Cancer Chemother Pharmacol (2001) 48: 435–445
DOI 10.1007/s002800100373

ORIGINAL ARTICLE

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Stereospecific antitumor activity of radicicol oxime derivatives

Received: 16 February 2001 / Accepted: 26 July 2001 / Published online: 3 October 2001

Abstract *Purpose*: Radicicol is a novel hsp90 antagonist, distinct from the chemically unrelated benzoquinone ansamycin compounds, geldanamycin and herbimycin. Both geldanamycin and radicicol bind in the aminoterminal nucleotide-binding pocket of hsp90, destabilizing the hsp90 client proteins, many of which are essential for tumor cell growth. We describe here antitumor activity of a novel oxime derivative of radicicol, KF58333. We also investigated the mechanism of antitumor activity of KF58333 in comparison with its oxime isomer KF58332. *Methods*: Antiproliferative activities were determined in a panel of breast cancer cell lines in vitro. We also examined inhibition of hsp90 function and apoptosis induction in erbB2-overexpressing human breast carcinoma KPL-4 cells in vitro. Direct binding activity to hsp90 was assessed by hsp90-binding assays using geldanamycin or radicicol beads. In animal studies, we investigated plasma concentrations of these compounds after i.v. injection in BALB/c mice and antitumor activity against KPL-4 cells transplanted into nude mice. Inhibition of hsp90 function and induction of apoptosis in vivo were investigated using tumor specimens from drug-treated animals. *Results*: KF58333 showed potent antiproliferative activity against all breast cancer cell lines tested in vitro, and was more potent than its stereoisomer KF58332. These results are consistent with the ability of KF58333 to deplete hsp90 client proteins and the induction of apoptosis in KPL-4 cells in vitro. Interestingly, KF58333, but not KF58332, showed significant in vivo antitumor activity accompanied by induction of apoptosis in KPL-4 human breast cancer xenografts. Although the plasma concentrations of these compounds were equivalent, KF58333, but not KF58332, depleted hsp90 client proteins such as erbB2, raf-1 and Akt in the tumor specimen recovered from nude mice. *Conclusions*: These results suggest that inhibition of hsp90 function, which causes depletion of hsp90 client proteins in tumor, contributes to the antitumor activity of KF58333, and that the stereochemistry of the oxime moiety is important for the biological activity of radicicol oxime derivatives.

Keywords Radicicol · erbB2 · Hsp90 · Apoptosis · Breast cancer

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Introduction

The 90-kDa heat shock protein family, including heat shock protein 90 (hsp90) and glucose-regulated protein 94 (grp94), are thought to play important roles as molecular chaperones that facilitate folding, renaturation and proper localization of a variety of proteins. Hsp90 binds to an array of specific proteins that require this interaction to execute their function (reviewed in references 2, 4, 16, 22, 26 and 34). These proteins include: serine/threonine kinases such as raf-1 [27] and cyclin-dependent kinase 4 (cdk4); non-receptor tyrosine kinases such as bcr-abl [33], v-src [37, 38] and src family kinases; ligand-dependent nuclear transcription factors such as steroid hormone receptors and aryl hydrocarbon receptors [23]; ligand-independent nuclear transcription

factors such as mutant p53 [19] and hypoxia-inducible factor 1α [17]; and active telomerase holoenzyme [9]. The stability and function of the receptor tyrosine kinase erbB2 and epidermal growth factor receptor (EGFR/erbB1) depend on hsp90 and its homologue grp94 [3, 18]. Geldanamycin and herbimycin A, both benzoquinone ansamycin antibiotics, bind hsp90 at its amino-terminal nucleotide-binding site, thus altering the assembly of the hsp90-containing multimolecular complex which includes the hsp90-associated signaling molecules (hsp90 client proteins) listed above, co-chaperones such as p23, p50, and Hop, and other family chaperones such as hsp70. Subsequently, these hsp90 client proteins become unstable and are rapidly degraded [20].

Recently, we have revealed using three different approaches that hsp90 family chaperone proteins are the major intracellular targets of radicicol. First, radicicol efficiently competes with solid-phase geldanamycin for binding to hsp90 [28]. Second, biotinylated radicicol is able to identify hsp90 in a pseudo-Western blot assay [32]. Finally, both biotinylated radicicol bound to streptavidin Sepharose and radicicol linked to Sepharose beads are able to capture the hsp90 family of proteins in solution [29]. It has been also revealed that, like geldanamycin, radicicol can directly bind to the aminoterminal ATP/ADP-binding pocket domain of hsp90 [24]. Using cultured rodent and human transformed cell lines, we have shown that radicicol can deplete hsp90 client proteins from these cells in vitro, suggesting that the inhibition of hsp90 function might be important for its anti-transformation effects [35].

Radicicol lacks anticancer activity in animal models because of its unstable chemical nature. Therefore, we generated a series of novel oxime derivatives of radicicol such as KF25706 (radicicol 6-oxime), which are more stable than radicicol in vivo. KF25706 exerts its anticancer activity in vitro as well as in vivo through the inhibition of hsp90 function [36]. Further structure-activity relationship studies of radicicol oxime derivatives furnished more potent analogues such as KF55823 (6-*O*-[2-(2-pyrrolidonyl)-ethyl] radicicol oxime), which is more active than KF25706 with regard to growth inhibition and depletion of hsp90 client proteins such as v-src and/or raf-1 in target cells. At first, radicicol oxime derivatives were synthesized as mixtures of E and Z oxime isomers, and then separated into single isomers by HPLC.

In this study, we compared the biological activities of the E and Z isomers of the radicicol derivative KF55823. Interestingly, the E isomer of radicicol oxime (KF58333) showed more potent antiproliferative activity as well as the ability to deplete hsp90 client proteins in vitro in target tumor cells. In addition, KF58333 was even more active than KF58332 in animal models. These studies, using novel radicicol oxime isomers, reinforce our original observation that the inhibition of the function of hsp90 contributes to their antitumor activity, and underscores the importance of the hsp90 family of molecular chaperones as key targets for cancer chemotherapy.

Materials and methods

Cell lines, drugs, and antibodies

SK-BR-3, BT-474, T-47D, MDA-MB-453, and MCF7 cells were purchased from the American Type Culture Collection through Dainippon Pharmaceutical Company (Osaka, Japan). KPL-1 and KPL-4 cells were obtained from Dr. Junichi Kurebayashi [13, 14]. SRC-3T3 cells were maintained as described previously [31]. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Xenograft cell lines were established by inoculation of the cultured cells into the flank of adult BALB/c *nu*/*nu* mice (Nippon Clea Company, Tokyo, Japan). These tumors were passaged in vivo using a trocar.

Radicicol was produced by fermentation, and its oxime derivatives (KF58333 and KF58332) were chemically synthesized an\d purified as oxime isomers.

Anti-raf-1 (C-12) and anti-cdk4 (C-22) rabbit-polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Phospho-specific Akt (Ser 473) antibody, and anti-Akt antibody were purchased from New England Biolabs (Beverly, Mass.). We also used anti-c-neu (erbB2) monoclonal antibody (clone 3B5; Oncogene Research Products, Cambridge, Mass.), anti-erk2 monoclonal antibody (clone 1B3B9; Upstate Biotechnology, Lake Placid, N.Y.), anti-hsp90 antibody (StressGen Biotechnologies Corporation, Victoria, British Columbia, Canada), and anti-grp94 antibody (a kind gift from Dr. Linda Hendershot, St. Jude Children's Research Hospital, Memphis, Tenn.).

In vitro antiproliferative activity

The cells were precultured in appropriate medium for 24 h in 96-well microwell plates (Nunc, Roskilde, Denmark). Drugs were added to the plate in serial threefold dilutions ($n = 3$), and the plates were incubated for another 72 h. After 72 h of treatment, cell viability was determined using a microculture tetrazolium (MTT) assay (Sigma) as described previously [36]. The concentration of drug required for 50% inhibition of cell growth (IC_{50}) was determined using the Softmax program (Wako Pure Chemical Industries, Osaka, Japan).

Cell lysis and Western blotting

Cells plated in 24-well tissue culture plates (Nunc) were used for each sample. After preculture for 8 h, drugs were added to each well without adding any fresh medium, and the cells were cultured for 40 h. After drug treatment, the cells were washed once with phosphate-buffered saline without calcium [PBS(–)] (ICN Biochemicals, Aurora, Ohio), and were lysed by the addition of 20 μl/well of ice-cold lysis buffer (50 m*M* Hepes-NaOH, pH 7.4, 250 m*M* NaCl, 1 m*M* EDTA, 1% Nonidet P-40, 1 m*M* dithiothreitol, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, 2 m*M* Na_3VO_4, 1 m*M* NaF, 10 m*M* β-glycerophosphate). The cells were lysed for 10 min on ice and clarified by centrifugation. Whole-cell lysate was heated in Laemmli gel loading buffer for 5 min at 95°C and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

For ex vivo analysis, tumor specimens or spleen organs were minced with a razor, and strained with a Falcon cell strainer (Becton Dickinson, Lincoln Park, N.J.). The cells were lysed and SDS-PAGE samples were prepared in the same way as samples from in vitro cultured cells. After SDS-PAGE, the protein was transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Tokyo, Japan) and immunoblotted with appropriate primary antibodies. For detection, the blots were incubated with

the appropriate secondary antibodies conjugated with horseradish peroxidase (Amersham Life Sciences, Little Chalfont, UK), and developed using an enhanced chemiluminescence detection system (Amersham), according to the instructions of the manufacturer.

Hsp90 binding assay

For the competition assay for hsp90 binding with geldanamycin, geldanamycin affinity beads were prepared as described previously [36]. SK-BR-3 cells were lysed in TNES buffer (50 m*M* Tris-HCl, pH 7.5, 1% Nonidet P-40, 2 m*M* EDTA, 100 m*M* NaCl) containing 1 m*M* sodium orthovanadate, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 1 m*M* PMSF. Total protein (100 μg) was incubated with dimethylsulfoxide (control), KF58333 or KF58332. After 30 min of end-over-end mixing, resins were washed three times in TNES buffer and boiled in Laemmli gel loading buffer. Affinity-purified proteins were separated on 8% SDS-polyacrylamide gels and visualized by silver staining. Bands were displayed and quantified using Adobe Photoshop and NIH image software.

For the radicicol beads competition assay, biotinylated radicicol-streptavidin agarose complex and hypotonic cell extract of SRC-3T3 cells were prepared as previously described [29]. Radicicol beads were used as an affinity matrix to capture hsp90 family chaperone from cell lysates. Briefly, 1.5 ml cell lysate was incubated with radicicol beads. For the competition assay, the cell lysates were preincubated with various concentrations of KF58333 and KF58332 for 2 h at 4°C prior to incubation with radicicol beads for 2 h at 4°C. After a total of 4 h treatment, proteins bound to the affinity matrix were collected by brief centrifugation and the beads were washed three times with PBS containing 0.2% Tween, resuspended in 50 μl of SDS sample buffer, and analyzed by Western blotting as described above using anti hsp90 antibody or grp94 antibody.

Assessment of apoptosis

For the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay, cells were processed according to the manufacturer's instructions using an ApopTag Direct Kit (Oncor, Gaithersburg, Md.). Briefly, cells were fixed in 1% formaldehyde solution for 15 min on ice, washed in PBS, suspended in 70% ethanol, and stored at –20°C. After washing in PBS, the cells were resuspended first in equilibration buffer for 15 min at room temperature, then terminal deoxynucleotidyl transferase (TdT) reaction buffer (50 μl) for 30 min at 37°C. After stopping the TdT reaction, the cells were incubated in PBS (1 ml) containing 10 μg/ml propidium iodide (PI) and 10 μg/ml RNase A for 15 min at room temperature in the dark. Bivariate analysis of apoptosis (green fluorescence of TdT-FITC staining) and DNA content (green fluorescence of PI staining) was performed flow cytometrically. Using the control (untreated) sample to define basal levels of green fluorescence, the R1 region was set. Cells with fluorescence within the R1 region were considered apoptotic. Data were analyzed using the Multi2D program (Phoenix Flow Systems).

For the analysis of apoptosis induction in tumor fragments, we stamped tumor sections on slide glass, and fixed the tumor cells on the slides with 1% paraformaldehyde solution for 15 min at room temperature, then with 70% ethanol for 24 h at –20°C. The slides were rinsed in PBS, then the cells were stained using an ApopTag Direct Kit, as described above. Briefly, the slides were incubated with equilibration buffer for 5 min at room temperature, then TdT reaction buffer for 1 h at 37°C. After stopping the TdT reaction, they were incubated in PBS containing 5 μg/ml PI and 1 mg/ml RNase A for 15 min at room temperature in the dark. Analyses were performed by laser scanning cytometry (LSC101; Olympus, Tokyo). We measured both DNA content and DNA strand breaks for each cell. We also analyzed the same slides by confocal laser scanning microscopy (CLSM; Leica, Hamburg, Germany), and investigated nuclear morphology, and TdT-FITC signal localization in each cell.

Evaluation of antitumor activity

For the evaluation of antitumor activity, tumor volumes were calculated from measurements of their length (mm) and width (mm) using the following formula, according to the method of the National Cancer Institute [7]:

$$Tumor\,volume\left(mm^3\right) = \left[Length \times width^2\right]/2$$

Drug efficacy was expressed as the ratio of the mean V/V_0 value against that of the control group (T/C), where V is the tumor volume on the day of evaluation and V_0 is the tumor volume on the day of initial treatment with the drug. KPL-4 cells (27 mm^3 fragments) were transplanted s.c. into the flanks of nude mice by trocar on day –31. Compounds were administered daily by intravenous (i.v.) injections for 5 days from day 0 to day 4.

Measurement of mouse plasma concentration

For the determination of plasma concentrations in mice, drugs were administered to BALB/c mice (Nippon Clea Company), and blood samples were collected from a femoral vein. Plasma was obtained by centrifugation (3300 *g*) for 10 min. Plasma samples were diluted by control mouse plasma and deproteinized by adding twice the volume of acetonitrile. The mixture was vigorously vortexed for 1 min and then stood on ice for 10 min. After centrifugation of the mixture (10,000 *g* for 10 min at 4°C), an aliquot (250 μl) of the supernatant was dried under nitrogen, and dissolved in the mobile phase buffer (10 m*M* pH 7.3 phosphate buffer/methanol 6:4). After centrifugation of the solution (10,000 *g* for 10 min at 4°C), an aliquot (250 μl) of the supernatant was analyzed by HPLC. The concentration of KF58333 or KF58332 was determined with standard curves prepared using blank plasma mixed with the standard compounds.

Results

Growth inhibition of human breast cancer cell lines

The chemical structures of radicicol and KF55823 (6-*O*-[2-(2-pyrrolidonyl)-ethyl] radicicol oxime), which is an E and Z mixture of the oxime isomer, are shown in Fig. 1A, B, respectively. KF58333 and KF58332 are oxime isomers (E and Z forms, respectively) of KF55823.

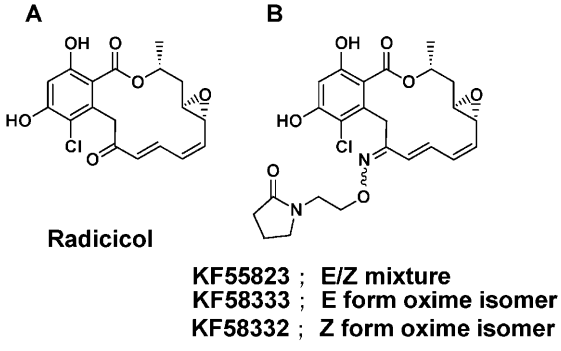

Fig. 1A, B Structures of radicicol (**A**) and its oxime derivative, KF55823 (6-*O*-[2-(2-pyrrolidonyl)ethyl] radicicol oxime) (**B**). KF58333 and KF58332 are oxime isomers of KF55823 (E form and Z forms, respectively)

To determine the differences in the growth-inhibitory activities of the E and Z isomers of KF55823, we used a panel of human breast cancer cell lines that express different levels of erbB2 oncoproteins [1, 5, 8, 11, 12, 13, 14, 15, 30]. As shown in Table 1, KF55823 and its oxime isomers (KF58333 and KF58332) showed potent antiproliferative activities (<330 n*M*) against all of these cell lines. All the compounds listed were more potent than the parent compound, radicicol. Importantly, KF58333 (E isomer) showed the most potent activities (<100 n*M*) against all of the cell lines tested, and was 2–13-fold more potent than the Z isomer KF58332 (see Z/E ratio in Table 1).

Interestingly, both compounds showed a tendency to inhibit the growth of erbB2-overexpressing breast cancer cell lines more effectively than those with low erbB2 expression levels. In particular, SK-BR-3 and KPL-4 cells, which over-express erbB2 but do not express estrogen receptor, were highly sensitive to KF58333 (IC_{50} values 8.72 and 9.97 n*M*, respectively). Differences in the expression levels of erbB2 are commonly found in numerous solid tumors, and overexpression of erbB2 has been associated with the poor prognosis of breast cancers, in particular. To further examine the effect of radicicol oxime derivatives on erbB2-overexpressing tumors, KPL-4 erbB2-overexpressing human breast cancer cells were used in the following studies.

Depletion of hsp90 client proteins in the KPL-4 cell line

We examined the effects of the radicicol oxime isomers, KF58333 (E) and KF58332 (Z), on the function of hsp90 family chaperones in KPL-4 cells using Western blot analysis. As shown in Fig. 2, KF58333 and KF58332 depleted erbB2 oncoprotein levels in a concentration-dependent manner. KF58333 was at least threefold more potent than KF58332 in this regard. This difference was consistent with the difference in their potencies in antiproliferative activity. KF58333 and KF58332 also depleted other well-known hsp90 client proteins such as raf-1 and cdk4 from the cells in a concentration-dependent manner, with KF58333 being more potent. However, they did not affect the expression levels of erk2 protein, which does not depend on the function of hsp90 family chaperones for its stability (Fig. 2). We also tested the effect of the two compounds on the expression levels of Akt kinase (protein kinase B) and its phosphorylated (activated) form because geldanamycin is known to deplete this kinase, which may be important for cell survival [10, 39]. As shown in Fig. 2, Akt protein, as well as its phosphorylated (activated) form, were downregulated after treatment with KF58333 or KF58332. In all cases, KF58333 was at least threefold more potent than KF58332.

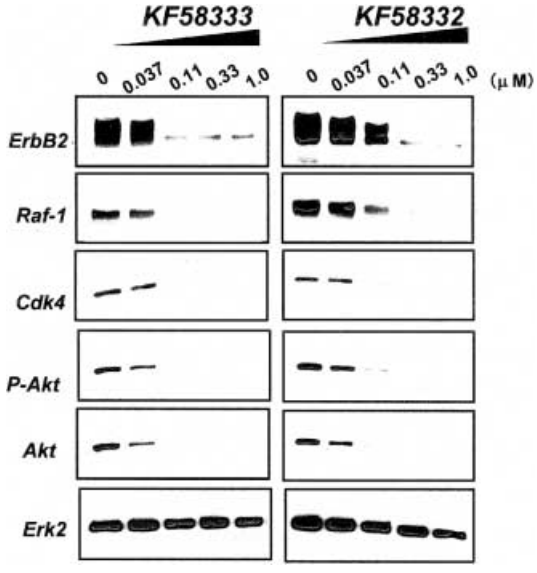

Fig. 2 Effects of KF58333 and KF58332 on the protein levels of hsp90 client proteins in vitro. Human breast carcinoma KPL-4 cells were treated with increasing concentrations of KF58333 or KF58332 for 40 h. The indicated hsp90 client proteins (erbB2, raf-1, cdk-4, and Akt) and erk2 (internal control) were analyzed by Western blotting using specific antibodies

Table 1 Antiproliferative activity of KF58333 (E isomer) and KF58332 (Z isomer) against human breast tumor cell lines in vitro. Each cell line was treated with the indicated compound for 72 h. Antiproliferative activity was determined by a microculture tetrazolium (MTT) assay as described in Materials and methods. Values are the means from two or three separate experiments

Cell line	Estrogen receptor status	IC_{50} (n*M*)				Ratio (Z/E)	Reference
		Radicicol	KF55823 (E/Z mixture)	KF58333 (E)	KF58332 (Z)		
ErbB2 low							
MCF7	+	59.7	35.8	12.5	36.3	2.9	1, 30
MDA-MB-231	–	6150	171	26.5	331	12.5	5, 15, 12
KPL-1	+	5170	191	72.7	166	2.3	13, 15
T47D	+	1450	109	30.2	129	4.3	15, 11
ErbB2 high							
KPL-4	–	2990	38.9	8.72	57.8	6.6	14, 15, 12
BT-474	+	90.2	83.3	21.7	89.5	4.1	30, 5, 8
SK-BR-3	–	85.4	26.5	9.97	25.9	2.6	30, 15

These results suggest that novel radicicol oxime compounds could inhibit the growth of erbB2-overexpressing KPL-4 cells through the depletion of oncoproteins, such as erbB2 and raf-1, cell cycle regulatory proteins such as cdk4, and cell survival signal molecules such as Akt. Similar results were obtained using another erbB2-overexpressing cell line, SK-BR-3 (data not shown). These results suggest that the difference in the growth-inhibitory activities of the two radicicol oxime isomers was mediated, at least in a part, through the extent of inhibition of hsp90 function.

Induction of apoptosis of KPL-4 cells

Since radicicol oxime derivatives at concentrations around100 n*M* depleted the Akt serine/threonine kinase which is important for cell survival, we tested both compounds to determine whether they would induce apoptosis in KPL-4 cells. To this end, KPL-4 cells were incubated with various concentrations of KF58333 or KF58332 for 40 h and analyzed for the induction of apoptosis by the TdT/PI double-staining method, using flow cytometry. As shown in Fig. 3, both KF58333 and KF58332 upregulated TUNEL-positive apoptotic cells and cells with a sub-G_1 DNA content (not related to any specific phase of the cell cycle) in a concentration-dependent manner. The percentages of TUNEL-positive cells (contained in the R1 regions in Fig. 3) after treatment with 1 μM KF58333 and KF58332 were 65.5% and 43.4%, respectively, suggesting that KF58333 was a more potent inducer of apoptosis than KF58332. This finding was consistent with the growth-inhibitory effects and the depletion of hsp90 client proteins in these cells.

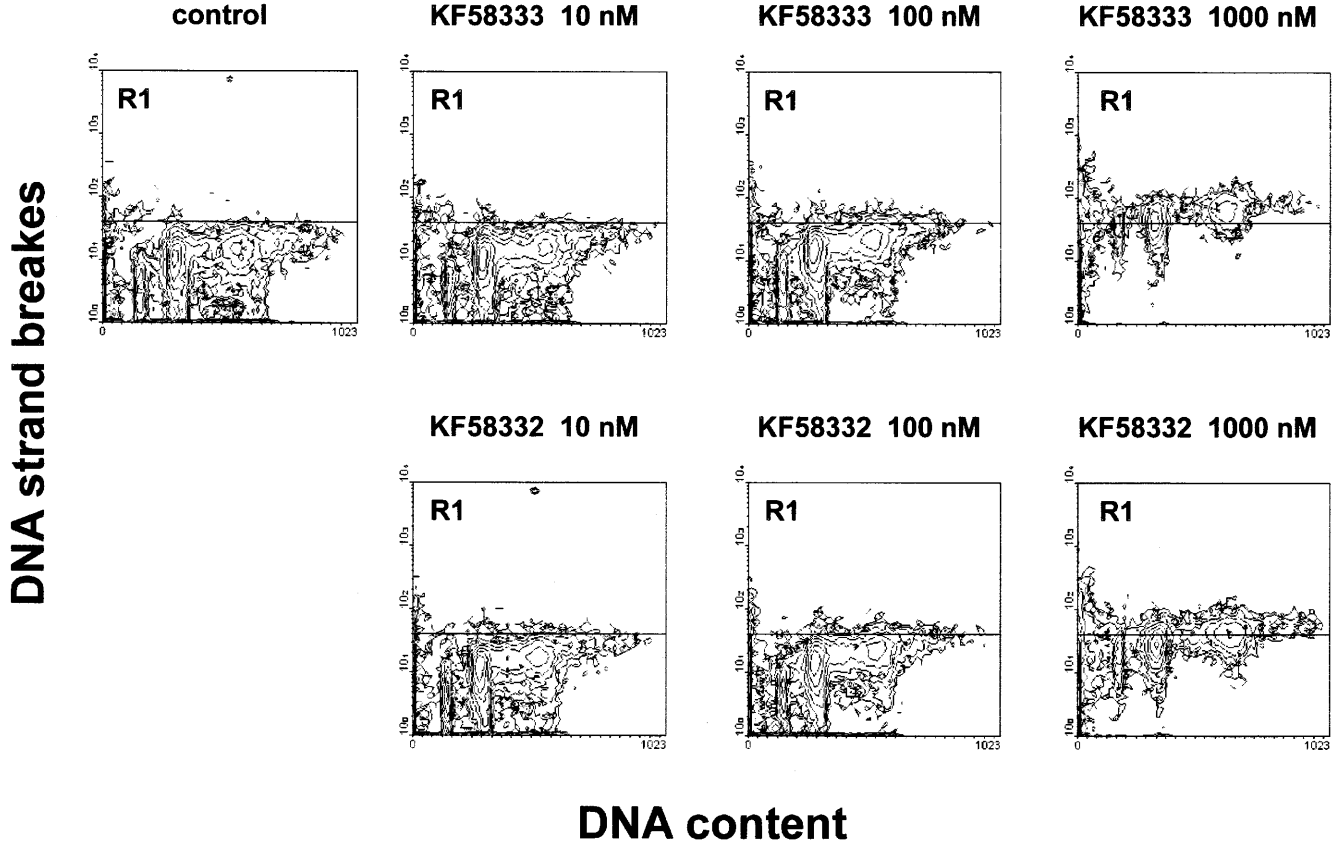

Fig. 3 Induction of apoptosis by KF58333 and KF58332. Cells were harvested after 40 h of treatment with 0, 10, 100, 1000 n*M* of the compounds and analyzed by flow cytometry, using the TUNEL technique as described in Materials and methods. Apoptotic cells were identified as those contained in the R1 region

Hsp90 binding affinities using geldanamycin and radicicol beads

The results described above suggest that each oxime isomer might have a different affinity for hsp90 family chaperone proteins, which could result in the different potencies in the depletion of hsp90 client proteins in the cells. To investigate this possibility, we examined the affinity of each compound for hsp90 using direct binding beads assays. As shown in Fig. 4A, B, both KF58333 and KF58332 blocked the binding of hsp90 to geldanamycin affinity beads with similar kinetics, with KF58333 showing about a twofold higher affinity than KF58332. We also tested the binding affinity to hsp90 using radicicol beads. However, KF58333 and KF58332

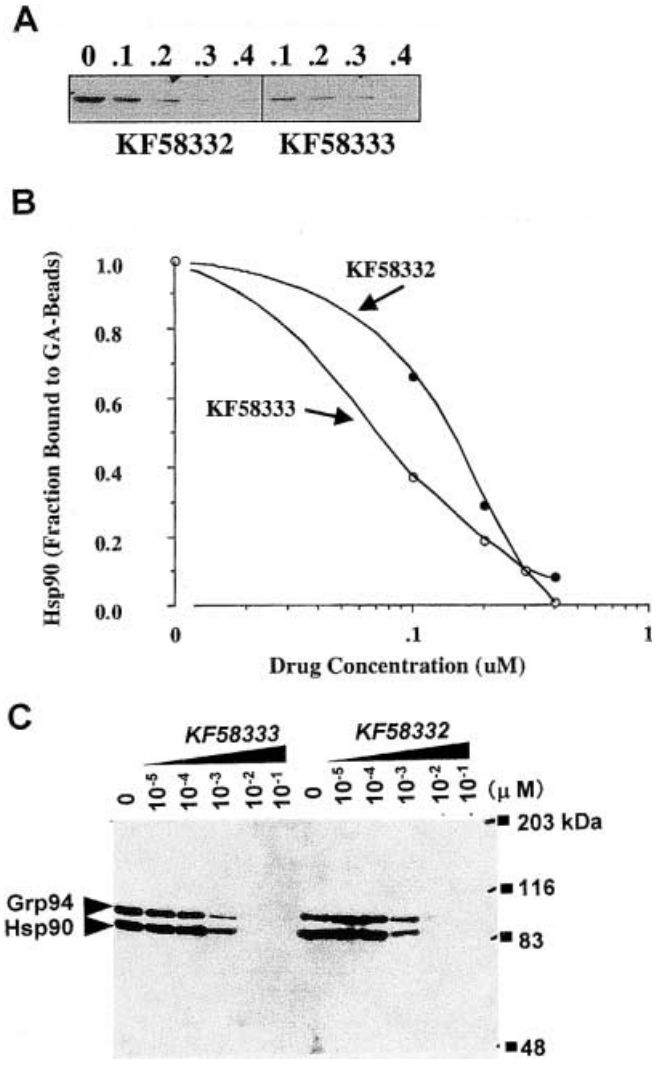

Fig. 4A–C Comparison of binding affinity of KF58333 and KF58332 to the hsp90 family chaperone. **A** Lysates from SK-BR-3 cells were incubated with geldanamycin affinity beads. Soluble KF58333 or KF58332 in increasing concentrations competed with hsp90 for binding to immobilized geldanamycin. Affinity-purified proteins were separated by SDS-PAGE, and hsp90 was detected by silver staining. **B** Band intensities were quantified and plotted against concentrations of KF58333 and KF58332. **C** The competition assay with radicicol beads was examined using SRC-3T3 cell lysates. Affinity-purified proteins were analyzed by sequential Western blotting with anti-hsp90 and anti-grp94 antibodies

blocked the binding of hsp90 and grp94 to radicicol affinity beads at very similar concentrations (Fig. 4C).

Therefore, the difference between the isomers cannot be fully explained by the difference in direct binding to hsp90 family chaperone proteins.

In vivo antitumor activity in the KPL-4 xenograft model

We next compared the antitumor activity of KF58333 and KF58332 in vivo using xenografted KPL-4 cells in nude mice. As shown in Fig. 5 and Table 2, KF58333

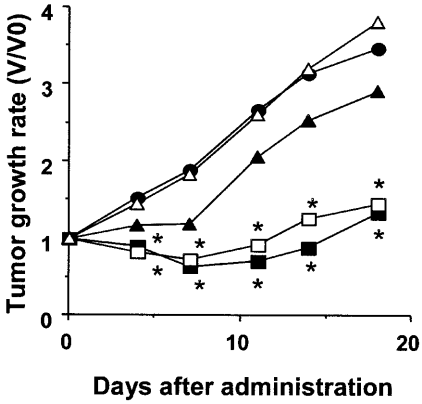

Fig. 5 Tumor growth inhibitory effect of KF58333 and KF58332 against KPL-4 xenografts. Human breast carcinoma KPL-4 cells (27 mm^3) were inoculated s.c. into nude mice ($n=5$) on day –31. The initial tumor volume on day 0 (V_0) was 155.1 ± 46.1 mm^3. Drugs were administered as five consecutive i.v. injections from day 0 to day 4. Tumor size was measured on the indicated days, and V/V_0 was calculated as described in Materials and methods (*filled circles* untreated control; *filled squares* KF58333, 50 mg/kg daily ×5 days; *open squares* KF58333, 25 mg/kg daily ×5 days; *filled triangles* KF58333, 12.5 mg/kg daily ×5 days; *open triangles* KF58332, 50 mg/kg daily ×5 days). *$P<0.02$ vs untreated control at the same time-point, Mann-Whitney *U*-test

showed significant and potent tumor growth-inhibitory activity ($T/C_{minimum} < 0.5$; Table 2) at doses of 25 and 50 mg/kg per day. In sharp contrast, KF58332 showed no effects on the growth of KPL-4 cells in vivo even at a dose of 50 mg/kg per day (Fig. 5, Table 2). KF58333 was at least fourfold more potent than KF58332 in the KPL-4 xenograft model.

KF58333 but not KF58332 induce apoptosis in the KPL-4 xenograft model

To determine whether the potent growth-inhibitory activity of KF58333 could result in the induction of apoptosis in vivo, as was the case in vitro (Fig. 3), we assessed apoptosis induction in tumor specimens using the TUNEL method. After i.v. administration of each drug at 50 mg/kg daily for five consecutive days from day 0 to day 4, the tumors were removed from the drug-treated and untreated animals on day 7. Induction of apoptosis in the tumor specimens was examined by the in situ TUNEL assay using LSC and CLSM analyses. As shown in Fig. 6A, DNA strand breaks, which could be detected by the TdT-FITC signal, were significantly elevated in the tumor specimens from mice treated with KF58333 but not in those from mice treated with KF58332 or the vehicle (Fig. 6A, left panel). No cell cycle stage specificity of the cells which underwent apoptosis was detected (Fig. 6A, KF58333, right panel).

The same tumor samples were also analyzed by CLSM (Fig. 6B). The results showed that KF58333 caused chromosome condensation (red; PI staining), upregulation of apoptosis (green; TdT-FITC staining),

Table 2 Antitumor activities against human breast carcinoma KPL-4 cells in vivo. BALB/c *nu/nu* tumor-transplanted mice ($n = 5$) were treated with KF58333 or KF58332 as daily i.v. injections for five consecutive days. T/C values were calculated as described in Material and methods

Compound	Dose (mg/kg/day)	$T/C_{minimum}$
KF58333	50	0.26*
	25	0.34*
	12.5	0.63
KF58332	50	0.95

*$P < 0.02$, Mann-Whitney *U*-test

and their colocalization (yellow; TdT and PI overlay) in the tumors. Such a staining pattern was not observed in tumors from mice treated with KF58332 or from untreated control mice. These results demonstrate that KF58333 effectively induced apoptosis of xenografted KPL-4 tumor cells, in a similar manner to its effect in the in vitro system, and this effect was in marked contrast to that of KF58332 at the same dose.

Comparison of pharmacokinetics of KF58333 and KF58332 in mice

As described above, the differences in the growth-inhibitory activities between the two oxime isomers, KF58333 and KF58332, were much more apparent in in vivo animal models than in in vitro cultured cells. One possible interpretation for this discrepancy is that KF58333 might have a quite different pharmacokinetic profile than KF58332 in mice after i.v. administration. To determine whether this was the case, we analyzed the mouse plasma concentration profile after a single i.v. injection of 50 mg/kg of KF58333 or KF58332. As shown in Fig. 7, the pharmacokinetic profiles of KF58333 and KF58332 were similar. In fact, KF58332 showed a slightly higher area under the blood concentration curve (AUC) value than KF58333, suggesting that the difference in the potency of the antitumor activity between KF58333 and KF58332 could not be explained by their pharmacokinetic behavior in mice.

Depletion of hsp90 family associated signaling molecules in vivo

In a previous study, we showed that KF25706, a novel radicicol oxime derivative, exhibits antitumor activity in the MX-1 human breast cancer xenograft model through putative depletion of hsp90 client proteins such as raf-1 and cdk4 in the tumor [36]. We hypothesized that inhibition of the function of hsp90 family chaperone proteins might be important for the antitumor activity of radicicol oxime derivatives. To further explore this hypothesis, we investigated the protein expression level of hsp90 client proteins in the tumor specimens

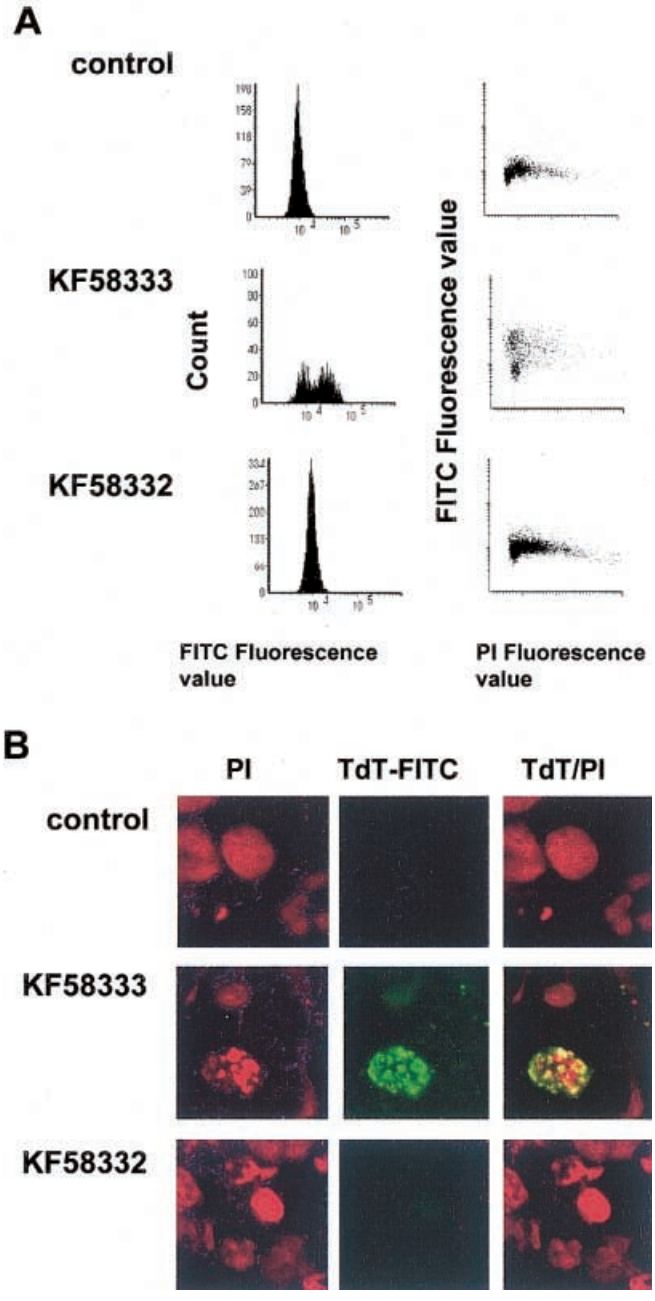

Fig. 6A, B Apoptosis induction in KPL-4 tumor fragments. Drugs were administered as five daily consecutive i.v. injections from day 0 to day 4. On day 7, tumors were removed. Cells from each tumor section were fixed on a glass slide and apoptotic cells were determined by the TUNEL method as described in Materials and methods. Each slide was analyzed by both LSC (**A**) and CLSM (**B**)

from KF58333- or KF58332-treated animals by Western blot analysis.

As shown in Fig. 8A, there were apparent decreases in hsp90 client proteins such as erbB2, raf-1 and Akt in tumor samples recovered from mice treated with KF58333, but not in samples from those treated with KF58332 (Fig. 8A, lane 2 and 3). Cdk4 was also slightly decreased in tumors from mice treated with KF58333,

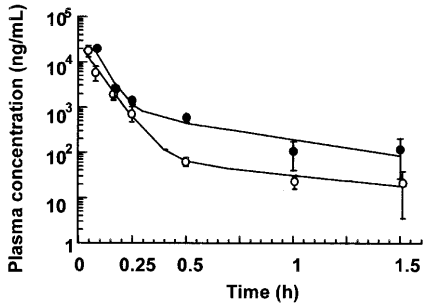

Fig. 7 Plasma concentration versus time profile of KF58333 (*open circles*) and KF58332 (*closed circles*) after a single i.v. administration to mice at a dose of 50 mg/kg. The *solid lines* represents the fitting curves of the data based on a two-compartment open model. Each point represents the mean ± SD from three animals

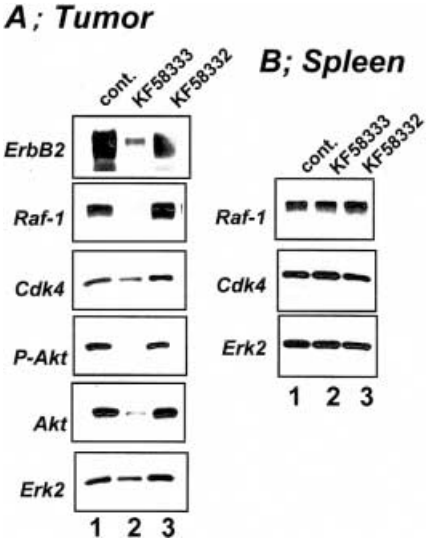

Fig. 8A, B KF58333 depletes hsp90 client proteins in tumor fragments. Drugs were administered to KPL-4 xenografted mice as five daily consecutive i.v. injections from day 0 to day 4. Tumor fragments and spleens were recovered at the same time from drug-treated or untreated animals on day 7. Total cell lysates of each tumor (**A**) and spleen tissue (**B**) were prepared as described in Materials and methods and analyzed by Western blotting using the indicated antibodies. Each lane number in **A** and **B** corresponds to the animal number

although the effect was less clear than in vitro (Fig. 2). These results suggest that KF58333, but not KF58332, showed antitumor activity through inhibition of hsp90 function in vivo. Similar effects were observed when the antitumor activity and the inhibition of hsp90 function in vivo were assessed in human epidermoid carcinoma A431 (which overexpresses EGFR) xenograft models (data not shown), suggesting that tumor-selective potency of KF58333 was not limited to one particular cell line.

In this experiment, we also recovered spleen from the same animals from which the tumors were obtained for analysis. Interestingly, neither KF58333 nor KF58332 had any effect on the expression levels of raf-1 and cdk4 proteins in spleens from tumor-bearing animals. This result suggests that hsp90 antagonists show tumor-selective depletion of hsp90 client proteins.

Discussion

In this study, we found that the E isomer of 6-*O*-[2-(2-pyrrolidonyl)-ethyl] radicicol oxime, KF58333, exhibited 2- to 13-fold more potent antiproliferative activity than its Z isomer, KF58332, in a panel of breast cancer cell lines (Table 1). KF58333 also depleted hsp90 client proteins in erbB2-overexpressing breast carcinoma KPL-4 cells at least threefold more potently than KF58332 (Fig. 2). Interestingly, this difference was more pronounced in xenografted KPL-4 tumor cells in nude mice in that KF58333, but not KF58332, showed significant and potent anticancer activity at the same dose of 50 mg/kg per day (Fig. 5). An apparent downregulation of the expression levels of hsp90 client proteins such as erbB2, raf-1 or Akt, was observed only in tumor tissue recovered from KF58333-treated mice (Fig. 8).

These results suggest that KF58333, but not KF58332, exhibited anticancer activity through the inhibition of hsp90 function in vivo in tumor cells. These findings are consistent with those of our previous study indicating that an active radicicol oxime KF25706, but not its inactive analogue KF29163, shows in vivo antitumor activity through the putative inhibition of the function of hsp90 family of chaperone proteins in tumor cells [36]. The reason why KF58332 lacked anticancer activity in vivo despite the compound's significant growth-inhibitory activity as well as its ability to deplete hsp90 client proteins in vitro (Table 1, Fig. 2), is unknown. It should also be noted that KF58333, but not KF58332, induced apoptosis in in vivo tumor specimens (Fig. 6). However, both isomers induced apoptosis at 1 μM in vitro, while KF58333 was still more active than KF58332 in this regard (Fig. 3).

To elucidate the reason why KF58333 is more active than KF58332, we compared the binding affinity of the two isomers to hsp90 and/or grp94 using geldanamycin and radicicol beads. The twofold higher affinity of KF58333 than KF58332 to hsp90 in the geldanamycin affinity beads assay might suggest the effect of stereochemistry of the oxime moiety on hsp90 binding. However, the difference was not fully consistent with the difference in hsp90 client depletion in vitro (more than threefold) and in antitumor activity in vivo (more than fourfold). These results suggest that another factor, or other factors, besides direct binding affinity to hsp90 family chaperone proteins, might affect their differential potency.

Hsp90 family chaperones and their client proteins are known to exist as multimolecular complexes which include other family chaperones and co-chaperones [2, 4, 20, 22, 26, 34]. Our studies do not rule out the possibility that KF58333 and KF58332 show different effects on

other co-chaperone(s) such as p23, Hip, and Hop, rather than for hsp90 itself. In the case of nuclear receptors, radicicol has been shown to block the ATP-dependent binding of the partner protein p23 to the hsp90-containing complex [28]. We have recently reported that KF58333 induces the dissociation of $p210^{Bcr\text{-}Abl}$ oncoprotein from the hsp90/p23 complex, and this increases the association of the kinase with hsp70 and Hop, in chronic myeloid leukemia K562 cells that overexpress $p210^{Bcr\text{-}Abl}$ [33]. In this regard, further studies are needed to determine the effects of both isomers on the binding of other cofactors (chaperones) to the multimolecular chaperone complex.

In the radicicol beads assay, there were no differences between the isomers in their affinity towards hsp90 family chaperone (Fig. 4C). Previous studies have shown that both geldanamycin and radicicol bind to the same aminoterminal nucleotide binding domain of hsp90, with subtle differences [28, 29, 32]. It might be possible that differences in the probe for hsp90 (geldanamycin or radicicol) affect the result of the competition assay. Recently, a co-crystallization study of radicicol and the hsp90 aminoterminal domain has revealed the nature of the interactions between radicicol and hsp90 [24]. Such direct structural analysis (co-crystallization study) between hsp90 and these isomers of radicicol oxime derivatives might be informative with respect to their differential effects.

Another possible reason for the difference in the in vivo anticancer activity between KF58333 and KF58332 could be that the isomers have different pharmacokinetic profiles in mice. To this end, we determined the plasma concentrations of KF58333 and KF58332 after a single i.v. injection of the drug. However, we did not observe any significant difference between the two isomers. In fact, the inactive analogue KF58332 showed an even higher AUC value than KF58333, the active analogue (Fig. 7), suggesting that differences in the plasma concentrations could not account for the observed differences in the anticancer activity of the radicicol oxime derivatives, at least in mice.

Are there other factors that could determine the in vivo selective anticancer activity of the two isomers? One possibility might be differential effect(s) of the two isomers on angiogenesis, since it has been reported that the parent compound, radicicol, inhibits angiogenesis in vivo [21]. We are currently investigating the effect(s) of KF58333 and KF58332 on angiogenesis in the KPL-4 tumor and other tumor models. The mechanism for the inhibitory effects of hsp90 antagonists on angiogenesis is suggested by the observation that hypoxia-inducible factor 1-α (HIF1-α), which is essential for vascular endothelial growth factor (VEGF) upregulation, is associated with hsp90, and that geldanamycin can inhibit HIF1 activation [17].

We have recently reported that KF58333 can induce apoptosis in K562 human chronic myeloid leukemia cell line in vitro at a concentration of 100 nM [33]. KF58333 induces G_1 phase accumulation of K562 cells at 50 nM. Our preliminary results showed that KF58333 could induce G_1 phase accumulation without induction of apoptosis in SK-BR-3 breast cancer cells that also overexpress erbB2 oncoprotein. In the present study, we investigated apoptosis induction by KF58333 and KF58332 using KPL-4 human breast carcinoma cells. Both compounds induced apoptosis in KPL-4 cells in a cell cycle-independent manner at 1 μM in vitro, which is in contrast to the results obtained with K562 and SK-BR-3 cells. At present, it is difficult to speculate how radicicol oxime derivatives could induce apoptosis. However, KF58333 depleted the serine/threonine kinase Akt, which is a key survival factor for inhibition of apoptosis signaling both in vitro and in vivo (Figs. 2 and 8). These results suggest that the effects of KF58333 on the Akt signaling pathway might be important for induction of apoptosis or G_1 phase accumulation (escape from apoptosis) by the drug.

Since hsp90 is a ubiquitous chaperone protein that associates with various substrate proteins in the cell, it might be possible that hsp90 inhibition causes adverse effect(s) in animals. However, KF58333 did not show liver or renal toxicity (data not shown), as is the case for the radicicol oxime derivative, KF25706 [36]. We also examined the effect of KF58333 and KF58332 on the expression levels of hsp90 client proteins in the spleen as an example of a normal tissue (Fig. 8B). Interestingly, KF58333 did not show any effects on the expression levels of raf-1 and cdk4 in spleens from tumor-bearing animals (compare Fig. 8A, B). This result suggests that the in vivo depletion of hsp90 client proteins by KF58333 might be a tumor-specific event. Several previous studies have shown that hsp90 family chaperone proteins are expressed at higher levels in tumor cells than in normal cells (reviewed in references 4 and 20). Many mutations in tumor suppressor genes and dominant activated oncogenes result in the expression of altered proteins in the tumor cells. Such alterations result, in some cases, in their unusual interaction with molecular chaperones. In this regard, it has been shown that only the mutated, but not the wild-type, p53 tumor suppressor gene product is stabilized by association with hsp90 (reviewed in reference 34).

Recently, the hsp90 antagonist 17AAG (17-allylamino-17-desmethoxygeldanamycin) has been reported to specifically accumulate in tumor tissue in mice after a single i.v. injection [6]. This finding might explain the tumor-specific effects of hsp90 antagonists in vivo. As discussed above, only the E isomer, KF58333, induced apoptosis in KPL-4 solid tumor specimens in the animal model (Fig. 6), although its plasma concentration declined to under 1 μM within 30 min of a single i.v. injection (Fig. 7). It is therefore possible that KF58333, but not KF58332, specifically accumulates in tumor cells. This hypothesis remains to be tested.

The erbB2 oncoprotein is a very important molecular target for anti-breast cancer drugs such as Herceptin, the humanized monoclonal anti-erbB2 antibody, as well as small molecular weight inhibitors of the erbB2 tyrosine

kinase. These results suggest that the described radicicol oxime derivatives may be effective and useful in the treatment of estrogen receptor-negative and erbB2-overexpressing breast cancers which are currently the most difficult breast cancers to treat. In this study, we showed that radicicol oxime derivatives exhibited potent growth-inhibitory activities against erbB2-overexpressing and estrogen receptor-negative breast cancer cell lines (KPL-4 and SK-BR-3). KF58333 inhibited the growth of erbB2-overexpressing KPL-4 tumor in nude mice through putative inhibition of hsp90 function (Fig. 8A). It would be interesting to combine radicicol oxime derivatives with standard chemotherapy drugs, such as Taxol or cisplatin, because Herceptin shows synergistic effects with these agents clinically [25].

In summary, KF58333, the E form oxime isomer of KF55823 (6-*O*-[2-(2-pyrrolidonyl)ethyl] radicicol oxime; E/Z mixture), exhibited specific and potent antitumor activity against erbB2-overexpressing tumors. We confirmed that depletion of hsp90-associated signaling molecules, including erbB2, plays an important role in the antitumor activity of radicicol derivatives. These agents may, therefore, be clinically useful in the treatment of estrogen-independent and erbB2-overexpressing, late-stage breast carcinoma.

Acknowledgements The authors thank Miyoko Asano and Yasuyo Kumazawa for excellent technical assistance, and Dr. Margaret Quinlan for a critical reading of the manuscript.

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