not expressed in these cell lines was the C-kit (CD117, stem cell factor receptor). PDGFR-α and -β were positive while c-Abl was strongly positive. There was no difference in expression of the IM's targets between the two cell lines. The results were the same for Y79 and WERI-RB-1 cell line in western Blot and in the cytospins. Even considering that these are preliminary studies, we suggest that the effects of IM on Rb cells are through PDGFR and c-Abl, and not C-kit. Further studies will have to be completed to determine which target is the one responsible for the effects that IM has on Rb cell lines. The presence or absence of only one of its targets is not enough to determine the potential effects of IM in a clinical setting. It is also known that IM may have others mechanisms of action other than the inhibitory effect on the growth kinases,[22] and further studies still have to be done to determine how that drug works in the distinctive types of tumors. Nevertheless, our preclinical results warrant further trials to study the effect of IM on Rb, specially in association with radiotherapy.

Summary

What was known before
- IM works as a radiosensitizing agent for some solid tumors.
- The effects of IM on Rb cell lines is unknown.

What this study adds
- Rb cell lines expresses bcr–abl, PDGFR but not c-kit.
- IM decreases proliferation, invasion and increases the radiosensitivity of Rb cell lines.

Conflict of interest

The authors declare no conflict of interest.

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