Appropriate inhibition of autophagy after treatment with imatinib mesylate enhances cytotoxicity in malignant peripheral nerve sheath tumor cells

Munehiro Okano

Department of Pediatrics, Kinki University Faculty of Medicine, Osaka, Osaka 589-8511, Japan

Abstract

Malignant peripheral nerve sheath tumor (MPNST), which is very rare in childhood, is a highly aggressive soft tissue tumor that is refractory to conventional therapy. Since this tumor expresses platelet-derived growth factor receptor, imatinib mesylate may be a novel therapeutic option. However, cellular reactions after the treatment of MPNST with imatinib are not fully understood. Therefore, we investigated the cellular reactions of imatinib in vitro using three MPNST cell lines. Imatinib induced cytotoxicity in vitro with variable IC_{50} values (11.7-> 30 μM). The induction of apoptosis was not a pivotal mechanism in the inhibitory effects. We found that the treatment of MPNST cell lines with imatinib induced autophagy. Suppression of the initiation of autophagy by 3-methyladenine or small interfering RNA (siRNA) against beclin-1 attenuated imatinib-mediated cytotoxicity. In contrast, blocking the formation of autophagosomes or development of autolysosomes using siRNA against microtubule-associated protein light chain 3B (LC3B) or bafilomycin A1 enhanced imatinib-induced cytotoxicity in MPNST cells. Our data showed that imatinib-mediated autophagy can function as a cytotoxic mechanism and that appropriate modulation of autophagy may sensitize MPNST cells to imatinib, which in turn may be a novel therapeutic strategy for MPNST.

Key words: malignant peripheral nerve sheath tumor, imatinib mesylate, platelet-derived growth factor receptor, autophagy

Introduction

Malignant peripheral nerve sheath tumor (MPNST) is very rare in the pediatric age group and involves a highly aggressive soft tissue tumor arising either from neurofibromatosis type I (NF1) or de novo from peripheral nerves. The overall 5-year survival rate has been reported to be approximately 30% because of resistance to conventional therapy. We experienced a 7-year-old NF1 patient with MPNST and a tumor refractory to conventional therapy. Therefore, we sought to develop a novel therapeutic strategy based on an investigation of the biology of the tumor.

Recently, platelet-derived growth factor receptor (PDGFR) and its ligands have been suggested to be involved in the malignant transformation and progression of MPNST. PDGFR is a kinase targeted by imatinib mesylate (imatinib), which provides great benefits to patients with gastrointestinal stromal tumors and chronic myelogenous leukemia (CML). Evidence that imatinib inhibits the cellular activity of several receptor kinases has led to an investigation of its use in various human cancers including MPNST. Since we examined and confirmed the expression of PDGFR-α and β in the tumor (Figure 1B), the administration of imatinib may be one of the therapeutic options
In this study, we investigated the cellular reactions of imatinib in vitro using MPNST cell lines. The present report is the first to indicate that autophagy is associated with imatinib-induced cytotoxicity and that appropriate modulation of autophagy is important for sensitizing MPNST to imatinib.

Materials and methods

Patient and tumor tissue
A 7-year-old boy with NF1 presented with a right neck tumor (Figure 1). A total excision was performed and pathological findings revealed a diagnosis of MPNST. The expressions of PDGFR-α and -β in the tumor tissue sample were examined by immunohistochemistry. The tumor was refractory to conventional therapy and metastasized to bilateral lungs (Figure 1).

Cell lines and culture
YST-1 was kindly provided by the RIKEN BioResource Center, and cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS) from Invitrogen (Karlsruhe, Germany). HS-PSS and HS-Sch-2, provided by the RIKEN BioResource Center, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% heat-inactivated FCS. Cells were cultured in RPMI 1640 or DMEM with 1% FCS when evaluating cell viability by the MTT assay.

Reagents
Anti-microtubule-associated protein light chain 3B (LC3B), β-actin, p62SQSTM1, poly(ADP-ribose) polymerase (PARP), cytochrome c, beclin-1 antibodies, siRNA against beclin-1, and a negative control were from Cell Signaling Technologies (Beverly, MA, USA). Anti-cleaved caspase-3 antibody was purchased from Trevigen (Gaithersburg, MD, USA). 3-Methylladenine (3-MA), pepstatin A, E-64d, and bafilomycin A1 were purchased from Sigma Aldrich (St. Louis, MO, USA). Imatinib mesylate was kindly provided by Novartis Pharma AG (Basel, Switzerland). Imatinib was dissolved in distilled water at a concentration of 10 mM. Small interfering RNA (siRNA) against LC3B was purchased from Qiagen (Hilden, Germany). 3-MA was dissolved in phosphate-buffered saline (PBS) and other reagents were dissolved in dimethyl sulfoxide (DMSO).
Reverse transcription polymerase chain reaction (RT-PCR)

RNA was extracted according to the manufacturers' procedure, and treated with a deoxyribonuclease for removing contaminated DNA. One microgram of RNA was reverse-transcribed into its complementary DNA (cDNA) using reverse transcriptase (Applied Biosystems, Foster City, CA). A small aliquot of cDNA (100 ng) was used for PCR. Sequences of cDNA were used for searches in Ensembl, and the primers' sequences were designed using Primer3 Plus software. The PCR product was detected using agarose gel electrophoresis.

MTT assay

Cells were cultured in 96-well culture plates and incubated with various reagents. Cell viability was analyzed using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) cell proliferation assay kit (Cayman Chemical Company, MI, USA) following the manufacturer's instructions.

Flow cytometry

For cell cycle analysis, incubated cells were fixed in 70% ethanol and then incubated in ribonuclease A solution at 37°C to remove RNA. After staining with propidium iodide (PI), cells were subjected to fluorescence-activated cell sorting (FACS).

Electron microscopy

HS-Sch-2 cells were treated with or without 20 μM imatinib for 48 h at 37°C. Cells were fixed in ice-cold 2.5% electron microscopy-grade glutaraldehyde in phosphate-buffered saline (PBS) overnight at 4°C. Fixed samples were washed three times in PBS, and then post-fixed in 1% osmium tetroxide containing 0.1% potassium ferricyanide for 1 h. Following three PBS washes, samples were dehydrated through a graded series of 30% to 100% ethanol, and then embedded in epoxy resin according to the standard procedure. Ultrathin (70 nm) sections were collected and stained with 2% uranyl acetate in 50% methanol for 10 min, followed by staining with 1% lead citrate for 7 min. Sections were imaged using a JH-7100 transmission electron microscope (Hitachi, Japan).

siRNA-mediated knockdown

Knockdown of siRNA was performed according to the manufacturer's instructions. To assess the effects of siRNA-mediated knockdown of beclin-1 or LC3B, transfected cells were subjected to western blots probed with anti-beclin-1 or LC3B antibodies.

Statistical analysis

Data are presented as mean ± standard error (SE). A paired Student's t test (2-tailed) was used for comparisons between 2 groups. Differences were considered significant when p was less than 0.05.

Results

Treatment of MPNST cell lines with imatinib decreased cell viability

First, we investigated PDGFR expression in the three cell lines by reverse transcription polymerase chain reaction (RT-PCR) and western blotting. YST-1 and HS-PSS cells expressed both PDGFR-α and β, and HS-Sch-2 expressed PDGFR-β (Figure 2A).

The MTT assay revealed that incubation of MPNST cells with various concentrations of imatinib for 72 h decreased the viability of these cells in a dose-dependent manner, with fifty percent inhibitory concentration (IC_{50}) values ranging from 11.7 (YST-1) to 29.3 μM (HS-PSS). However, the IC_{50} of HS-Sch-2 cells was over 30 μM (Figure 2B). In the most sensitive YST-1 cells, cell cycle analysis by flow cytometry after propidium iodide staining revealed that 48-h treatment with 10 μM imatinib significantly increased the G0/G1 population (45.2% ± 3.2% vs. 56.3% ± 9.9%, p<0.05), whereas there was no difference in the subG1 population between untreated and treated cells (Figure 2C). To determine whether the cytotoxicity of imatinib was involved in the induction of apoptosis, we performed western blotting following treatment.
with imatinib. The amount of cytochrome c, the cleaved form of PARP (89 kDa), or the cleaved form of caspase-3 (17 kDa) was slightly increased in YST-1 cells after the 72-h treatment (Figure 2D), but was not in HS-PSS or HS-Sch-2 cells (data not shown). The presence of the pan-caspase inhibitor z-Val-Ala-Asp (Ome)-CH₂F (zVAD-fmk) had no impact on imatinib-mediated cytotoxicity in YST-1 (Figure 2E). These findings suggest that the induction of apoptosis was not substantially involved in the cytotoxicity of imatinib following treatment in MPNST, and non-apoptotic cell death mechanisms may be associated. We mainly used two cell lines, YST-1 as an imatinib-sensitive cell line and HS-Sch-2 as a resistant cell line, in further experiments.

**Treatment of MPNST cell lines with imatinib induced autophagy**

Next, we investigated whether imatinib induced autophagy in MPNST cells. During autophagy, LC3B is conjugated to phosphatidylethanolamine, creating a more rapidly migrating band (LC3B-II) that can be detected in western blots. LC3B can serve as the most reliable marker for autophagy. Western blotting probed with the anti-LC3B antibody showed that the treatment of HS-Sch-2 or YST-1 cells with various concentrations of imatinib resulted in the upregulation of LC3B-II, which was detectable within 24 h of imatinib treatment of cells and was still present after 72 h (Figure 3A). These findings suggest that the induction of apoptosis was not substantially involved in the cytotoxicity of imatinib following treatment in MPNST, and non-apoptotic cell death mechanisms may be associated. We mainly used two cell lines, YST-1 as an imatinib-sensitive cell line and HS-Sch-2 as a resistant cell line, in further experiments.

**Treatment of MPNST cell lines with imatinib induced autophagy**

Next, we investigated whether imatinib induced autophagy in MPNST cells. During autophagy, LC3B is conjugated to phosphatidylethanolamine, creating a more rapidly migrating band (LC3B-II) that can be detected in western blots. LC3B can serve as the most reliable marker for autophagy. Western blotting probed with the anti-LC3B antibody showed that the treatment of HS-Sch-2 or YST-1 cells with various concentrations of imatinib resulted in the upregulation of LC3B-II, which was detectable within 24 h of imatinib treatment of cells and was still present after 72 h (Figure 3A). Similar results were obtained using HS-PSS (data not shown). We also confirmed the formation of autophagosomes and autolysosomes in HS-Sch-2 cells treated with imatinib by electron microscopy (Figure 3B). We next examined whether there was induction of an autophagic flux in imatinib-treated HS-Sch-2 cells.

**Fig. 2** Imatinib induced cytotoxicity in MPNST cell lines, but apoptosis was partially involved

A: PDGFR-α and -β expression in MPNST cell lines was examined by RT-PCR according to the materials and methods. The sequences of primers are as follows: PDGFR-α (F) TGGGGTCTCAAGAGATGG (R) CAGCTTCACACATGGCCTCC, PDGFR-β (F) TGCTTACGATGTGTCACC, β-actin (F) GGCTTCAGCAAGAGATGG, (R) AGACGTGTTGTCCGTACAG..

PDGFR-α and -β expression in MPNST cell lines was examined by western blot according to the materials and methods.

B: YST-1, HS-PSS, or HS-Sch-2 cells were incubated with various concentrations of imatinib (STI) for 72 h, and cell viability was analyzed by the MTT assay. Results are presented as means ± S.E. Similar results were obtained from three independent experiments. Paired *t*-test analysis comparing untreated cells versus imatinib-treated cells demonstrated a paired value of $p < 0.05$ (*) or $p < 0.01$ (**).

C: YST-1 cells were incubated with imatinib for 48 h, and cell cycle analysis was carried out according to the Material and Methods. * denotes significant differences from untreated controls ($p < 0.05$) and n.s. denotes a difference that was not significant using paired *t*-test analysis.

D: YST-1 cells were incubated with various concentrations of imatinib for the indicated time, and cytochrome c, cleaved PARP, and cleaved caspase-3 were examined by western blotting.

E: YST-1 cells were incubated with zVAD-fmk (20 μM) and imatinib with or without zVAD-fmk for 72 h, and cell viability was then examined by the MTT assay. The control sample contained DMSO as a vehicle. Results are presented as means ± S.E. Similar results were obtained from three independent experiments.
iments employing the lysosome-specific protease inhibitors, pepstatin A and E64-d, we found an increase in LC3B-II levels, indicating that the inhibitors blocked LC3B-II lysosome-dependent degradation (Figure 3C). 3-MA, a class III phosphoinositide 3-kinase inhibitor, suppresses

Fig. 3 Imatinib induced autophagy in MPNST cell lines
A: HS-Sch-2 or YST-1 cells were incubated with imatinib (STI) at the indicated concentrations from 24 h to 72 h, as indicated. Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-LC3 or anti-β-actin antibodies.
B: Electron microscopic assessment of autophagy formation. Untreated HS-Sch-2 cells (a); HS-Sch-2 cells were treated with imatinib (20 μM) for 60 h. Autophagosomes (black arrowheads), autolysosomes (white arrowheads) (b); high magnification. Autophagosomes (white arrowheads), autolysosomes (black arrowheads) (c); high magnification. Double-membrane autophagosome sequestering a damaged mitochondrion. (d); N: nucleus, M: mitochondrion.
C: HS-Sch-2 cells were pre-treated for 60 min with pepstatin A (10 μM) and E64d (10 μM) and subsequently treated for 72 h with imatinib (30 μM) in the continuous presence or absence of inhibitors, as indicated. DMSO as a vehicle was added in the indicated samples because pepstatin A and E64d were dissolved in DMSO. Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-LC3 or anti-β-actin antibodies. Signals for the bands corresponding to LC3B-II and β-actin were quantitated using Image J software and data are expressed as ratios of LC3B-II to β-actin. Similar results were obtained in two other independent experiments as well as the one shown.
D: HS-Sch-2 cells were pre-treated for 60 min with 3-MA (1 mM) or bafilomycin A1 (BFA, 3 nM), and were subsequently treated for 72 h with imatinib (30 μM) in the continuous presence or absence of inhibitors, as indicated. DMSO as a vehicle was added in the indicated samples. Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-LC3 or anti-β-actin antibodies. Signals for the bands corresponding to LC3B-II and β-actin were quantitated using Image J software and data are expressed as ratios of LC3B-II to β-actin. Similar results were obtained in two other independent experiments as well as the one shown.
E: HS-Sch-2 cells were treated for 48 h with various concentrations of imatinib as indicated. Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-p62SQSTM1 or anti-β-actin antibodies. Similar results were obtained in two other independent experiments as well as the one shown.
F: YST-1 or HS-Sch-2 cells were treated with various concentrations of imatinib for 48 h and then stained with acridine orange for quantitation of the formation of acidic vesicular organelles (AVOs). An increase in AVO formation was accompanied by an increase in FL3 fluorescence, reflecting the induction of autophagy (right panel). AVOs were observed by fluorescence microscopy (left panel).
autophagy at an early stage before the conversion of LC3B-I to LC3B-II, and bafilomycin A1, a vacuolar-type H+ -ATPase inhibitor, inhibits the degradation of autolysosome content by inhibiting acidic lysosomal protease, resulting in the accumulation of LC3B-II-positive autophagosomes. In the presence of 3-MA, the signal of the LC3B-II band induced by imatinib treatment decreased, but it markedly increased in the presence of bafilomycin A1 in HS-Sch-2 cells (Figure 3D). The protein level of p62/SQSTM1, an LC3-interacting protein, which was degraded in autolysosomes23 and detected by western blotting, decreased in a dose-dependent manner following the treatment of HS-Sch-2 cells with imatinib for 48 h, indicating an autophagic flux (Figure 3E). Next, in fluorescence microscopy and flow cytometry by acridine orange staining,20 imatinib increased the strength of bright red fluorescence in MPNST cells, indicating the development of AVOs (Figure 3F). Altogether, these results indicated that the treatment of MPNST cells with imatinib induced autophagy.

Modulation of autophagy by pharmacological inhibitors or siRNA-mediated knockdown affected imatinib-induced cytotoxicity in MPNST cell lines

To determine whether the induction of autophagy by imatinib initiated MPNST cell death, we first examined the effect of inhibition of autophagy at an early stage using 3-MA or beclin-1-specific siRNA (siBeclin-1). In the presence of 1 mM 3-MA, we confirmed the inhibition of conversion of LC3B-I to LC3B-II after treatment with imatinib in HS-Sch-2 cells (Figure 3D). In the MTT assay, co-treatment with 3-MA and imatinib attenuated imatinib-mediated cytotoxicity significantly in MPNST cell lines (Figure 4A). Beclin-1, a member of the family of Atg proteins, plays a key role in the induction of autophagy.14 The expression of beclin-1 protein in siBeclin-1-transfected MPNST cells was lower than that in cells transfected with siRNA of the negative control (Figure 4B upper panels). Next, we examined how the depletion of beclin-1 affected imatinib-mediated cytotoxicity in the sensitive cell line, YST-1, using the MTT assay. The percent survivals of samples transfected with siBeclin-1 and those transfected with negative control siRNA followed by treatment with imatinib for 72 h were 95.4% and 62.9%, respectively, and were significantly different (p = 0.0007) (Figure 4B, lower graph), suggesting that siRNA knockdown of beclin-1 resulted in attenuation of the imatinib-induced inhibitory reaction. Similar results were obtained using the HS-Sch-2 cell line. These findings suggest that the induction of autophagy was associated with imatinib-mediated cytotoxicity in MPNST cells.

There is considerable debate regarding the role of autophagy in cell death. Recently, autophagy, while recognized as type II programmed cell death, has been shown to play a role in prosurvival or protective reactions in certain circumstances.24,25 Next, we assumed that the steps of generating autophagosomes and degrading or recycling damaged organelles in autolysosomes during autophagy may exert protective effects against imatinib-mediated cytotoxicity. Since LC3B plays an important role in the formation of autophagosomes, we examined cell viability following the knockdown of LC3B protein in the resistant cell line, HS-Sch-2. Transient transfection of siRNA against LC3B resulted in a decrease in the amount of LC3B protein (Figure 4C, upper panel). In this setting, imatinib-induced cytotoxicity in HS-Sch-2 cells was significantly augmented (Figure 4C, lower graph). Next, we examined the effect of bafilomycin A1 on imatinib-mediated autophagy. In the presence of 3 nM bafilomycin A1 in HS-Sch-2, we confirmed that the imatinib-induced increase in LC3B-II was augmented by bafilomycin A1, consistent with an inhibitory effect of bafilomycin A1 on the formation of autolysosomes where LC3B-II should be degraded. In the MTT assay, imatinib-induced cytotoxicity was significantly augmented in the presence of bafilomycin A1 in MPNST cell lines (Figure 4D).

Altogether, these findings suggest that autophagy played an important role in the exertion of imatinib-mediated cytotoxicity in MPNST cells because blocking the initiation of autophagy using 3-MA or siRNA targeting beclin-1 attenuated imatinib-mediated cytotoxicity. In contrast, the knockdown of LC3B and bafilomycin A1 inhibited the protective part of the autophagic machinery, which caused sensitization of MPNST cells to imatinib.

Effect of bafilomycin A1 on imatinib-mediated cytotoxicity in YST-1

We showed an augmentation in imatinib-mediated cytotoxicity upon co-treatment of MPNST cells with imatinib and bafilomycin A1. Western blotting probed with anti-cytochrome c
Fig. 4  Blocking of autophagy using pharmacological inhibitors or siRNA-mediated knockdown of beclin-1 or LC3B affected imatinib-induced cytotoxicity in MPNST cells

A : MPNST cells were pre-treated with 3-methyladenine (3-MA, 1 mM) for 60 min, and subsequently treated for 72 h with imatinib (STI 10 μM for YST-1, 20 μM for HS-PSS, 300 μM for HS-Sch-2) in the continuous presence or absence of inhibitors, as indicated. Cell viability was measured by the MTT assay. The control sample contained PBS as a vehicle. Results are presented as means ± S.E. in triplicate. Similar results were obtained from three independent experiments. * denotes significant differences from untreated controls (p<0.05) ; ** (p<0.01) using paired t-test analysis.

B, C : Cells were transfected with the negative control, beclin-1, or LC3B siRNA as indicated. After 24 h of incubation, beclin-1 siRNA-transfected cells were treated with imatinib (20 μM) for 24 h, and then total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-beclin-1, anti-LC3B, or anti-β-actin antibodies. We defined the ratio of the viability of cells treated with and without imatinib as the percent survival and compared it between samples transfected with siRNA targeting Beclin-1 (siBeclin-1) or siRNA targeting LC3B (siLC3B) and those transfected with control siRNA (siCtrl). Results are presented as means ± S.E. from three independent experiments. Paired t-test analysis comparing imatinib-treated control siRNA-transfected cells versus imatinib-treated beclin-1 or LC3B siRNA-transfected cells demonstrated a paired value of p<0.05 (*) or p<0.01 (**) .

D : MPNST cells were pre-treated with bafilomycin A1 (3 nM) for 60 min and subsequently treated for 72 h with imatinib (20 μM) in the continuous presence or absence of inhibitors, as indicated. Cell viability was measured by the MTT assay. The control sample contained DMSO as a vehicle. Results are presented as means ± S.E. Similar results were obtained from three independent experiments. Paired t-test analysis comparing cells treated with imatinib and those with imatinib in addition to BFA demonstrated a paired value of p<0.01 (**) and p<0.05 (*) .
Fig. 5 Effect of bafilomycin A1 on the imatinib-mediated inhibitory effect
YST-1 cells were pre-treated with bafilomycin A1 (BFA, 3 nM) for 60 min and subsequently treated for 48 h with imatinib (20 μM) in the continuous presence or absence of inhibitors, as indicated. Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-cytochrome c, anti-PARP, or anti-β-actin antibodies. Similar results were obtained in two other independent experiments as well as the one shown.

or PARP antibody revealed that the amount of cytochrome c or cleaved form of PARP (89 kDa) was higher in samples treated with imatinib and bafilomycin A1 than that in samples treated with imatinib alone in YST-1 cells (Figure 5). These findings suggest that inhibition of autophagy with the treatment of bafilomycin A1 activated the apoptosis pathway, which resulted in the enhancement of imatinib-mediated cytotoxicity in YST-1. In HS-Sch-2, this activation of apoptosis when co-treated with imatinib and bafilomycin A1 was not observed (data not shown).

Discussion

The present study is the first to show that the imatinib-mediated autophagic response is associated with cytotoxicity in MPNST cells. There is emerging evidence that autophagy plays critical roles in the generation of anti-tumor responses and mediates caspase-independent tumor cell death.16 There is debate regarding the role of autophagy in cell death. Autophagy may have a protective effect in malignant cells or exhibit the opposite effects; this is dependent on the malignant cells and/or initiated treatments (reviewed in25). For example, autophagy promoted the resistance of breast cancer cells to anti-HER2 monoclonal antibody (trastuzumab).24 Inhibition of autophagy appears to enhance the effects of BCR-ABL kinase inhibitors on Ph1(+) leukemic cells.28 In contrast, recent studies have established that autophagy mediates the cell death of acute lymphoblastic leukemia by dexamethasone,27 chondrosarcoma cell lines by histone deacetylase inhibitors,28 and acute myelogenous leukemia by arsenic trioxide.29 In the present study, the treatment of MPNST cell lines with 3-MA or knockdown of beclin-1 using siRNA attenuated imatinib-induced cytotoxicity, suggesting that the initiation of autophagy is indispensable for imatinib-induced cytotoxicity. We also showed the possibility that blocking of the degradation process of damaged organelles using the knockdown of LC3B or bafilomycin A1 can promote the augmented cytotoxicity of imatinib against MPNST cells. Therefore, the steps of generating autophagosomes and autolysosomes during the late stage of autophagy may be associated with the establishment of drug resistance. Similar effects were observed when MPNST cells were treated with histone deacetylase inhibitors.30

In MPNST cell lines, IC50 values evaluated by the MTT assay ranged from 11.7 to 29.3 μM and were higher than those in CML cell lines.31 However, similar studies also used more than 10 μM imatinib to induce an inhibitory effect in MPNST cells,7,8 glioma cells,14 and neuroectodermal tumor cells,23 which implies that the inhibitory effect of imatinib does not depend on the inhibition of tyrosine kinases such as PDGFR or c-Kit because lower concentrations of imatinib (0.1-1.0 μM) are sufficient to inhibit the phosphorylation of PDGFR or c-Kit.23 However, in vitro experiments with uncertain factors in the FCS medium may influence the effects of imatinib; for example, alpha 1 acid glycoprotein, one of the plasma proteins, is known to bind to imatinib with high affinity and inhibit imatinib activity in vitro and in vivo.24 Therefore, we reduced the concentration of FCS to 1% when measuring cell viability after exposure to imatinib. Sensitivity to imatinib varied among the three MPNST cell lines used in this study, which may be dependent on the expression of imatinib-sensitive receptors. YST-1, the most sensitive cell line, expressed PDGFR-α, -β, and c-kit (data not shown), and the next most sensitive cell line, HS-PSS, expressed both PDGFR-α and -β, but not c-kit. The HS-Sch-2 cell line that expressed PDGFR-α and c-kit was less sensitive.

Bond et al. performed a pharmacokinetic study in pediatric patients12 and showed that the
Autophagy modulation in MPNST

through plasma imatinib concentration obtained on day 8 with the administration of 440 mg/m²/day was 6.1 μM (median, range 0.5-21.4 μM), suggesting that the inhibitory effect of imatinib may be clinically demonstrated in patients with MPNST if some type of enhancement is present. Treatment with bafilomycin A1 resulted in the accumulation of LC3B-II and augmentation of imatinib-induced cytotoxicity. It has been reported that the inhibition of imatinib-induced autophagy by bafilomycin A1 enhanced apoptosis associated with mitochondrial permeabilization and the release of cathepsin D from mitochondria, which is consistent with the data in this study from YST-1 cells. In YST-1 cells, it subsequently activated the apoptosis pathway with the enhancement of imatinib-mediated cytotoxicity because there is crosstalk between the induction of apoptosis and autophagy. In contrast, in HS-Sch-2 cells, in which the late stage of autophagy is impeded, non-apoptotic cell death was enhanced without the activation of apoptosis because HS-Sch-2 cells may have a p53 mutation.

As with bafilomycin A1, chloroquine (CQ), a well-known compound clinically used for the treatment of malaria, prevented the fusion of lysosomes to autophagosomes, causing an accumulation of ineffective autophagic vesicles that promoted cell death in cells dependent on autophagy for survival. CQ represents a promising drug for co-treatment with chemotherapeutic agents, and several clinical trials are underway.

In conclusion, imatinib may be an appropriate candidate for novel therapeutic strategies because MPNST expresses imatinib-sensitive tyrosine kinase receptors. We demonstrated that imatinib-mediated autophagy in MPNST cells, which was associated with the cytotoxicity of imatinib. However, the subsequent autophagic machinery, the formation of autophagosomes and autolysosomes in which the degradation process occurs, may play an important role in imatinib-resistance. We have provided evidence that impeding this process by genetic and pharmacological approaches sensitized MPNST cells to imatinib, resulting in the activation of apoptosis or non-apoptotic cell death pathways (Figure 6). Thus, appropriate autophagy modulation is very important for cancer treatment because the induction of autophagy has a double-edged sword function. In order for us to develop successful autophagy-modulating strategies against cancer, we need to better understand how the roles of autophagy differ depending on the tumor cell type and genetic factors, and we need to determine how specific pathways of autophagy are activated or inhibited by various anti-cancer therapies. Further research will be required for the development of new combinatorial therapeutic strategies that will hopefully sensitize tumor cells to anti-cancer therapy.

Acknowledgments

We thank Shinji Kurashimo (Kinki University Life Science Research Institution) for his technical support during flow cytometry analysis, Yoshitaka Horiuchi (Kinki University Life Science Research Institution) for his technical support for imaging by transmission electron microscopy, and Mikiko Aoki (Department of Pathology, Fukuoka University School of Medicine) for analyzing the expression of PDGFR-β in the tumor samples.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest are declared.

References


Fig. 6 Appropriate inhibition of autophagy mediated by imatinib enhanced cytotoxicity in MPNST
Autophagy modulation in MPNST