A Novel Compound, NK150460, Exhibits Selective Antitumor Activity against Breast Cancer Cell Lines through Activation of Aryl Hydrocarbon Receptor

Kazuteru Fukasawa1,2, Shigehide Kagaya1, Sakiko Maruyama1, Shunsuke Kuroiwa3, Kuniko Masuda1, Yoshio Kameyama1, Yoshitaka Sato1, Yuichi Akatsu1, Arieiro Tomura1, Kiyohiro Nishikawa1, Shigeo Horie2, and Yuh-ichiro Ichikawa3

Abstract

Antiestrogen agents are commonly used to treat patients with estrogen receptor (ER)-positive breast cancer. Tamoxifen has been the mainstay of endocrine treatment for patients with early and advanced breast cancer for many years. Following tamoxifen treatment failure, however, there are still limited options for subsequent hormonal therapy. We discovered a novel compound, NK150460, that inhibits 17β-estradiol (E2)–dependent transcription without affecting binding of E2 to ER. Against our expectations, NK150460 inhibited growth of not only most ER-positive, but also some ER-negative breast cancer cell lines, while never inhibiting growth of non–breast cancer cell lines. Cell-based screening using a random shRNA library, identified aryl hydrocarbon receptor nuclear translocator (ARNT) as a key gene involved in NK150460’s antitumor mechanism. siRNAs against not only ARNT but also its counterpart aryl hydrocarbon receptor (AhR) and their target protein, CYP1A1, dramatically abrogated NK150460’s growth-inhibitory activity. This suggests that the molecular cascade of AhR/ARNT plays an essential role in NK150460’s antitumor mechanism. Expression of ERs was decreased by NK150460 treatment, and this was inhibited by an AhR antagonist. Unlike two other AhR agonists now undergoing clinical developmental stage, NK150460 did not induce histone H2AX phosphorylation or p53 expression, suggesting that it did not induce a DNA damage response in treated cells. Cell lines expressing epithelial markers were more sensitive to NK150460 than mesenchymal marker-expressing cells. These data indicate that NK150460 is a novel AhR agonist with selective antitumor activity against breast cancer cell lines, and its features differ from those of the other two AhR agonists. Mol Cancer Ther; 14(2); 343–54. ©2014 AACR.

Introduction

Breast cancer is a common disease, and its incidence is increasing worldwide (1). However, during the past two decades the mortality rate in the United States has declined as a result of improvements in treatment and early detection (2).

Despite advances in the treatment of early-stage disease, approximately 40% of patients will experience recurrence, and 35% to 40% will eventually progress to metastatic disease (3). The treatment strategy for advanced/metastatic cancers is commonly decided based on the estrogen receptor (ER), progesterone receptor (PgR), and Her2 statuses determined for resected or biopsied tumor specimens. Recently, the Ki-67 index is also used as a biomarker for deciding the treatment (4). Most breast cancers (at least 30%–50% in premenopausal patients and 80% in postmenopausal patients) express ER or PgR, or both (5). For patients with ER- and/or PgR-positive disease without life-threatening metastases, endocrine treatment is strongly recommended. For premenopausal patients with breast cancer, combination of tamoxifen with a luteinizing hormone-releasing hormone (LH-RH) agonist should be administered as initial treatment. For postmenopausal patients with breast cancer, aromatase inhibitors are commonly used as initial treatment. Although these agents possess certain effectiveness, most patients experience disease progression during treatment. Switching to another type of antihormone agent is the preferred option for such patients, provided that the disease is still not life threatening. Taxane- and/or anthracycline-based chemotherapies should be administered to patients with life-threatening metastasis during adjuvant hormone therapy. Advanced disease with ER, PgR, and Her2-negative (triple-negative) properties, or advanced disease that failed to respond to previous antihormone therapies (6). However, these chemotherapeutic agents are often accompanied by various adverse events such as myelosuppression, nausea and vomiting, peripheral sensory neuropathy, heart failure, and so on. To overcome these problems, new treatment agents having different mechanisms from the conventional antihormone therapies have been strongly desired.

ER is a member of the nuclear receptor superfamily and is activated by its ligand, 17β-estradiol (E2; ref. 7). ER is known to be a transcription factor. Once activated by E2, ER is translocated

1Pharmaceutical Research Laboratories, Research and Development Group, Nippon Kayaku Co., Ltd., Kita-ku, Tokyo, Japan. 2Department of Urology, Graduate School of Medicine, Juntendo University, Bunkyo-ku, Tokyo, Japan.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Kazuteru Fukasawa, Nippon Kayaku Co., Ltd., 3-31-12, Shimo, Kita-Ku, Tokyo 155-8588, Japan. Phone: 81-3-3598-5883; Fax: 81-3-3598-5422; E-mail: kazuteru.fukasawa@nipponkayaku.co.jp

doi: 10.1158/1535-7163.MCT-14-0158

©2014 American Association for Cancer Research.

www.aacjournals.org
into the nucleus. Then dimerized ER molecules bind to the motif sequence, estrogen-responsive element (ERE), on DNA to upregulate several target genes, including trefoil factor 1 precursor (pTFF2; ref. 8) and cathepsin D (9). During this step, ERs bind with various transcriptional cofactors, forming huge protein complexes (10). CBP/p300 (11), SRC-1 (12), GRIP1 (13), and AIB1 (14) are transcriptional cofactors and have histone acetyltransferase activity. As another example, ATP-dependent chromatin-remodeling complexes, such as Switch/Sucrose Nonfermentable (SWI/SNF), are recruited to the hormone-responsive element together with several transcription factors (15). Although these proteins are potential new pharmaceutical targets, no agents have been developed to date to overcome resistance to conventional antihormone therapies.

We screened various chemical compounds and extracts from natural products for the ability to inhibit E2-dependent transcriptional upregulation via ERE without competing for E2/ER binding. As a result, we discovered a novel compound, NK150460 (5S,7S)-7-methyl-3-(3-(trifluoromethyl)phenyl)-5,6,7,8-tetradrocinnolin-5-ol (Fig. 1A), that shows selective growth-inhibitory activity for breast cancer cells. To identify the key genes involved in NK150460’s action mechanism and elucidate the precise mechanism, we carried out cell-based screening using a random shRNA library. We found that NK150460 is a novel aryl hydrocarbon receptor (AhR) agonist whose growth-inhibitory activity is strongly dependent on AhR/ARNT (aryl hydrocarbon receptor nuclear translocator) and one of their target proteins, cytochrome P450 1A1 (CYP1A1).

Materials and Methods

Compounds

NK150460 was synthesized at Nippon Kayaku Co., Ltd. (Fig. 1A). Tamoxifen citrate, 3-methylcholanthrene (3MC), α-naphthoflavone (ANF), β-naphthoflavone (BNF), and IC1182,780 were purchased from Sigma-Aldrich. Lactacytin and Z-Leu-Leu-Leu-H (aldehyde; MG-132; proteasome inhibitor) were purchased from Peptide Institute, Inc.

Cell lines and culture

All cell cultures were performed in accordance with Nippon Kayaku’s internal standard operating procedure. All cell lines except KPL-1 were purchased from the ATCC. All cell lines were cultured in plates in accordance with the ATCC’s instructions. A tamoxifen-resistant human breast cancer cell line, KPL-1, was kindly provided by Dr. J. Kurebayashi of Kawasaki Medical School (Kurashiki, Japan; ref. 16). All cell lines were authenticated morphologically. Cells were initially grown and multiple aliquots were frozen and stored at −80°C for future use. All cell lines were obtained more than 1 year ago from each experiment. All cell lines were used at low passage in our laboratory and were tested regularly to confirm the absence of Mycoplasma infection. In estrogen or AhR agonist induction experiments, cells were cultured in phenol red-free RPMI-1640 supplemented with 10% charcoal-stripped FBS. After 2 days, [1H]-β estradiol (final concentration, 3 nmol/L) and various concentrations of E2, tamoxifen, or NK150460 were added to each culture medium, followed by incubation for 1 hour. The plates were placed on ice, and the culture media were aspirated off. After washing the wells with PBS (−) containing 0.5% BSA and 10% glycerol, ethanol was added to the wells, and the plates were incubated at room temperature for 1 hour. Cellular radioactivity was measured with a liquid scintillation counter.

Growth-inhibition assay

Growth-inhibition assay was performed according to the manufacturer’s instructions (17). Briefly, cultured cells were fixed with methanol and stained for 30 minutes with 0.05% methylene blue in 10 mmol/L Tris-HCl (pH 8.5). After three washes with water, the stain was extracted with 3% HCl, and the cells were analyzed with a microplate reader using a 660-nm test filter.

Xenografts in nude rats

ZR-75-1 human breast cancer cells (18), established as tumor xenografts in female athymic mice, were transplanted subcutaneously at bilateral dorsal sites of F344/N-rnu/rnu nude rats (CLEA). Slow-release 17β-estradiol pellets (Innovative Research of America) were implanted on the same day. Eight to 9 days after implantation, rats with good growth of implanted tumors were divided into three groups (control and two NK150460 treatment arms) of 4 rats each. NK150460 was administered orally every day for 24 days in a dose of 20 or 100 mg/kg. Tumor size was measured periodically with calipers, and each tumor volume was calculated using the formula (width2 × length)/2. Differences in the relative tumor volume in the NK150460-treated groups compared with the control group on day 25 were performed using one-way ANOVA with the two-tailed Dunnett post hoc test.

Random shRNA screening

Cell-based screening using a random shRNA library was performed by GenoFunction Inc. In brief, a random shRNA library was transfected into MCF-7 cells by vesicular stomatitis virus-G lentivirus vector infection. An shRNA-targeting EGFP virus-G lentivirus vector was selected with blasticidin S, were treated with 10 μmol/L NK150460 for 8 days, exchanging the medium and drug every day. Then shRNA fragments in the remaining cells were collected and used to generate the next shRNA library. This cycle was repeated until obvious NK150460-resistant transfectants appeared.

Microarray analysis

Three 150460-sensitive cell lines, T-47D, MCF-7, and SK-BR-3, and an NK150460-insensitive cell line, MDA-MB-231, were treated with 2.5 μmol/L NK150460 for 0, 3, or 6 hours. Total RNA at each time point was purified with an RNeasy Mini Kit (QIAGEN) according to the instructions, and gene expression was analyzed using Gene Chip Human Genome Focus Arrays (Affimetrix, Inc.), with a total of approximately 8,500 probe sets, at Bio Matrix Research, Inc. Gene-expression data were analyzed with the GeneSpring 7.2 program. The data were assigned GEO accession number as GSE61548.

Plasmid construction, cell transfection, and RNA interference

Plasmid vectors containing three tandem consensus EREs–TATA–luciferase cDNA (19), as well as vectors containing three
tandem consensus xenobiotic-responsive elements (XRE)–TATA-luciferase cDNA (20), were kindly provided by Dr. F. Ohtake of the University of Tokyo (Tokyo, Japan). CYP1A1 complementary DNA was amplified by RT-PCR and subcloned into the pcDNA3 vector (Invitrogen Corp.). Plasmids were transfected with Lipofectamine 2000 transfection reagent (Invitrogen) that had been preincubated for 20 minutes at room temperature. Luciferase assay

Plasmid DNA containing EREs–TATA-luciferase was transfected into T-47D, and stable transfectants were established. Samples for luciferase assay were prepared using the Luciferase Assay System (Promega). In brief, $1 \times 10^4$ cells per well were plated into 96-well plates, and compounds were added on the next day. After 24 hours, cells were lysed with Cell Culture Lysis Reagent, and the cell lysates were transferred to black 96-well plates. The luciferase activity in each well was measured with a luminometer.
Western blot analysis and quantitative reverse transcription-PCR

Cells were lysed in RIPA buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, and 1 mmol/L EDTA). The cell lysates were stored at −80°C until SDS-PAGE, after which Western blot analysis was performed according to the manufacturer's instructions.

Total RNA was purified from cultured cells with ISOSGEN (Nippon Gene) and then reverse-transcribed with oligo-dt primer and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. mRNAs were quantified using the TaqMan assay (Applied Biosystems) on an ABI prism 7000 apparatus (Applied Biosystems).

Chromatin immunoprecipitation assay

MCF-7 cells were cultured on plates with phenol red-free RPMI-1640 supplemented with charcoal-stripped FBS, and NK150460 or 3MC was added to the culture medium. Soluble chromatin from the MCF-7 cells was prepared with acetyl-histone H4 immunoprecipitation assay kit (Upstate Biotechnology). In brief, 0, 30, 45, 60, 75, and 90 minutes later, protein/DNA complexes were crosslinked with formalin at 37°C for 15 minutes. After lysing the cells, DNA was fragmented by sonication, and DNA–protein complexes were coimmunoprecipitated with antibodies against AhR, ARNT, ERα, or p300. The ERE motif in the pS2 promoter and the XRE motif in the CYP1A1 promoter were visualized with ethidium bromide under UV light.

Detection of cellular metabolites

NK150460-treated SK-BR-3 cells were washed with ice-cold PBS(−) and then suspended in acetonitrile/acetic acid solution. After incubation on ice, the cell extract was centrifugally removed and the supernatant was concentrated. The concentration of M-2 was identified by nuclear magnetic resonance (NMR).

Statistical analysis

All data are expressed as the mean ± SD. Statistical significance was performed one-way ANOVA with post hoc testing using the two-tailed Dunnett multiple comparison test for comparison with the control group (Fig. 1B, C, and F) or two-way ANOVA with post hoc testing using the two-tailed Dunnett multiple comparison test for comparison with the nonsilencing control siRNA-transfected group (Fig. 2C). Significance was set at 0.05. The SAS version 9.2 GLM procedure was applied for statistical analysis.

Results

NK150460 inhibited estrogen-dependent transactivation and exhibited selective growth-inhibitory activity

We screened chemical compounds or extracts from natural products for the ability to inhibit E2-dependent transcriptional upregulation via ERE by means of a different mechanism compared with conventional antagonists like selective ER modulators (SERM). As a result, we discovered a novel compound, NK150460 (Fig. 1A). NK150460 inhibited E2-dependent transactivation via ERE in a dose-dependent manner, and its inhibitory activity was comparable with that of tamoxifen (Fig. 1B). NK150460 also inhibited two E2-induced endogenous ER target genes, PgR (Fig. 1C, ref. 21). To determine whether NK150460 inhibits E2-dependent transactivation by a different mechanism from that of tamoxifen, we examined whether NK150460 competes with E2 binding to ER in vitro. As expected, tamoxifen competed with endogenous E2 binding in a dose-dependent manner, whereas NK150460 did not (Fig. 1D), indicating that it inhibits E2-dependent transactivation by a different mechanism from that of tamoxifen. Next, we examined the growth-inhibitory activity of NK150460 against various breast cancer cell lines (Fig. 1E). As expected, both NK150460 and the comparator, tamoxifen, inhibited proliferation of two ER+/PgR+ cell lines, MCF-7 and T-47D. To our surprise, NK150460 inhibited proliferation even of some ER−/PgR− cell lines, that is, MDA-MB-453, MDA-MB-468, and SK-BR-3, whereas tamoxifen did not. Proliferation of the other ER+ /PgR− cell lines, that is, MDA-MB-231 and MDA-MB-4358, was not inhibited by either NK150460 or tamoxifen (Fig. 1E). We also examined NK150460's growth-inhibitory activity against various tumor cell lines, including non–breast cancer cell lines. NK150460 inhibited all of the ER+ breast cancer cell lines we examined, including the tamoxifen-resistant cell line KPL-1, but did not inhibit proliferation of the non–breast cancer cell lines we examined (Supplementary Tables S1 and S2). Finally, we examined NK150460 for antitumor activity against the ZR-75-1 (ER+ /PgR+) breast cancer cell line in vivo. Orally administered NK150460 significantly inhibited growth of ZR-75-1 in a nude rat xenograft model (Fig. 1F).

AhR, ARNT, and CYP1A1 are essential for NK150460's growth-inhibitory activity

To elucidate the mechanism of antitumor activity of NK150460, we used a random shRNA library. We hypothesized that once an shRNA knocks down expression of a gene that plays a crucial role in the growth-inhibitory activity of NK150460, that cell will become resistant to NK150460. To maximize the screening sensitivity, we first set the screening conditions as follows: MCF-7 cells were treated with 10 μmol/L NK150460 for 8 days, exchanging the culture medium and NK150460 every day. On the basis of colony formation after replating cells, more than 99% of MCF-7 cells were killed under these conditions. Even though less than 1% of cells survive even under these conditions, we predicted that nonspecific shRNA selection could be minimized if we repeated this selection process several times. We introduced a random shRNA library into MCF-7 using lentivirus vectors, and stable transfectants were treated with NK150460 under the conditions described above. After repeating the screening three times, obvious cell colonies were seen compared with the control group, so we continued the NK150460 treatment for 6 more days (Fig. 2A). shRNA fragments were recovered from the remaining cells, and shRNA-specific sequences were identified. Of note, 72 of 169 colonies (42.6%) having shRNA-sequence (Fig. 2B). Next, we examined whether ARNT is essential for the growth-inhibitory activity of NK150460 by using ARNT-specific siRNA. ARNT belongs to the basic-helix-loop-helix-PAS protein family and functions as an essential dimerization partner of AhR transcription factor (22).
NK150460: A Potential Antitumor Drug Activating AhR

We also examined AhR for a possible role in NK150460-dependent growth inhibition using the same siRNA transfection assay. Both ARNT and AhR siRNA significantly canceled the growth-inhibitory activity of NK150460 (Fig. 2C). It is known that ligand-activated AhR/ARNT heterodimers activate transcription of target genes such as CYP1A1 (23), CYP1A2 (24), and CYP1B1 (25). Accordingly, we also examined for possible involvement of CYP1A1, CYP1A2, and CYP1B1 in the growth-inhibitory activity of NK150460. Interestingly, similar to AhR and ARNT siRNAs, introduction of CYP1A1 siRNA dramatically abrogated NK150460’s inhibition of cell growth, but transfection of siRNA for CYP1A2 (Fig. 2C) or CYP1B1 (Supplementary Fig. S1) had no effect. The knockdown effect of each siRNA was confirmed at both the mRNA (Supplementary Fig. S2) and protein (Fig. 2D) levels. These data indicate that at least three proteins, AhR, ARNT, and CYP1A1, are crucial for the growth-inhibitory activity of NK150460.

NK150460 induced CYP1A1 gene expression in NK150460-sensitive cell lines

Our findings strongly suggested that the AhR/ARNT transcription factors are involved in the growth-inhibitory activity of NK150460, so we used microarrays to search for NK150460-dependent changes in gene expression in NK150460-sensitive and -insensitive breast cancer cell lines. Among approximately 8,500 examined genes, only CYP1A1 and CYP1B1 mRNAs—only
in NK150460-sensitive cell lines—were clearly induced by NK150460 treatment for 3 hours (Supplementary Table S3). We next investigated the reproducibility of NK150460-dependent CYP1A1 mRNA induction using 9 cell lines, that is, three ER-positive/NK150460-sensitive cell lines (MCF-7, T-47D, and KPL-1), three ER-negative/NK150460-sensitive cell lines (SK-BR-3, MDA-MB-435, and MDA-MB-468), and three ER-negative/NK150460-insensitive cell lines (MDA-MB-231, MDA-MB-435, and Hs0578T). CYP1A1 mRNA, measured by quantitative reverse transcription-PCR (qRT-PCR), was specifically induced only in the NK150460-sensitive cell lines, regardless of their ER status (Fig. 2E). CYP1A1 induction in MCF-7 by NK150460 was also confirmed by Western blotting (Fig. 2F). Ligand-activated AhR/ARNT complexes are known to activate the transcription of target genes through XREs. Thus, to monitor the transactivation function of endogenous AhR, we transfected luciferase reporter plasmids bearing three XRE consensus-binding elements into MCF-7 cells and treated the cells with NK150460 or a known AhR agonist, that is, 3MC or BNF. NK150460 induced XRE-driven luciferase activity, but not as strongly as by 3MC and BNF (Supplementary Fig. S3). We also confirmed that NK150460 induced gene expression of CYP1A2 and CYP1B1 as well as CYP1A1 in MCF-7 cells (Supplementary Fig. S4).

Cross-talk between NK150460-dependent AhR-mediated signaling and ER-mediated estrogen signaling

Several AhR agonists, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 6-methyl-1,3,8-trichlorodibenzo-p-dioxin (M-2), benz[a]pyrene (BaP), degrade ERα through proteasome activation in breast cancer cell lines (26, 27). We examined whether NK150460 shows similar activity on cellular ERα protein. Endogenous ERα was clearly decreased in MCF-7 cells following treatment with NK150460, and this was a posttranslational event because it was strongly inhibited by two proteasome inhibitors, MG-132 and lactacystin (Fig. 3A; ref. 28). To elucidate whether AhR is involved in NK150460-dependent downregulation of endogenous ERα, we treated MCF-7 cells simultaneously with NK150460 and an AhR antagonist, ANF (29). ANF inhibited that downregulation in a dose-dependent manner (Fig. 3B). Two other AhR antagonists, BNF (30) and 3MC (31), also decreased endogenous ERα, and they were also inhibited by ANF (Fig. 3C). TCDD-induced degradation of ERα was inhibited by transfection of AhR siRNA (26), suggesting that NK150460's mechanism for ERα degradation is similar to that of TCDD and other AhR agonists. IC1182,780 (fulvestrant) is known to be a selective ER downregulator (SERD; ref. 32). NK150460 has similar characteristics with IC1182,780, because it inhibits E2-dependent transactivation and decreases cellular ERα protein in breast cancer cell lines. However, the mechanism of ERα downregulation by IC1182,780 is totally different from that of NK150460 because ERα degradation by IC1182,780 was not affected by ANF treatment (Fig. 3C).

Next, we performed chromatin immunoprecipitation (ChIP) assay to investigate whether AhR, ERα, and related proteins were recruited onto the XRE motif sequence in the pS2 promoter and the XRE motif sequences in the CYP1A1 promoter. We used 3MC as a positive control AhR agonist, because it is known to recruit ERα, AhR, and ARNT onto the XRE motif in the –449 to –241 region of the pS2 promoter and onto the XRE motif in the –1155 to –775 region of the CYP1A1 promoter (33). Recruitment of AhR and ERα onto the XRE motif in the CYP1A1 promoter was seen by 30 minutes after NK150460 addition, and ARNT and p300 recruitment was seen by 45 minutes. On the contrary, AhR, ARNT, ERα, and p300 were all recruited onto the XRE motif by 30 minutes after 3MC addition. p300 had already been recruited onto the XRE motif in the pS2 promoter under ligand-free conditions. When NK150460 was added, AhR and ERα recruitment was seen by 30 minutes, and then ARNT recruitment was seen by 45 minutes (Fig. 3D).

Identification of potential CYP1A1-dependent active metabolite of NK150460

The mechanisms of the growth-inhibitory activity of AhR agonists, including ER-dependent and ER-independent mechanisms, have been extensively investigated (34). One of the features of NK150460's growth-inhibitory action mechanism is that not only AhR/ARNT, but also CYP1A1, play crucial roles. Two AhR agonists, that is, AFPA64 (a lysyl prodrug of aminoflavone) and Phortress (L-lysylamide prodrug of 5F-203), have already advanced to clinical development (35). Both aminoflavone and 5F-203 are known to be degraded by metabolizing enzymes, including CYP1A1 and/or SULT1A1, and active metabolites are essential for their antitumor activity (36–38). To examine whether CYP1A1 metabolizes NK150460 to active metabolites, we first transiently overexpressed human CYP1A1 in an NK150460-insensitive cell line, HEK293. As expected, NK150460 inhibited proliferation of CYP1A1-overexpressing HEK293 (Fig. 4A). This growth inhibition by NK150460 in CYP1A1-overexpressing HEK293 cells was abrogated by the CYP1A1 inhibitor, ANF (39), in a dose-dependent manner (Fig. 4A). ANF is also known to be an AhR antagonist, so we performed a similar experiment using AhR siRNA. As anticipated, the growth-inhibitory activity of NK150460 in CYP1A1-overexpressing HEK293 was not inhibited by AhR siRNA transfection (Supplementary Fig. S5). These findings suggested that CYP1A1 produces an active metabolite that is independent of AhR in NK150460-insensitive HEK293 cells. LC/MS-MS showed two main peaks of hydroxylated NK150460, which we named M-1 and M-2, in NK150460-treated SK-BR-3 (Fig. 4B). We predicted that boda fde active metabolite(s) would be reduced if we knocked down AhR or CYP1A1 mRNA expression. We found that the intracellular level of M-2 was drastically reduced by AhR or CYP1A1 knockdown, but it was not affected by CYP1A2 or CYP1B1 siRNA transfection (Fig. 4C). We purified M-2 from rat urine and identified its chemical structure by NMR (Fig. 4D). The chemical shifts of NK150460 and M-2 analyzed with NMR are shown in Supplementary Fig. S6. M-2 inhibited cell proliferation of both the NK150460-sensitive and -insensitive cell lines we examined, but its growth inhibitory activity was weaker than that of NK150460 (Fig. 4E). These data support the existence of active metabolites of NK150460, and M-2 appears to be one.

Absence of a DNA damage response in NK150460-treated cells

Several studies have shown that active metabolite(s) of aminoflavone or 5F-203 metabolized by CYP1A1 and/or SULT1A1 form DNA adducts (36–38). To further clarify the characteristics of NK150460, we examined whether it induces a DNA damage response in sensitive cell lines. Aminoflavone was reported to induce dose- and time-dependent phosphorylation of histone H2AX (pH2AX; ref. 40). 3MC and 5F-203 induced pH2AX and expression of p53 and its target gene, p21, in MCF-7 cells. In contrast, NK150460 did not show such induction (Fig. 5), suggesting that it does not form DNA adducts in cells. This feature is a big difference from aminoflavone and 5F-203.
Difference in cell morphology between NK150460-sensitive and -insensitive cell lines, and possibility of identification of NK150460-sensitive cells using epithelial and mesenchymal markers

A key remaining issue is how to identify NK150460-sensitive tumor cells. We examined the expression of AhR mRNA in various breast cancer cell lines, but none showed a correlation between NK150460 sensitivity and AhR mRNA expression (Supplementary Table S4). We next examined whether there were any differences in cell morphology between NK150460-sensitive and -insensitive cell lines. NK150460-sensitive cell lines like MCF-7 and T-47D were round cells with close cell–cell adhesions. On the contrary, the cell shapes of NK150460-sensitive cell lines like MDA-MB-231 and Hs0578T were elongated and cell–cell adhesions were very rare (Fig. 6A). We thus speculated that NK150460 sensitivity can be identified using epithelial or mesenchymal features. We examined expression of several epithelial- and mesenchymal-related genes in both NK150460-sensitive and -insensitive cell lines. As we expected, mRNA expression for some epithelial markers (CLDN7, KRT19, PROM2, RAB25, SPDEF, and STARD) was higher in NK150460-sensitive cell lines than in insensitive cell lines, and conversely some mesenchymal markers (CAV1, COL4A1, IGFBP7, SPARC, VIM, and TGFB1) showed higher expression in insensitive cell lines than in sensitive cell lines (Fig. 6B). These findings suggest that NK150460-sensitive cells might be able to be identified using epithelial- and mesenchymal-related markers.

Discussion

Breast cancer cells grow estrogen dependently, and several antihormone therapies are playing important roles in current clinical practice. The first class of antihormone agents includes SERMs such as tamoxifen, toremifene, and raloxifene. The next class is aromatase inhibitors, including anastrozole, letrozole, and...
exemestane. The third class is SERD, which is fulvestrant (41). Here, we have shown that NK150460 inhibits E2-dependent transactivation, without affecting E2/ER binding. Its mechanism appears to be totally different from those of the currently available antihormone agents, suggesting that NK150460 could become a fourth class of antihormone therapy in the future.

Although we discovered the novel compound NK150460, its precise mechanism was unclear. To solve this problem, we carried

Figure 4.
The antitumor activity of NK150460 depends on its being metabolized by CYP1A1. A, Cyp1A1-dependent antitumor activity of NK150460 in an insensitive cell line, HEK293. Mock plasmid vector (pcDNA3) or CYP1A1 cDNA-inserted vector was transfected into HEK293. Twenty-four hours later, the cells were plated onto 96-well plates and various concentrations of NK150460 alone (left graph) or NK150460 plus ANF (right graph) were added to each well. Cell growth was determined by methylene blue staining at 72 hours after adding the compounds. The inset shows the expression of Cyp1A1 protein at 24 hours after transfection of the mock vector (1) or CYP1A1 cDNA-inserted vector (2), detected by Western blotting. B, the peaks of NK150460 metabolites in SK-BR-3. Cells were treated with NK150460 for 6 hours, and cellular extracts were prepared. The LC/MS-MS chart shows two main peaks of hydroxylated NK150460 (indicated as M-1 and M-2). C, metabolism of NK150460 to M-2 was dependent on AhR and CYP1A1. CYP1A1, CYP1A2, CYP1B1, and AhR siRNAs were transfected into SK-BR-3 cells. Nonsilencing control siRNA was used as a negative control. After transfection, 10 μmol/L NK150460 was added, followed by incubation for 6 hours. Cellular extracts were prepared, and peaks of NK150460 and its metabolites were measured by LC/MS-MS. The area of each peak is expressed as fold induction compared with the nonsilencing control siRNA group. D, chemical structure of M-2. E, comparison of antitumor activities of NK150460 and M-2 against various cell lines, including NK150460-sensitive and -insensitive lines. Cells were treated with either NK150460 or M-2 for 72 hours, and cell growth was determined by methylene blue staining.
out several comprehensive analyses, including random shRNA library screening and microarray analysis. We identified a key molecule, ARNT, by random shRNA library screening, using NK150460's growth-inhibitory activity as an indicator. Also, using microarray chips, we found that mRNA expression of only CYP1A1 and CYP1B1 was clearly induced, only in NK150460-sensitive cell lines. CYP1A1 and CYP1B1 are well known as AhR/ARNT target genes. CYP1A2 expression is also induced by AhR (Supplementary Fig. S4), but we could not examine its induction because the Gene Chip Human Genome Focus Array does not include a CYP1A2 gene probe. On the basis of the results of these two comprehensive analyses, we were convinced that the AhR/ARNT pathway plays a critical role in the action mechanism of NK150460. We also showed that these comprehensive analyses are very effective for elucidating unknown molecular mechanisms of compounds in which target molecules are unknown. Before starting the random shRNA screening, we set very strict conditions for growth inhibition by NK150460 because we wanted to exclude nonspecific selection of genes. The conditions we set, resulted in more than 99% of MCF-7 cells' being killed by NK150460. We surmised that these conditions would be very effective for our purpose. In fact, after three repetitions of the screening, we were able to identify ARNT as a key protein in NK150460's antitumor activity.

Like other AhR agonists, ERα and AhR/ARNT were recruited to both the ERE motif of the pS2 promoter and the XRE motif of the CYP1A1 promoter (Fig. 3D), and these recruitments were seen by 30 minutes after NK150460 treatment. This strongly suggested that these prompt recruitments directly and simultaneously regulated NK150460's inhibition of E2-dependent...
gene transactivation and induction of XRE-mediated transactivation. However, further investigation of these transcriptional regulation mechanisms of NK150460 is necessary. The level of XRE-mediated transactivation by NK150460 was lower than those seen with 3MC and BNF (Supplementary Fig. S3), suggesting that the mechanisms for transcriptional regulation differ among AhR agonists. Matthews and colleagues (42) reported that TCDD recruited ERα to the pS2 promoter with maximal recruitment at 30 minutes after treatment, and to the CYP1A1 promoter with maximal recruitment at 60 minutes. Interestingly, recruitment of ERα onto the CYP1A1 promoter was still present at 120 minutes after TCDD treatment, but no longer in the case of the pS2 promoter. In our experiment, recruitment of transcription factors by NK150460 was not followed beyond 90 minutes, so longer observation and comparison with other AhR agonists is necessary. Gillesby and colleagues (43) reported that the 3' flanking region of the pS2 gene has three motifs that resemble XRE core sequences. One of the motifs, motif 1 (−520 to −517), is necessary for inhibiting E2-induced transactivation by TCDD, and a TCDD-induced protein/motif 1 complex was confirmed (43). In our experiment, we did not investigate NK150460-dependent recruitment of AhR/ARNT to XRE motif 1 of the pS2 promoter, and this warrants examination.

CYP1A1 transfection converted NK150460-insensitive cells to sensitive, suggesting the existence of active metabolites of NK150460. We found one putative active metabolite of NK150460, which we named M-2. M-2 showed antiproliferation activity in both NK150460-sensitive and -insensitive cell lines, but its activity was weaker than that of NK150460. At least two possibilities might explain this. One possibility is a difference in cell penetrability between NK150460 and M-2. When SK-BR-3 cells were treated with NK150460 in vitro, NK150460 and M-1 were each detected in both the cellular extract and culture medium, but the level of M-2 in the culture medium was quite low compared with that in the cellular extract (Supplementary Fig. S7). The second possibility is the existence of yet undetected, more powerful active metabolites of NK150460. At the same time, we need to consider other possibilities besides active metabolites of NK150460, which we named M-2. M-2 showed antiproliferation activity in both NK150460-sensitive and -insensitive cell lines, but the level of M-2 in the culture medium was quite low compared with that in the cellular extract (Supplementary Fig. S7). The second possibility is the existence of yet undetected, more powerful active metabolites of NK150460. At the same time, we need to consider other possibilities besides active metabolites of NK150460 as playing a key role in NK150460's antitumor activity. We have not analyzed for cellular metabolites in cells other than SK-BR-3. To conclude, NK150460's precise mechanism of antitumor activity, we need to perform further investigations in various other cell lines, including ER-positive and ER-negative cells. In addition, it is well known that several AhR agonists also inhibit growth of ER-negative breast cancer cell lines (44–46). Considering the shared mechanisms among various AhR agonists, NK150460 itself might exhibit antitumor activity in some other cell lines.

Clinical trials have been conducted on two AhR agonists, AFP-464 and Phortress. A phase II study of AFP-464 with and without Faslodex was conducted in patients with ER-positive breast cancer (ClinicalTrials.gov Identifier: NCT01233947), but detailed results have yet to be disclosed. A phase I trial against solid tumors (NCT00348699) is now ongoing. As for Phortress, a phase I study was started, but it was terminated because of lack of efficacy (47). The administration route of these drugs is intravenous. Oral administration of NK150460 showed antitumor activity against a breast cancer cell line, ZR-75-1, in a xenograft model (Fig. 1F). Furthermore, unlike aminoflavone and 5F-203, NK150460 did not induce a DNA damage response in sensitive cell lines (Fig. 5), meaning that it might be able to be administered orally for a long period, like conventional antiestrogen agents. These points suggest that NK150460 has favorable properties as an anti-breast cancer agent. Another point we would like to stress is that CYP1A2 and CYP1B1 are not involved in NK150460's antitumor activity. Even though CYP1A1 plays essential roles in bioactivation of 5F-203 and aminoflavone, there have been no published reports regarding possible involvement of CYP1A2 and CYP1B1.

NK150460 showed antiproliferation activity even against some ER-negative cell lines. This indicates that NK150460 may have not only an ER-dependent, but also an ER-independent, mechanism for expressing its antitumor effect through AhR activation. This, in turn, suggests the possibility that NK150460 could also be used to treat ER-negative breast cancer, including triple-negative breast cancer (TNBC). Among the NK150460-sensitive cell lines we examined, MDA-MB-43 and MDA-MB-468 are classified as TNBC. There is a lack of effective nonchemotherapeutic agents for TNBC (48). Although several molecular-targeting agents, including PARP inhibitors (49, 50), EGFR inhibitors (51, 52), and tyrosine kinase inhibitors (53–55), have undergone clinical studies against TNBC, they have not yet been demonstrated to be clinically useful. Thus, there is an “unmet medical need” for treatment of TNBC, and NK150460 may be able to meet that need.

One of the challenges to showing clinical usefulness of NK150460 will be to establish a method for identifying NK150460-sensitive breast cancers. Neve and colleagues (56) analyzed the gene-expression patterns in 51 breast cancer cell lines and identified three subtypes (i.e., Neve’s subtyping), called luminal, basal A, and basal B. Most of the NK150460-sensitive cell lines we tested were clustered in the luminal and basal A subtypes. We showed that several epithelial- and mesenchymal-related markers may be useful for identifying NK150460 sensitivity (Fig. 6B). These findings strongly support identification of NK150460 sensitivity by using gene-expression patterns. In clinical practice, breast cancer can be classified into at least five subtypes, luminal A, luminal B, ErbB2, basal-like, and normal-like, based on their gene-expression patterns (57). This classification is very commonly used in current medical practice. Lehmann and colleagues (58) showed that TNBC is a highly diverse group of cancers, and their gene-expression profiles allow them to be clustered into six subtypes (i.e., Lehmann’s subtyping). They comprise two basal-like subtypes (BL1 and BL2), an immunomodulatory subtype (IM), a mesenchymal subtype (MSL), and a luminal androgen receptor (LAR) subtype. Interestingly, most TNBC cell lines assigned to basal B in Neve’s subtyping belong to M or MSL in Lehmann’s subtyping. On the basis of this background and our results, M and MSL in TNBC might be insensitive to NK150460. Gene-expression patterns might become a powerful tool for patient selection, and some TNBC might respond to NK150460.

AhR was recently shown to negatively regulate epithelial-to-mesenchymal transition (EMT) in primary human keratinocytes and a mouse epithelial cell line (59). AhR deficiency in the mouse epithelial cells triggered morphologic and phenotypic changes indicative of EMT. Interestingly, in those epithelial cells AhR bound to and colocalized with both E-cadherin and β-catenin, key molecules for epithelial cell adhesion. It is possible that AhR is functionally active in epithelial cells—but not mesenchymal-like breast cancer cells—by forming complexes with key epithelial proteins, and that NK150460 works through “active” AhR only in sensitive breast cancer cells.
In conclusion, we discovered a novel compound, NK150460, which inhibits E2-dependent transactivation. NK150460 activates AhR/ARNT-dependent signaling and induces CYP1A1 expression, and these three genes play essential roles in the antitumor activity of NK150460. Furthermore, AhR activated by NK150460 interacts with ERα-dependent signaling. Thus, we anticipate that NK150460 will exhibit both ER-dependent and ER-independent antitumor activity.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: K. Fukasawa, S. Kagaya, S. Manuyama, S. Kuroiwa, A. Tomura, K. Nishikawa
Development of methodology: K. Fukasawa, S. Kagaya, A. Tomura
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Fukasawa, S. Kagaya, S. Manuyama, S. Kuroiwa, K. Masuda, Y. Kameyama, Y. Akatsu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Fukasawa, S. Kagaya, S. Kuroiwa, K. Nishikawa, S. Horie
Writing, review, and/or revision of the manuscript: K. Fukasawa, Y. Kameyama, A. Tomura, K. Nishikawa, S. Horie
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Fukasawa, S. Maruyama, Y. Satoh
Study supervision: K. Fukasawa, S. Kagaya, K. Nishikawa, Y.-i. Ichikawa

Acknowledgments
The authors thank Iwao Nozawa, Yousuke Suzuki, Hidekazu Ruma, and Seiji Kinoshita (Genofunction Inc.) for collaborating on the random shRNA technology. The authors thank their colleagues, Takahiro Watanabe, Takashi Aoki and Eri Ussuzaka, for providing key data and helpful discussions on this project. The authors thank their colleague, Takayuki Hirai, for performing the statistical analyses.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 24, 2014; revised November 20, 2014; accepted November 25, 2014, published OnlineFirst December 18, 2014.

References
10. Arpino G, De Angelis C, Giuliano M, Giordano A, Falato C, De Laurentiis K. Masuda, Y. Kameyama, Y. Akatsu (Genofunction Inc.) for collaborating on the random shRNA tech-
23. Sogawa K, Fuji-Kuriyama Y. Ah receptor, a novel ligand-activated tran-
24. Okano ST, Quattrocchi LC, Pooler D, Iwahashi M, Diahy R. A dioxin-


Molecular Cancer Therapeutics

A Novel Compound, NK150460, Exhibits Selective Antitumor Activity against Breast Cancer Cell Lines through Activation of Aryl Hydrocarbon Receptor

Kazuteru Fukasawa, Shigehide Kagaya, Sakiko Maruyama, et al.

Mol Cancer Ther 2015;14:343-354. Published OnlineFirst December 18, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-0158

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2014/12/18/1535-7163.MCT-14-0158.DC1

Cited articles
This article cites 58 articles, 24 of which you can access for free at:
http://mct.aacrjournals.org/content/14/2/343.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mct.aacrjournals.org/content/14/2/343.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.