Research Article

Novel Application of Radotinib for the Treatment of Solid Tumors via Natural Killer Cell Activation

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Radotinib (Supect™; C27H21F3N8OHCl, 4-methyl-N-[3-(4-methyl-imidazol-1-yl)-5-trifluoromethyl-phenyl]-3-(4-pyrazin-2-yl-pyrimidin-2-ylamino)-benzamide hydrochloride) was developed to treat chronic myeloid leukemia (CML) as a BCR-ABL1 tyrosine kinase inhibitor (TKI). Other TKIs, including imatinib and nilotinib, were also developed for treatment of CML, and recent studies were increasing about the therapeutic effects of other TKIs on solid tumors. However, the effect of radotinib on solid tumors has not yet been investigated. In this study, radotinib killed CML cell line K562 directly; however, radotinib did not enhance NK cell cytotoxicity against K562 cells. Because K562 is known as a Fas-negative cell line, we investigated whether radotinib could regulate cell cytotoxicity against various Fas-expressing solid cancer cell lines. Radotinib dramatically increased NK cell cytotoxicity against various Fas-expressing solid cancer cells, including lung, breast, and melanoma cells. Additionally, the efficiency of radotinib-enhanced cytotoxicity was lower in Fas siRNA-transfected cells than in negative controls, suggesting that Fas signaling might be involved in the radotinib-enhanced NK cell cytotoxicity. This study provides the first evidence that radotinib could be used as an effective and strong therapeutic to treat solid tumors via upregulation of NK cell cytotoxicity, suggesting that radotinib has indirect killing mechanisms via upregulation of antitumor innate immune responses as well as direct killing activities for CML cells.

1. Introduction

Radotinib (Supect™, C27H21F3N8OHCl, 4-methyl-N-[3-(4-methyl-imidazol-1-yl)-5-trifluoromethyl-phenyl]-3-(4-pyrazin-2-yl-pyrimidin-2-ylamino)-benzamide hydrochloride) was developed to treat chronic myeloid leukemia (CML) as a breakpoint cluster region-Abelson (BCR-ABL) 1 tyrosine kinase inhibitor (TKI). Other TKIs, including imatinib and nilotinib, were also developed for treatment of CML, and recent studies were increasing about the therapeutic effects of other TKIs on solid tumors. However, the effect of radotinib on solid tumors has not yet been investigated. In this study, radotinib killed CML cell line K562 directly; however, radotinib did not enhance NK cell cytotoxicity against K562 cells. Because K562 is known as a Fas-negative cell line, we investigated whether radotinib could regulate cell cytotoxicity against various Fas-expressing solid cancer cell lines. Radotinib dramatically increased NK cell cytotoxicity against various Fas-expressing solid cancer cells, including lung, breast, and melanoma cells. Additionally, the efficiency of radotinib-enhanced cytotoxicity was lower in Fas siRNA-transfected cells than in negative controls, suggesting that Fas signaling might be involved in the radotinib-enhanced NK cell cytotoxicity. This study provides the first evidence that radotinib could be used as an effective and strong therapeutic to treat solid tumors via upregulation of NK cell cytotoxicity, suggesting that radotinib has indirect killing mechanisms via upregulation of antitumor innate immune responses as well as direct killing activities for CML cells.

Several studies have reported the effect of imatinib and/or nilotinib on natural killer (NK) cell activity [2–4]. Imatinib specifically at all treatment concentrations does not affect interferon-γ (IFN-γ) production or NK cell cytotoxicity in the CML cell line, K562. However, high concentrations of nilotinib inhibit IFN-γ production, resulting in decreased NK cell activity [2]. Nilotinib induces cell death of CD56brightCD16− NK cells, well-known as a cytokine-producing NK cell subset. These results suggest that the suppression of IFN-γ production by nilotinib is due to cytokine-producing NK cell death [2]. Additionally, imatinib and nilotinib do not regulate granule expression,
such as perforin [3]. Other study has suggested that ima-
tinib and nilotinib do not affect activating or inhibitory
receptors on NK cells; however, chemokine receptor
CXCR4 are increased by treatment of imatinib or nilotin-
ib, suggesting the off-target effects of TKIs on immune
cells [4].

NK cells, CD56+CD3+ cytotoxic lymphocytes in the
blood, play a critical role in the innate immune system
through spontaneous elimination of cancerous and
virus-infected cells. The cytolytic activity of NK cells is
mediated by Fas/Fas ligand interaction, granule exocytosis,
and antibody-dependent cell-mediated cytotoxicity [5]. Fas
is part of a death receptor containing a conserved death
domain in its intracytoplasmic domain. Activated NK cells
express Fas ligand and recognize Fas-expressing target cells
via Fas/Fas ligand interaction. This interaction leads to
activation of a caspase cascade and ultimately apoptotic
mechanisms in target cells [6, 7].

Although other TKIs, such as imatinib and nilotinib, do
not enhance NK cell activity, the effect of radotinib on NK
cell cytotoxicity has not been investigated. In this study, we
demonstrate anticancer effects of radotinib via upregulation
of NK cell cytotoxicity against Fas-expressing cancer cells.

2. Materials and Methods

2.1. Cell Culture and siRNA Transfection. The human
CML cell line K562, human lung carcinoma cell lines
A549 and NC1-H460, human melanoma cell lines A375
and SK-MEL-5, and human breast cancer cell lines
MDA-MB-231 and MCF-7 were purchased from ATCC
(Manassas, VA, USA). K562 cells were cultured in a
RPMI-1640 medium (Gibco), and other cells were cultured
in Dulbecco's Modified Eagle Medium. Both media were
supplemented with 2 mM L-glutamine, 100 U/ml penicillin,
100 μg/ml streptomycin, and 10% heat-inactivated fetal
bovine serum. Cells were maintained in a 5% CO_2
incubator at 37°C.

At approximately 70% confluence, A549 cells were trans-
fected with 50 pmole Fas siRNA using Lipofectamine RNAi-
MAX (Invitrogen, Carlsbad, CA, USA) per manufacturer’s
instructions. Commercially available human Fas siRNA and
negative control siRNA were purchased from Santa Cruz
Biotechnology (Santa Cruz Biotechnology, CA, USA).
Transfection efficiency was confirmed by surface staining
analysis using a FACS Calibur (BD Biosciences, San Jose, CA, USA)
using phycoerythrin- (PE-) conjugated Fas antibody (BD
Biosciences) or PE-conjugated mouse IgG isotype control.

2.2. Isolation of Human Peripheral Blood Lymphocytes and
NK Cells. Human blood samples were obtained from Inje
University Busan Paik Hospital (Korea). All studies using
human subjects were approved by the Institutional Review
Board (Inje IRB/1). Peripheral blood mononuclear cells
(PBMC) were isolated from the blood by density gradient
centrifugation using Ficoll-Paque (Sigma, St. Louis, MO,
USA), and then peripheral blood lymphocytes (PBLs) were
collected after monocyte depletion. Briefly, PBMC were
resuspended in a RPMI1640 medium supplemented with
10% fetal bovine serum (FBS), and incubated on plastic cul-
ture dishes in 5% CO_2 incubator at 37°C for overnight. Sus-
pended cells including PBLs were collected. Human
primary NK cells were isolated from PBLs using MACS NK
cell isolation kit (Miltenyi Biotec, Bergisch Gladbach,
Germany) as per the manufacturer’s recommendation.

2.3. Cytotoxicity Assay. A cytotoxicity assay was performed as
previously described [8]. Briefly, effector cells, such as isola-
ted PBLs or purified NK cells, were treated with radotinib
at indicated concentrations or with recombinant human
interleukin- (IL-) 2 (50 U/ml) for 48 h. Target cells were
stained with carboxyfluorescein diacetate succinimidylester
(Molecular Probes Inc., USA) for five min at 37°C. After three
washes with cold complete medium, the labeled target cells
were incubated with effector cells. The assay was performed
in triplicate with various effector to target cell (E:T)
ratios. After incubation at 37°C in 5% CO_2 for 2 h, the target
cell lysis was analyzed by 7-aminoactinomycin D (7-AAD)
(BD Biosciences) staining using a FACS Calibur (BD Biosci-
ces) with Cell Quest software.

To block the Fas-Fas ligand interactions, approximately
0.5–2 μg of recombinant human soluble Fas (R&D systems)
was incubated with resting or radotinib-treated NK cells
before the cytotoxicity assay. After preincubation for 1 h,
the cytotoxicity assay was performed as described above
without washing.

2.4. Statistical Analysis. All values were analyzed with an
unpaired Student’s t-test. Statistical analyses were performed
using GraphPad Prism 5 (GraphPad Software, La Jolla,
CA, USA).

3. Results and Discussion

Radotinib, a novel BCR-ABL1 TKI, is an effective therapeutic
drug for treating CML [1]. BCR-ABL1 fusion is the main
pathogenesis of CML, leading to uncontrolled proliferation.
Therefore, TKIs targeting BCR-ABL1 have been developed
d as therapeutics for CML because of their inhibition of uncon-
trolled proliferation. To confirm the effect of radotinib on
CML cells, we used a representative CML cell line, K562. Cell
viability of K562 cells was significantly reduced even at the
lowest concentration of radotinib (12.5 μM), indicating that
radotinib kills K562 cells directly (Figure 1(a)). It has been
reported that another standard TKI, imatinib, inhibits cancer
cell proliferation via the regulation of cell cycle [9]. There-
fore, our data suggest that radotinib is able to inhibit cell pro-
liferation and induce cell apoptosis by inhibiting BCR-ABL1
as well as imatinib.

To determine the ability of radotinib to kill K562 cells via
the cytolytic activity of peripheral blood lymphocytes (PBLs),
we performed a cytotoxicity assay using radotinib-treated
PBLs as effector cells and K562 cells as target cells. Although
radotinib directly and effectively killed K562 cells, it did
not enhance the cytolytic activity of PBLs against K562,
whereas IL-2 significantly stimulated cytotoxicity of PBLs
(Figure 1(b)). Because K562 cells are Fas-negative cells
[10–12], we hypothesized that radotinib may regulate cell
cytotoxicity against certain types of tumor cells, such as Fas-expressing cells. To confirm the effect of radotinib on the cytotoxicity of PBLs against Fas-expressing cells, we determined the Fas expression in A549 cell lines. As shown in Figure 2(b), A549 cells highly expressed the Fas receptor. Consistent with these differences in Fas expression, radotinib dramatically increased the cytolytic activity of PBLs only in A549 cells (Figure 1(b)) suggesting a novel therapeutic effect of radotinib on solid tumor other than CML.

We next isolated human primary NK cells from PBLs to determine whether the cytolytic activity of PBLs was mediated by NK cell cytotoxicity. The purified NK cells (purity = 96.6% as shown in Figure 1(a)) were incubated with various doses (0, 12.5, 25, 50, 100, and 200 μM) of radotinib, which did not affect the viability of NK cells (data not shown). NK cell cytotoxicity was markedly upregulated upon radotinib treatment. This significant increase in cytotoxicity with radotinib treatment was dose-dependent (Figure 2(b)). To determine the involvement of Fas-FasL interaction in radotinib-enhanced NK cell cytotoxicity, the A549 cells were transiently transfected with Fas-specific siRNA or negative control siRNA. Surface staining and flow cytometry were then performed to detect Fas expression on the transfected cells. As shown in Figure 2(c), surface expression of Fas decreased in the A549 cells transfected with Fas-specific siRNA compared to controls. Figure 2(d) shows that the efficiency of radotinib-enhanced cytotoxicity was lower in the Fas-specific siRNA-transfected A549 cells than in the control cells. To further confirm this result, recombinant human soluble Fas was used to block Fas-FasL interaction. The soluble Fas was preincubated with NK cells to bind Fas ligands on their surface before the cytotoxicity assay. Figure 2(e) shows that the efficiency of radotinib-enhanced cytotoxicity was significantly decreased by the preincubation with soluble Fas.

Additionally, the expression of Fas ligand on NK cells was confirmed to determine the effect of radotinib on Fas ligand expression of NK cells. Fas ligand expression was slightly enhanced by radotinib treatment on NK cells. Primary NK cells were isolated from nine healthy donors and then incubated with 100 μM of radotinib for 48 h. The average of mean fluorescence intensity (MFI) of Fas ligand expression in all nine samples increased about 10–20% as shown in Supplementary Figure S1. Fas ligand expression on NK cells tended to be increased by radotinib treatment; however, this increase has been shown in part of the donors (3 individuals among 9 donors). Nevertheless, it is certain that radotinib significantly enhances NK cell cytotoxicity against various solid cancer cell lines even in donors whose Fas ligand does not increase. NK cell cytotoxicity of radotinib in the other Fas-expressing cell lines was tested by using a different lung cancer cell line, NCI-H460; the melanoma cell lines, SK-MEL-5 and A375; and the breast cancer cell lines MCF-7 and MDA-MB-231. These cell lines were used as target cells in which Fas ligand expression was confirmed by flow cytometry (Figure 3(a)). Figure 3(b) shows that radotinib-stimulated NK cells effectively killed all the solid tumor cells tested.

Thus, it could be a difference by NK subsets in the individuals or the other NK-activating receptors. Several studies have reported various NK cell subsets, such as CD56bright or CD56dim. It is well-known that CD56dim/CD16+ NK cells are the major population in the peripheral blood having cytolytic activity [13, 14]. Especially, it was reported that Fas
In addition to Fas-FasL interaction, it is known that NK cell activation is regulated by the balance of activating and inhibitory receptors; the expression of several receptors on NK cells was determined, as shown in Supplementary Figure S2. Both activating (NKP46, NKP44, NKP80, and NKG2D) and inhibitory (CD94, KIR2DL1, KIR2DL2/DL3, and KIR3DL2) receptors were not affected by treatment with radotinib. A recent study has investigated the effect of other TKIs including imatinib and nilotinib, resulting that both TKIs also do not enhance activating or inhibitory receptors on NK cells. However, these TKIs increase the chemokine receptor CXCR4 on the surface of NK cells.

Figure 2: Radotinib enhances cytolytic activity of NK cells against Fas-expressing A549 cells. (a) Primary NK cells were isolated from healthy donors to perform NK cell cytotoxicity assay. The purity of CD3 CD56dim NK cells was 96.6%. (b) To determine the effect of radotinib on the cytolytic activity of NK cells against A549 cells, the cells were treated with various concentrations of radotinib (0, 12.5, 25, 50, 100, and 200 μM) for 48 h and the cytotoxicity assay was performed (E:T ratio = 5:1). (c) To determine if the effect of radotinib on NK cytotoxicity was mediated by the Fas receptor, Fas expression was transiently downregulated by Fas siRNA transfection into A549 cells. At approximately 70% confluence, A549 cells were incubated with 50 pmole Fas-specific siRNA or negative control siRNA using Lipofectamine RNAiMAX. Surface expression of Fas on A549 cells was determined by staining with PE-conjugated Fas antibody (solid line). PE-conjugated mouse IgG antibody was used as an isotype control (dotted line). (d) The effect of radotinib on NK cytolytic activity against Fas siRNA-transfected A549 cells was determined by cytotoxicity assay. Radotinib-treated NK cells were used as effector cells, and Fas siRNA-transfected A549 cells or control cells were used as target cells (E:T ratio = 2:1). All values were normalized relative to the control (radotinib 0 μM). The relative level was set to 1 for the control. (e) To further confirm the involvement of Fas-Fas ligand interaction in the radotinib-enhanced NK cytotoxicity, recombinant human soluble Fas was used to block Fas ligand on NK cells. Various concentrations of soluble Fas were preincubated with resting NK cells or radotinib-treated NK cells for 1 h, and then cytotoxicity assays were performed (E:T ratio = 2:1). All values were normalized relative to the control (radotinib 0 μM). The relative level was set to 1 for the control. Data are reported as mean ± SD. All values were analyzed by unpaired Student’s t-tests using GraphPad Prism 5. *P < 0.05, **P < 0.01, and ***P < 0.001. All data presented are representative of three independent experiments.

Ligand-mediated NK cell cytotoxicity depends on the expression of CD2 on CD56dim NK cells. CD2−CD56dim NK subsets highly express Fas ligand, implying that the degree of Fas ligand expression is different in the subdivided NK subsets [15]. Additionally, 2B4 and LFA-1 also upregulate Fas ligand expression on NK cells while CD94/NKG2A inhibits Fas ligand-mediated cytotoxicity [16]. Therefore, further study will be required to demonstrate the effect of radotinib on Fas ligand expression by subdividing the NK subset in the individuals for understanding radotinib-enhanced NK cell killing activity to design future clinical application for therapy.
and monocytes [4]. Thus, it is needed to investigate the effect of radotinib on the expression of chemokine receptors or adhesion molecules.

Here, we confirmed that radotinib enhanced NK cell cytotoxicity against Fas-expressing cancer cells, but not CML cell line K562, providing new insight into the mechanisms of the antitumor effects of radotinib against solid tumors. These results, indicating that radotinib does not induce K562 cell death through PBL-mediated cytotoxicity, are comparable to those of other TKIs, including imatinib and nilotinib, that do not enhance the cytolytic activity of PBLs against K562 cells [2]. Therefore, in order to examine whether imatinib can also kill Fas-expressing cancer cells mediated by PBL cytotoxicity, PBLs were treated with imatinib mesylate (100 μM; Sigma-Aldrich) to enhance cytolytic activity. Cytotoxicity against A549 cells increased slightly upon imatinib mesylate treatment (Figure 4). However, enhancement of cytolytic activity upon radotinib treatment was greater.

In conclusion, this study provides the first evidence that radotinib, which was developed as a therapeutic drug for CML, can be used for a novel application for treatment of solid tumors. This study shows a strong evidence that radotinib enhances NK cytolytic activity, resulting in antitumor effects against various solid cancer cell lines. It suggests that

\[ \text{Figure 3: Radotinib enhances cytolytic activity of NK cells against various cancer cells expressing Fas receptor. To further explore the involvement of the Fas receptor in cytolysis, various cancer cell lines were used to analyze the radotinib-enhanced cytolytic activity of NK cells. (a) Surface expression of Fas on various cancer cells, including A549, NCI-H460, A375, SK-MEL-5, MDA-MB-231, and MCF-7, was determined by staining with PE-conjugated Fas antibody (solid line). PE-conjugated mouse IgG antibody was used as an isotype control (dotted line). (b) The effect of radotinib on NK cytolytic activity against various cancer cell lines was determined by cytotoxicity assays. Radotinib-treated NK cells were used as effector cells, and cancer cell lines were used as target cells (E : T ratio = 2 : 1). Data are reported as mean ± SD. All values were analyzed by unpaired Student’s t-tests using GraphPad Prism 5. **P < 0.01 and ***P < 0.001. All data presented are representative of three independent experiments.} \]

\[ \text{Figure 4: A comparison of the cytolytic activity of radotinib and imatinib mesylate. A comparison of the cytolytic activity of radotinib and imatinib mesylate, a TKI for treating CML, against Fas-expressing A549 cells was performed using radotinib- or imatinib mesylate-treated PBLs as effector cells and A549 cells as target cells in a cytotoxicity assay. Data are reported as mean ± SD. All values were analyzed by unpaired Student’s t-tests using GraphPad Prism 5. *P < 0.05 and ***P < 0.001. All data presented are representative of three independent experiments.} \]
radotinib has indirect killing mechanisms via upregulation of antitumor innate immune responses as well as direct killing activities for BCR-ABL1-positive CML cells. These off-target effects of radotinib in immune cells indicate that radotinib could be the most effective and strongest therapeutics for solid tumors. Additionally, radotinib has been approved and currently used for treating CML patients and has these off-target effects without killing of normal immune cells as shown in our study. Therefore, we expect to be able to apply it directly to the treatment of other solid cancers.

**Data Availability**

The data used to support the findings of this study are included within the article.

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

**Authors’ Contributions**

Kyung Eun Kim, Sunyoung Park, Hyun Jeong Park, and Daeho Cho contribute to this work equally. Kyung Eun Kim and Sunyoung Park are equally contributed to this work as co-first authors.

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**Supplementary Materials**

Supplementary Figure 1: effect of radotinib on surface expression of Fas ligand in primary NK cells. To determine the effect of radotinib on Fas ligand expression of NK cells, primary NK cells were isolated from nine healthy donors and then incubated with 100 μM of radotinib for 48 h. (A) Surface expression of Fas ligand on NK cells was determined by staining with PE-conjugated Fas ligand antibody (solid line). PE-conjugated mouse IgG antibody was used as an isotype control (dotted line). Data presented is a representative of three donors showing increased expression of Fas ligand. (B) The average of mean fluorescence intensity (MFI) of Fas ligand expression in all nine samples was shown. All values were normalized relative to the control (radotinib 0 μM). The relative level was set to 1 for the control. Supplementary Figure 2: effect of radotinib on the expression of activating or inhibitory receptors. To determine the effect of radotinib on activating or inhibitory receptors of NK cells, surface FACS staining was performed as described in Materials and Methods. Resting NK cells or radotinib-treated NK cells were incubated with antibodies as follows: PE-conjugated NKp44, PE-conjugated NKp46, PE-conjugated NKp80, PE-conjugated NKG2D, FITC-conjugated CD94, APC-conjugated KIR2DL1, PE-conjugated KIR2DL2/DL3, PE-conjugated KIR3DL2 (solid line), or isotype controls (dotted line). The expression level of surface molecules was detected using FACSCalibur (BD Biosciences); then data was analyzed using FlowJo software. All data presented are representative of three independent experiments. (Supplementary Materials)

**References**


