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NK150460: a potential antitumor drug activating AhR

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Abbreviation lists:

3MC	3-methylcholanthrene
AhR	aryl hydrocarbon receptor
ANF	α-naphthoflavone
ANOVA	analysis of variance
ARNT	aryl hydrocarbon receptor nuclear translocator
ATCC	American Type Culture Collection
BaP	benzo[α]pyrene
BNF	β-naphthoflavone
ChIP	chromatin immunoprecipitation
CYP1A1	cytochrome P450 1A1
E2	17b-estradiol
EMT	epithelial-to-mesenchymal transition
ER	estrogen Receptor
ERE	estrogen-responsive element
FBS	fetal bovine serum
HAT	histone acetyltransferase
Her2	human epidermal growth factor receptor type 2
MCDF	6-methyl-1,3,8-trichlorodibenzofuran
MG-132	Z-Leu-Leu-Leu-H (aldehyde)
PgR	progesterone receptor
pH2AX	phosphorylation of histone H2AX
pS2	trefoil factor 1 precursor
RT-PCR	reverse transcription-polymerase chain reaction
SERD	selective estrogen receptor down-regulator
SERM	selective estrogen-receptor modulators

TAM	Tamoxifen citrate
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TNBC	triple negative breast cancer
XRE	xenobiotic responsive element

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All authors except Shigeo Horie (SH) are employees of Nippon Kayaku Co., Ltd. and have no other relevant conflicts of interest to disclose. SH also has no conflicts of interest to disclose.

Abstract

Anti-estrogen 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 ERα 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.

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 35-40% 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 human epidermal growth factor receptor type 2 (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 breast cancer patients, combination of tamoxifen with an LH-RH agonist should be administered as initial treatment. For postmenopausal breast cancer patients, 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

anti-hormone 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 anti-hormone 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 anti-hormone therapies have been strongly desired.

ER is a member of the nuclear receptor superfamily and is activated by its ligand, 17β-estradiol (E2) (7). ER is known to be a transcription factor. Once activated by E2, ER is translocated into the nucleus. Then dimerized ER molecules bind to the motif sequence, estrogen-responsive element (ERE), on DNA to up-regulate several target genes, including trefoil factor 1 precursor (pS2) (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 (HAT) activity. As another example,

ATP-dependent chromatin-remodeling complexes, such as 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 anti-hormone 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-tetrahydrocinnolin-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 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 (TAM), 3-methylcholanthrene (3MC), α-naphthoflavone (ANF), β-naphthoflavone (BNF) and ICI182,780 were purchased from Sigma-Aldrich (St. Louis, MO). Lactacystin and Z-Leu-Leu-Leu-H (aldehyde) (MG-132) (proteasome inhibitors) were purchased from Peptide Institute, Inc. (Osaka, Japan).

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 American Type Culture Collection (ATCC). All cell lines were cultured in plates in accordance with the ATCC's instructions. A TAM-resistant human breast cancer cell line, KPL-1, was kindly provided by Dr. J. Kurebayashi of Kawasaki Medical School (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 one 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 PRMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum (FBS). Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

In vitro competition assay

MCF-7 cells were plated onto 24-well plates and cultured in phenol red-free PRMI-1640 supplemented with 10% charcoal-stripped FBS. After 2 days, [^{3}H]-β estradiol (final concentration: 3 nM) and various concentrations of E2, TAM 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 min with 0.05% methylene blue in 10 mM 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, Japan). Slow-release 17β-estradiol pellets (Innovative Research of America, FL) were implanted on the same day. Eight to nine days after implantation, rats with good growth of implanted tumors were divided into three groups (control and two NK150460 treatment arms) of four 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 $(\text{width}^2 \times \text{length})/2$. Differences in the relative tumor volume in the NK150460-treated groups compared with the control group on Day 25 were performed using one-way analysis of variance (ANOVA) with two-taied Dunnett's post hoc test.

Random shRNA screening

Cell-based screening using a random shRNA library was performed by GenoFunction Inc. (Ibaraki, Japan). In brief, a random shRNA library was transfected into MCF-7 cells by vesicular stomatitis virus-G lentivirus vector infection. An shRNA targeting EGFP was used as a control. Stable transfectants, selected with blasticidin S, were treated with 10 μM 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 μM 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. (Chiba, Japan). Gene expression data were analyzed with the GeneSpring 7.2 program. The data was 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 (XREs)-TATA-luciferase cDNA (20), were kindly provided by Dr. F. Ohtake of the University of Tokyo. CYP1A1 complementary DNA was amplified by RT-PCR and subcloned into the pcDNA3 vector (Invitrogen Corp., Carlsbad, CA). Plasmids were

transfected with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

All siRNAs, including the negative-control siRNA (Stealth™ RNAi Negative Control Low GC Duplex), were purchased from Invitrogen and transfected into cultured cell lines with Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. All experiments were performed in triplicate. Briefly, for cell growth assay, 24 hours before transfection, $5x10^4$ SK-BR-3 cells were plated onto 24-well culture dishes. Next, 20 pM of siRNA duplexes and 1 μL of Lipofectamine 2000 in Opti-MEM Reduced-Serum Medium (Invitrogen) that had been preincubated for 20 minutes at room temperature were added to each well. The dishes were incubated at 37°C for 24 hours, followed by replacement with fresh culture medium. Then, to study the effect of siRNA on the growth-inhibitory activity of 150460, various concentrations of the compound were added to the wells, and the cells were cultured for another 48 hours.

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, Madison, WI). In brief, $1x10^4$ 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-polymerase chain reaction (RT-PCR)

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM 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 ISOGEN (Nippon Gene, Tokyo, Japan) 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, Foster City, CA) on an ABI prism 7000 apparatus (Applied Biosystems).

Chromatin immunoprecipitation (ChIP) 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 an acetyl-histone H4

immunoprecipitation assay kit (Upstate Biotechnology, Lake Placid, NY). 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 co-immunoprecipitated with antibodies against AhR, ARNT, ERα or p300. The ERE motif in the pS2 promoter and the XRE motif in the CYP1A1 promoter were amplified by PCR using specific primers. The specific primer pairs were 5'-CTG CGC CAG GCC TAC AAT TT ATT-3' and 5'-TCC TAC TCA TAT CTG AGA GGC CCT-3' for the *pS2* promoter, and 5'-TTC GCC ATC CAT TCC GAT CCT TCA-3' and 5'-GGG ACT CCT CTT CGT CAT TTTT TGC-3' for the *CYP1A1* promoter. The PCR products were subjected to 1.5% agarose gel electrophoresis and 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 filtered. Cellular metabolites in the extract were detected with an LC-MS/MS system composed of Shimadzu LC-10AD pumps (Shimadzu, Kyoto, Japan) coupled to an API-4000^{TM} mass spectrometer (Applied Biosystems). The chemical structure of M-2 was identified by nuclear magnetic resonance (NMR).

Statistical Analysis

All data are expressed as the mean ± standard deviation. Statistical significance was performed one-way ANOVA with post hoc testing using two-tailed Dunnett's multiple comparison test for comparison with the control group. (Figure 1B, C and F) or two-way ANOVA with post hoc testing using two-tailed Dunnett's multiple comparison test for comparison with non-silencing control siRNA transfected group (Figure 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 (SERMs). 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 to that of TAM (Fig. 1B). NK150460 also inhibited two E2-induced endogenous ER target genes, PgR (21) (Fig. 1C). To determine whether NK150460 inhibits E2-dependent transactivation by a different mechanism from that of TAM, we examined whether NK150460 competes with E2 binding to ER *in vitro*. As expected, TAM 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 TAM. Next, we examined the growth-inhibitory activity of NK150460 against various breast cancer cell lines (Fig. 1E). As expected, both NK150460 and the comparator, TAM, 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, i.e., MDA-MB-453, MDA-MB-468 and SK-BR-3, whereas TAM did not. Proliferation of the other ER-/PgR- cell lines, i.e., MDA-MB-231 and MDA-MB-435S, was not inhibited by either NK150460 or TAM (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 TAM-resistant cell line KPL-1, but did not inhibit proliferation of the non-breast cancer cell lines we examined (Supplementary Tables 1 and 2). 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 utilized 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 μM NK150460 for 8

days, exchanging the culture medium and NK150460 every day. Based on 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 non-specific 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 to 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. 72 of 169 colonies (42.6%) having shRNA-specific sequences contained a portion of the *ARNT* gene 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). 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. 1) had no effect. The knockdown effect of each siRNA was confirmed at both the mRNA (Supplementary Fig. 2) 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 3). We next investigated the reproducibility of NK150460-dependent CYP1A1 mRNA induction

using 9 cell lines, i.e., 3 ER-positive/NK150460-sensitive cell lines (MCF-7, T-47D and KPL-1), 3 ER-negative/NK150460-sensitive cell lines (SK-BR-3, MDA-MB-453 and MDA-MB-468) and 3 ER-negative/NK150460-insensitive cell lines (MDA-MB-231, MDA-MB-435 and Hs0578T). CYP1A1 mRNA, measured by quantitative RT-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 transactivational 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, i.e., 3MC or BNF. NK150460 induced XRE-driven luciferase activity, but not as strongly as by 3MC and BNF (Supplementary Fig. 3). We also confirmed that NK150460 induced gene expression of *CYP1A2* and *CYP1B1* as well as *CYP1A1* in MCF-7 cells (Supplementary Fig. 4).

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-trichlorodibenzofuran (MCDF) and benzo[α]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 post-translational event because it was strongly inhibited by two proteasome inhibitors, MG-132 and lactacystin (28) (Fig. 3A). To elucidate whether AhR is involved in NK150460-dependent down-regulation of endogenous ERα, we treated MCF-7 cells simultaneously with NK150460 and an AhR antagonist, ANF (29). ANF inhibited that down-regulation in a dose-dependent manner (Fig. 3B). Two other AhR agonists, 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. ICI182,780 (fulvestrant) is known to be a selective estrogen receptor down-regulator (SERD) (32). NK150460 has similar characteristics with ICI182,780 because it inhibits E2-dependent transactivation and decreases cellular ERα protein in breast cancer cell lines. However, the mechanism of ERα down-regulation by ICI182,780 is totally different from that of NK150460 because ERα degradation by ICI182,780 was not affected by ANF treatment (Fig. 3C).

Next, we performed ChIP assay to investigate if AhR, ERα and related proteins were recruited onto the ERE 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 ERE 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 ERE 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, i.e., AFP464

(a lysyl pro-drug of aminoflavone) and Phortress (L-lysylamide pro-drug 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 if 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. 5). 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 bona fide 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. 6. 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 CYP1As 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-dependent and time-dependent phosphorylation of histone H2AX (pH2AX) (40). 3MC and 5F-203 induced pH2AX and expression of p53 and its target gene, p21, in MCF-7 cells. In contrast, NK150460 showed no 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 4). We next examined if 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-insensitive 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 anti-hormone therapies are playing important roles in current clinical practice. The first class of anti-hormone agents includes selective estrogen-receptor modulators (SERM) 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 anti-hormone agents, suggesting that NK150460 could become a fourth class of anti-hormone therapy in the future.

Although we discovered the novel compound NK150460, its precise mechanism was unclear. To solve this problem, we carried 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. 4), but

we could not examine its induction because the Gene Chip Human Genome Focus Array does not include a CYP1A2 gene probe. Based on 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 whose 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 non-specific 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. 3), suggesting that the mechanisms for transcriptional regulation differ among AhR agonists. Matthews et al. 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 (42). 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 et al. reported that the 5' flanking region of the pS2 gene has three motifs that resemble XRE core sequences (43). 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 anti-proliferation 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 in the cellular extract (Supplementary Fig. 7). 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 NK105460 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 2 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 1 trial against solid tumors (NCT00348699) is now ongoing. As for Phortress, a Phase 1 study was started, but it was terminated due to 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 anti-proliferation 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-453 and MDA-MB-468 are classified as TNBC. There is a lack of effective non-chemotherapeutic agents for TNBC (48). Although several molecular targeting agents, including poly(ADP-ribose) polymerase 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 et al. 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 (56). 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 et al.

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 (M), a mesenchymal stem–like subtype (MSL) and a luminal androgen receptor (LAR) subtype (58). Interestingly, most TNBC cell lines assigned to Basal B in Neve's subtyping belong to M or MSL in Lehmann's subtyping. Based on 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 morphological and phenotypical changes indicative of EMT. Interestingly, in those epithelial cells AhR bound to and co-localized 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.

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Figure Legends

Figure 1. (A) Chemical structure of NK150460. (B) 17β estradiol (E2)-dependent ERE activity was inhibited by NK150460. Stable transfectants of the T-47D cell line were cultured in phenol red-free medium containing dextran-coated, charcoal-treated FBS. E2 and various concentrations (0.01, 0.04, 0.16, 0.63 and 2.5 μM) of NK150460 or tamoxifen were added to the medium, followed by continuous culture for 24 hours. ERE activities were measured by luciferase assay. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, vs vehicle-control group (Dunnett's multiple comparison test). (C) E2-dependent progesterone receptor (PgR) mRNA expression was inhibited by NK150460. MCF-7 was cultured in phenol red-free medium containing dextran-coated, charcoal-treated FBS. E2 and either NK150460 or tamoxifen (0.01, 0.5 and 2.5 μM) were simultaneously added and incubated for 16 hours. PgR gene expression was quantified by quantitative RT-PCR. ** $P<0.01$, *** $P<0.001$, vs vehicle-control (Dunnett's multiple comparison test). (D) *In vitro* competition assay for E2 and its receptors. MCF-7 cells were incubated with [^{3}H]-β estradiol and either E2, tamoxifen (TAM) or NK150460 (NK) for one hour. After washing the cells, the cellular radioactivity was counted with a liquid scintillation counter. (E) NK150460 exhibited selective antitumor activity against breast cancer cell lines. MCF-7 and T-47D are ER-positive cell lines.

MDA-MB-453, MDA-MB-468, SK-BR-3, MDA-MB-231 and MDA-MB-435S are ER-negative cell lines. (F) Effect of NK150460 on the growth of ZR-75-1 tumor xenografts transplanted to rats. NK15040 (20 mg/kg or 100 mg/kg) or vehicle was administered orally every day for 24 days. ** $P<0.01$, *** $P<0.001$, vs vehicle-control group (Dunnett's multiple comparison test).

Figure 2. (A) Screening for candidate genes essential for growth-inhibitory activity of NK150460 using a random shRNA library. Random shRNA-transfected MCF-7 cells were plated and cultured with NK150460 for 8 days, and the remaining shRNAs were recovered. EGFP shRNA was used as a reference control. After repeating the screening three times, NK150460-resistant colonies were seen on the plates (left photo). On the other hand, no obvious colonies were seen in the control group (right photo). (B) shRNAs were recovered from the remaining cells after the 3rd screening and subcloned into plasmids for transformation of *E. coli*. 384 colonies were picked up. The shRNA sequence was able to be analyzed for 169 colonies, and 72 contained a portion of the ARNT sequence. (C) Abrogation of growth-inhibitory activity of NK150460 by transfection of siRNA. The NK150460-sensitive cell line SK-BR-3 was transfected with ARNT siRNA, AhR siRNA or non-silencing control siRNA (left graph), or transfected

with two independent Cyp1A1 siRNAs, two independent Cyp1A2 siRNAs or non-silencing control siRNA (right graph). At 24 hours after transfection, various concentrations of NK150460 were added to the cell cultures. Two days later, the culture medium was removed, the cells were stained with methylene blue and the absorbance at 660 nm of each sample was measured. * $P<0.05$, *** $P<0.001$, vs non-silencing control siRNA-transfected group (Dunnett's multiple comparison test). (D) Knockdown of target genes by each siRNA. AhR (upper left), ARNT (upper right) and CYP1A1 (lower) were detected by western blotting. Cellular extracts were prepared at 24 hours after siRNA transfection into SK-BR-3 cells. To detect CYP1A1, 1 μM NK150460 was added to the culture medium on the day after siRNA transfection, followed by incubation for 24 hours. (E) Induction of CYP1A1 mRNA in various breast cancer cell lines. Each cell line was treated with 0.5 (open bar), 2.5 (grey bar) or 12.5 (closed bar) μM NK150460. Total RNA was collected at 3 hours after starting the NK150460 treatment. CYP1A1 mRNA in each sample was measured by quantitative RT-PCR. (F) Induction of CYP1A1 protein in MCF-7 by treatment with NK150460. MCF-7 cells were treated with various concentrations of NK150460 for 8 hours, and then both the CYP1A1 and β-actin levels were examined by western blotting.

Figure 3. Modulation of ERα by NK150460. (A) NK10460-dependent ERα suppression was inhibited by proteasome inhibitors. MG132 (10 μM) or lactacystin (10 μg/mL) (proteasome inhibitors) was added to MCF-7 cells, and 30 min later 10 μM NK150460 was added. Incubation was for 8 hours. Whole-cell lysates were extracted, and ERα expression in each sample was determined by western blotting. (B) NK10460-dependent ERα suppression was inhibited by an AhR antagonist, α-naphthoflavone (ANF). MCF7 cells were treated simultaneously with NK150460 and ANF for 24 hours. Whole-cell lysates were extracted, and ERα expression in each sample was determined by western blotting. (C) Inhibition of ERα degradation by ANF was specific for AhR agonists, not SERD. MCF-7 cells were treated with NK150460, an AhR agonist (BNF or 3MC) or ICI172,780 combined with 10 μM ANF for 24 hours. ERα expression in each sample was determined by western blotting. (D) Ligand-dependent recruitment of ER or AhR-related protein onto XRE or ERE. MCF-7 cells were treated with NK150460 or 3MC for 0, 30, 45, 60, 75 and 90 min. Protein/DNA complexes were co-immunoprecipitated with antibodies against AhR, ARNT, ERα or p300. The XRE motif in the *CYP1A1* promoter and ERE motif in the *pS2* promoter were amplified using specific primers.

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 insert 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. Non-silencing control siRNA was used as a negative control. After transfection, 10 μM 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 Non-silencing 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.

Figure 5. The antitumor activity of NK150460 was independent of a DNA damage response. MCF-7 cells were treated with various concentrations of NK150460 (20, 100 μM) or other AhR agonists, i.e., 3MC (0.8, 4, 20, 100 μM), BNF (0.8, 4, 20, 100 μM) or 5F-203 (0.04, 0.2, 1 μM), for 24 hours. Cellular proteins were extracted, and p53, p21 and histone H2AX (pH2AX) were detected by western blotting.

Figure 6. (A) Morphology of NK150460-sensitive (MCF-7 and T-47D) and -insensitive (MDA-MB-231 and Hs0578T) cell lines. The length of the scale bar is 100 μm. (B) Expression patterns of epithelial- and mesenchymal-related genes in NK105460-sensitive and -insensitive cell lines. Total RNA was extracted from 6 cell lines, and gene expressions were determined by quantitative RT-PCR. Representing epithelial-related genes, the quantities of CLDN7, KRT19, PROM2, RAB25, SPDEF and STARD mRNAs in each cell line are expressed as relative values compared to

MCF-7's respective values as 100. Representing mesenchymal-related genes, the quantities of CAV1, COL4A1, IGFBP7, SPARC, VIM and TGFB1 mRNAs in each cell line are expressed as relative values compared to MDA-MB-231's respective values as 100. Abbreviations on the horizontal bar are as follows. A: MCF-7, B: T-47D, C: SK-BR-3, D: MDA-MB-231, E: MDA-MB-435S, F: Hs0578T.

Figure 1

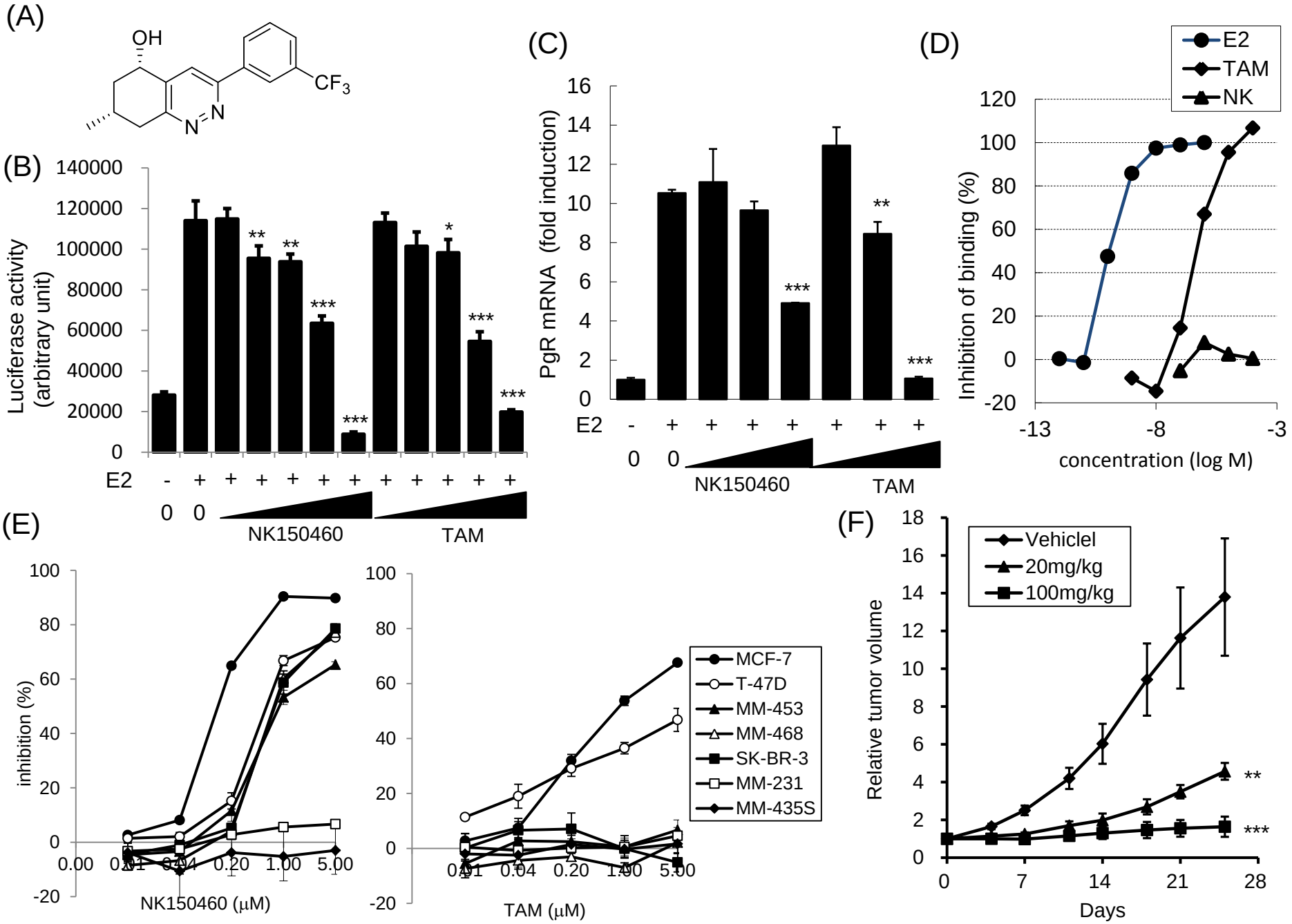
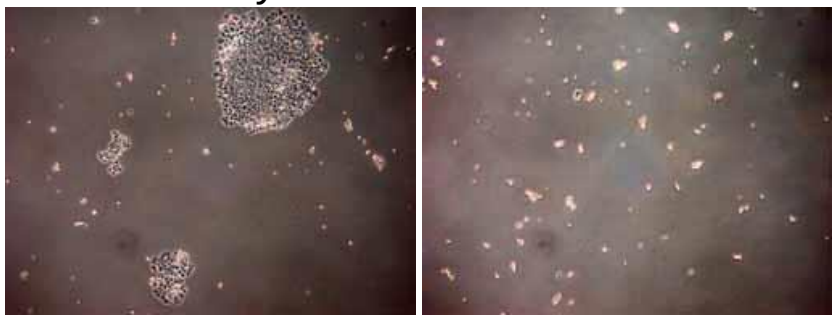
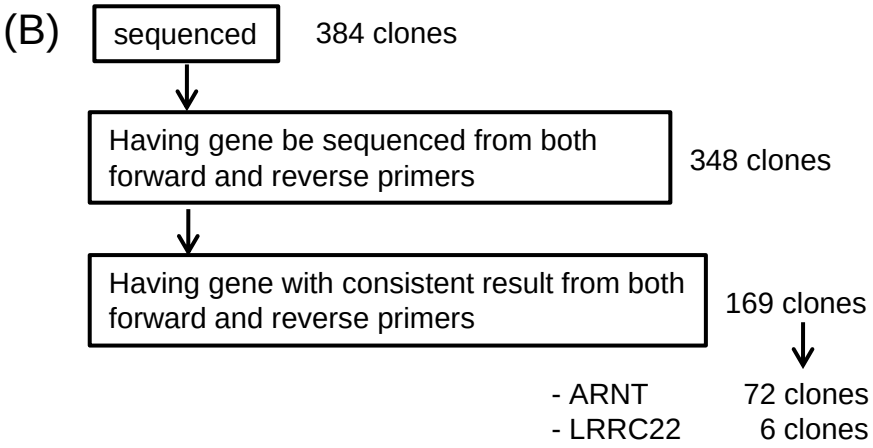
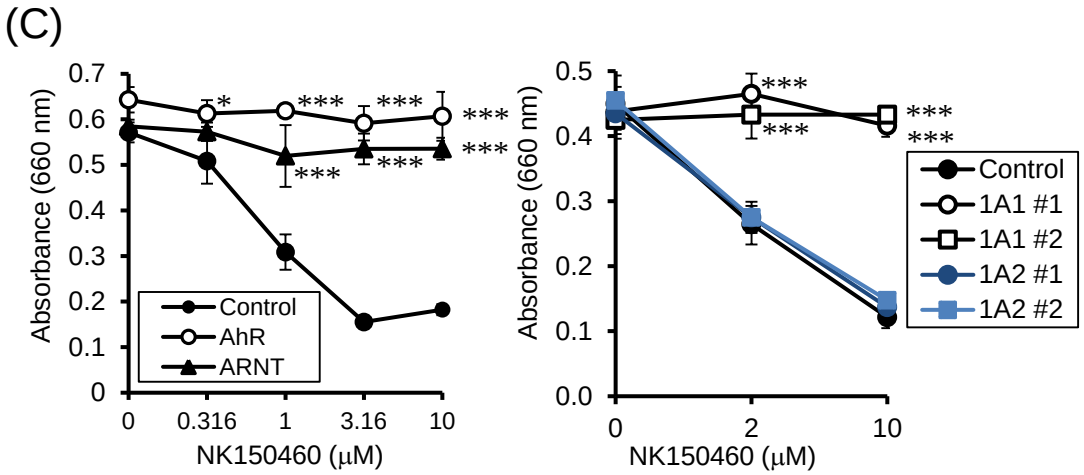
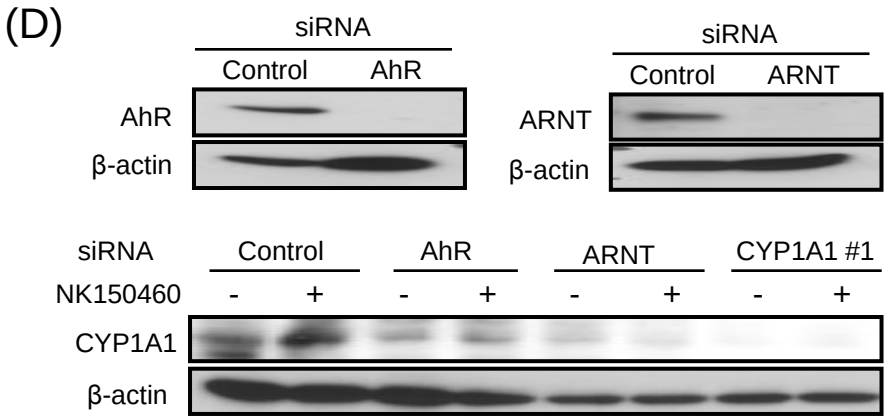

(A)
OH
CF3
N
N
(B)
Luciferase activity (arbitrary unit)
140000
120000
100000
80000
60000
40000
20000
0
E2
0 0
NK150460
TAM
(C)
PgR mRNA (fold induction)
16
14
12
10
8
6
4
2
0
E2
0 0
NK150460
TAM
(D)
E2
TAM
NK
Inhibition of binding (%)
120
100
80
60
40
20
0
-20
-13
-8
-3
concentration (log M)
(E)
inhibition (%)
100
80
60
40
20
0
-20
0.00
0.01
0.04
0.20
1.00
5.00
NK150460 (μM)
TAM (μM)
MCF-7
T-47D
MM-453
MM-468
SK-BR-3
MM-231
MM-435S
(F)
Relative tumor volume
18
16
14
12
10
8
6
4
2
0
Vehiclel
20mg/kg
100mg/kg
0
7
14
21
28
Days

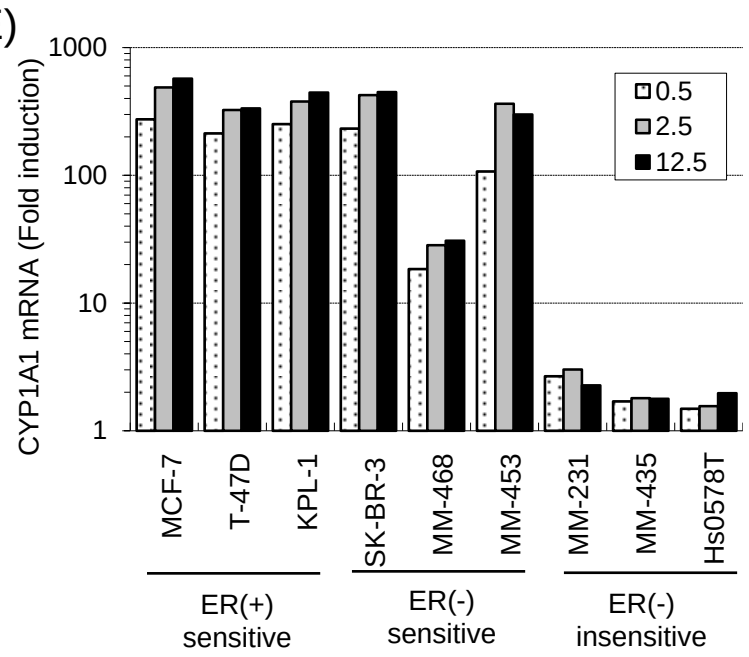
Figure 2


(A)
3rd library
EGFP


(B)
sequenced
384 clones
Having gene be sequenced from both forward and reverse primers
348 clones
Having gene with consistent result from both forward and reverse primers
169 clones
- ARNT
72 clones
- LRRC22
6 clones


(C)
Absorbance (660 nm)
NK150460 (μM)
Control
AhR
ARNT
1A1 #1
1A1 #2
1A2 #1
1A2 #2


(D)
siRNA
Control
AhR
ARNT
β-actin
CYP1A1 #1
NK150460
CYP1A1


(E)
CYP1A1 mRNA (Fold induction)
0.5
2.5
12.5
MCF-7
T-47D
KPL-1
SK-BR-3
MM-468
MM-453
MM-231
MM-435
Hs0578T
ER(+) sensitive
ER(-) sensitive
ER(-) insensitive

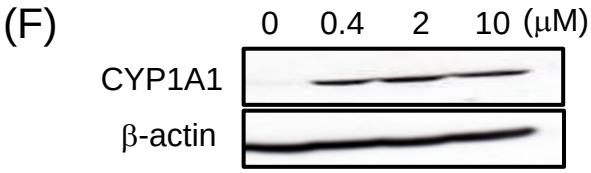

(F)
0 0.4 2 10 (μM)
CYP1A1
β-actin

Figure 3

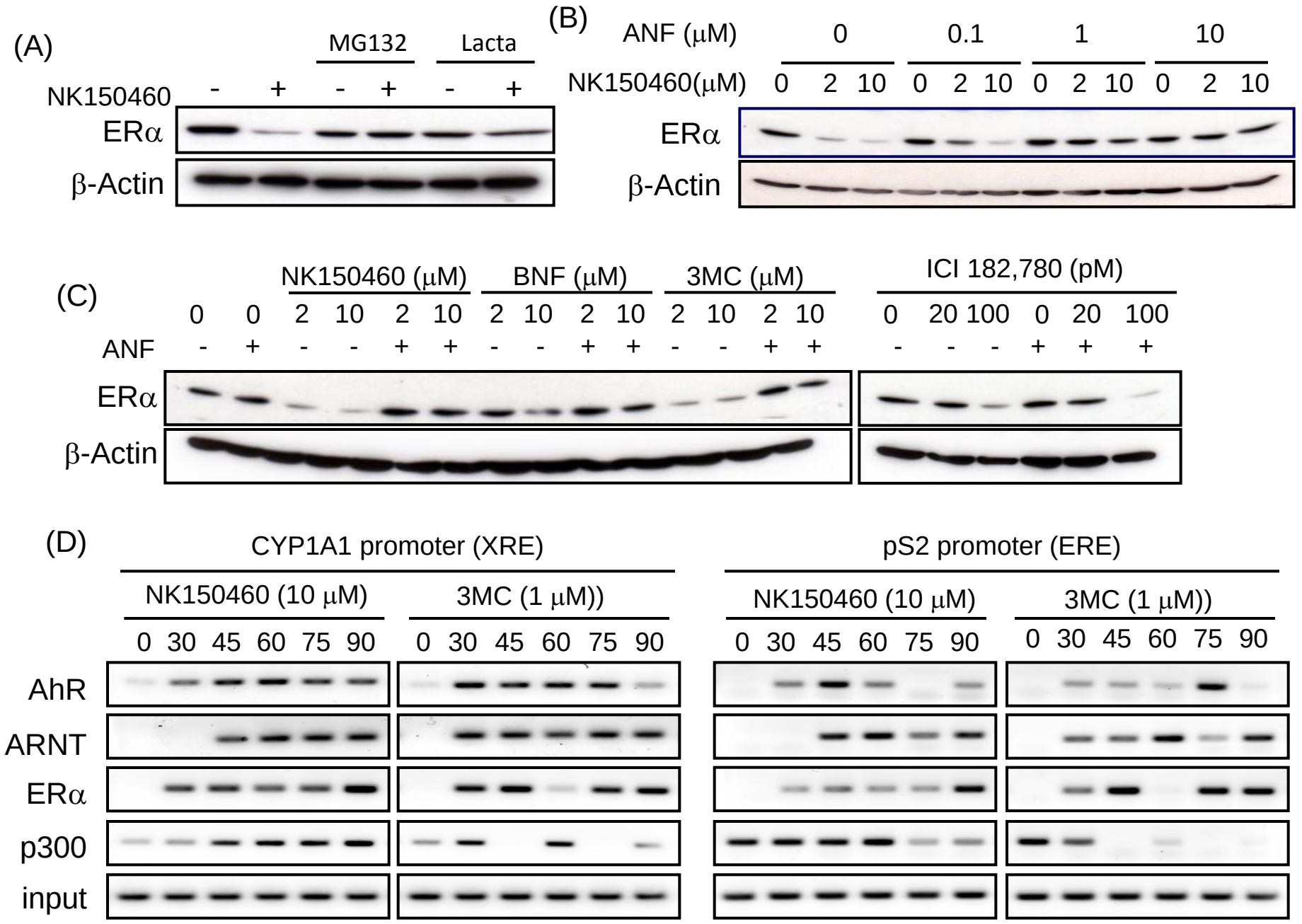
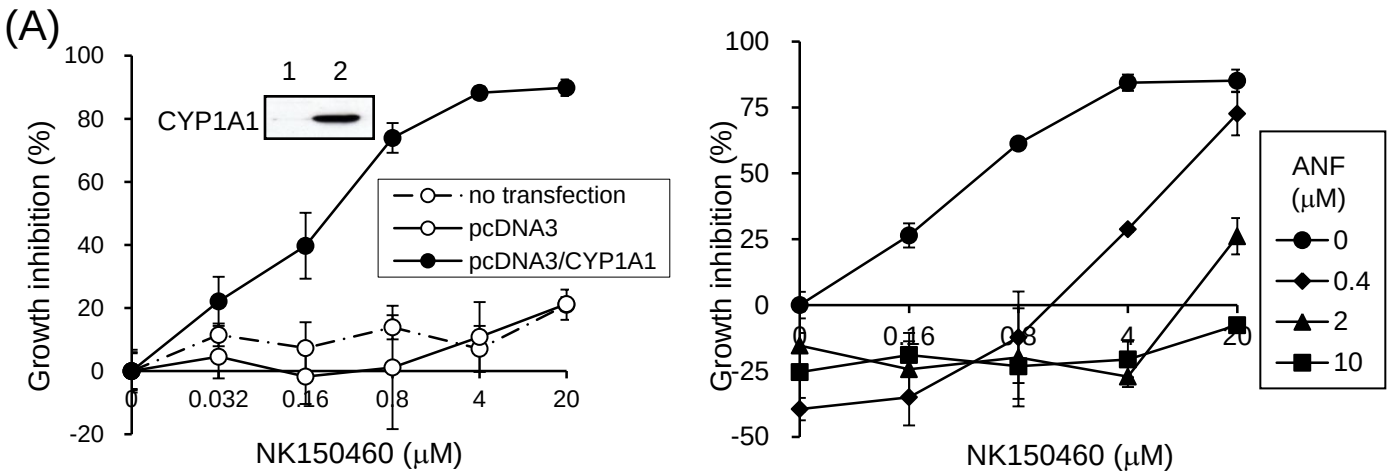
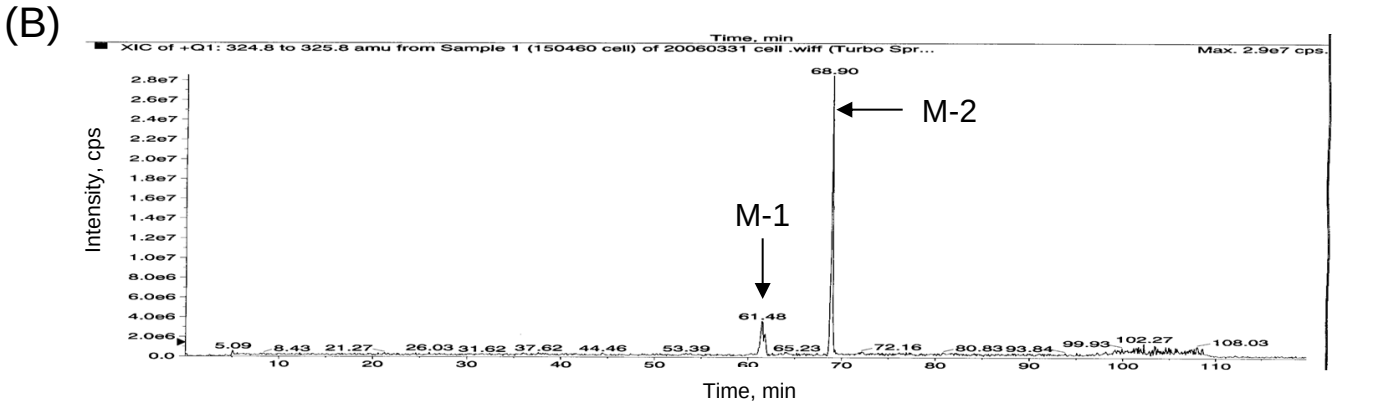
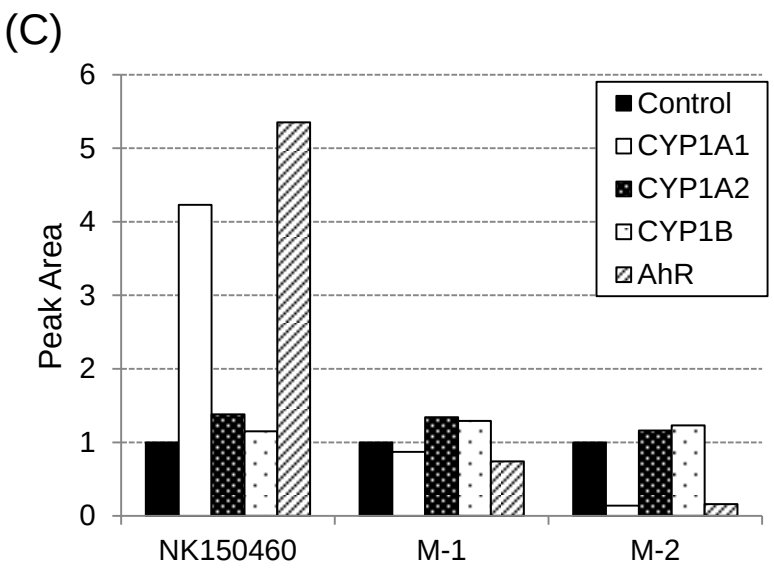
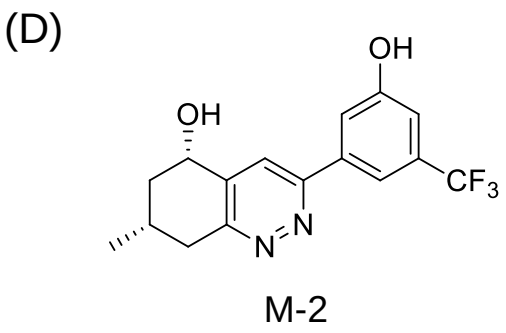

(A)
MG132
Lacta
NK150460
- + - + - +
ERα
β-Actin
(B)
ANF (μM)
0
0.1
1
10
NK150460(μM)
0 2 10 0 2 10 0 2 10 0 2 10
ERα
β-Actin
(C)
NK150460 (μM)
BNF (μM)
3MC (μM)
ICI 182,780 (pM)
0 0 2 10 2 10 2 10 2 10 2 10 2 10
0 20 100 0 20 100
ANF
- + - - + + - - + + - - + + - - - + + +
ERα
β-Actin
(D)
CYP1A1 promoter (XRE)
pS2 promoter (ERE)
NK150460 (10 μM)
3MC (1 μM))
0 30 45 60 75 90
AhR
ARNT
ERα
p300
input

Figure 4


(A)
CYP1A1
1 2
no transfection
pcDNA3
pcDNA3/CYP1A1
Growth inhibition (%)
NK150460 (μM)
ANF (μM)
0
0.4
2
10


(B)
Time, min
XIC of +Q1: 324.8 to 325.8 amu from Sample 1 (150460 cell) of 20060331 cell .wiff (Turbo Spr...
Max. 2.9e7 cps.
Intensity, cps
M-1
M-2
Time, min


(C)
Peak Area
Control
CYP1A1
CYP1A2
CYP1B
AhR
NK150460
M-1
M-2


(D)
OH
OH
CF3
N
N
M-2

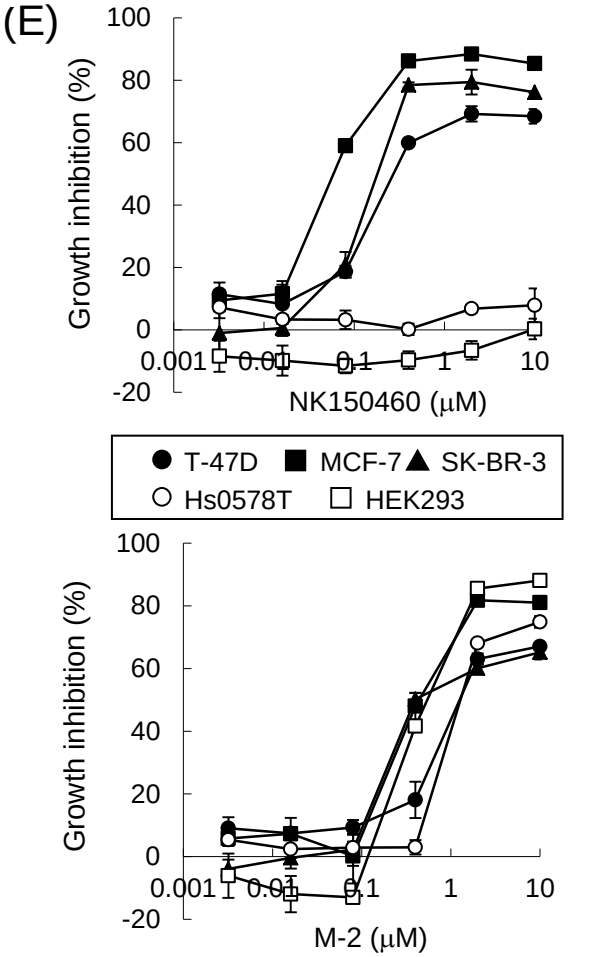

(E)
Growth inhibition (%)
NK150460 (μM)
T-47D
MCF-7
SK-BR-3
Hs0578T
HEK293
M-2 (μM)

Figure 5

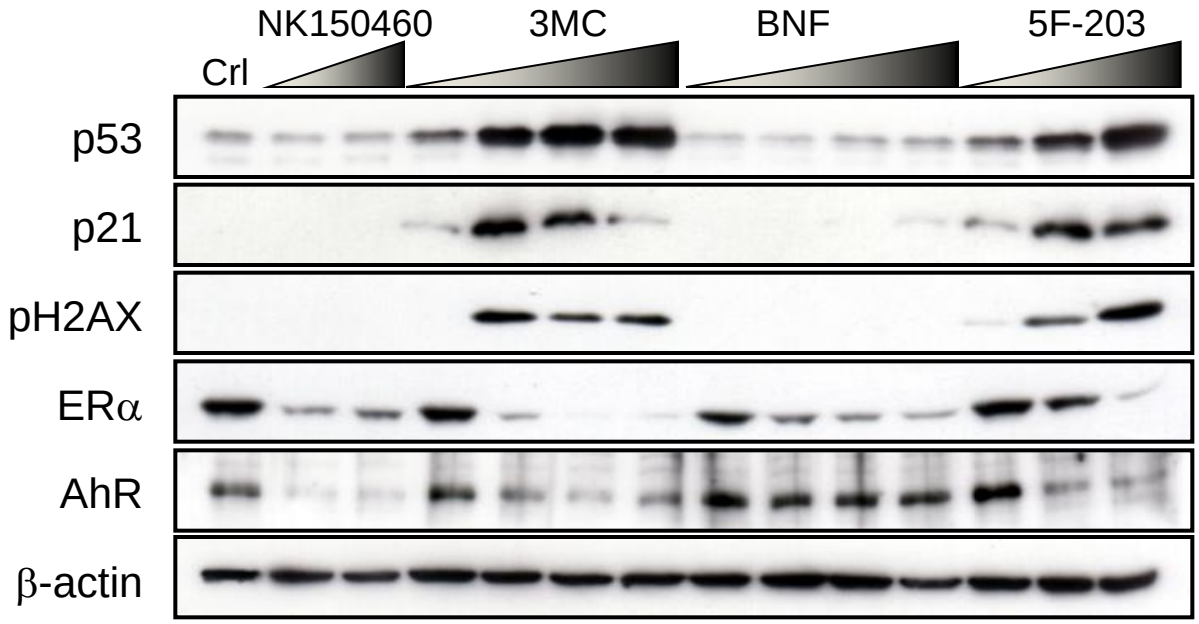
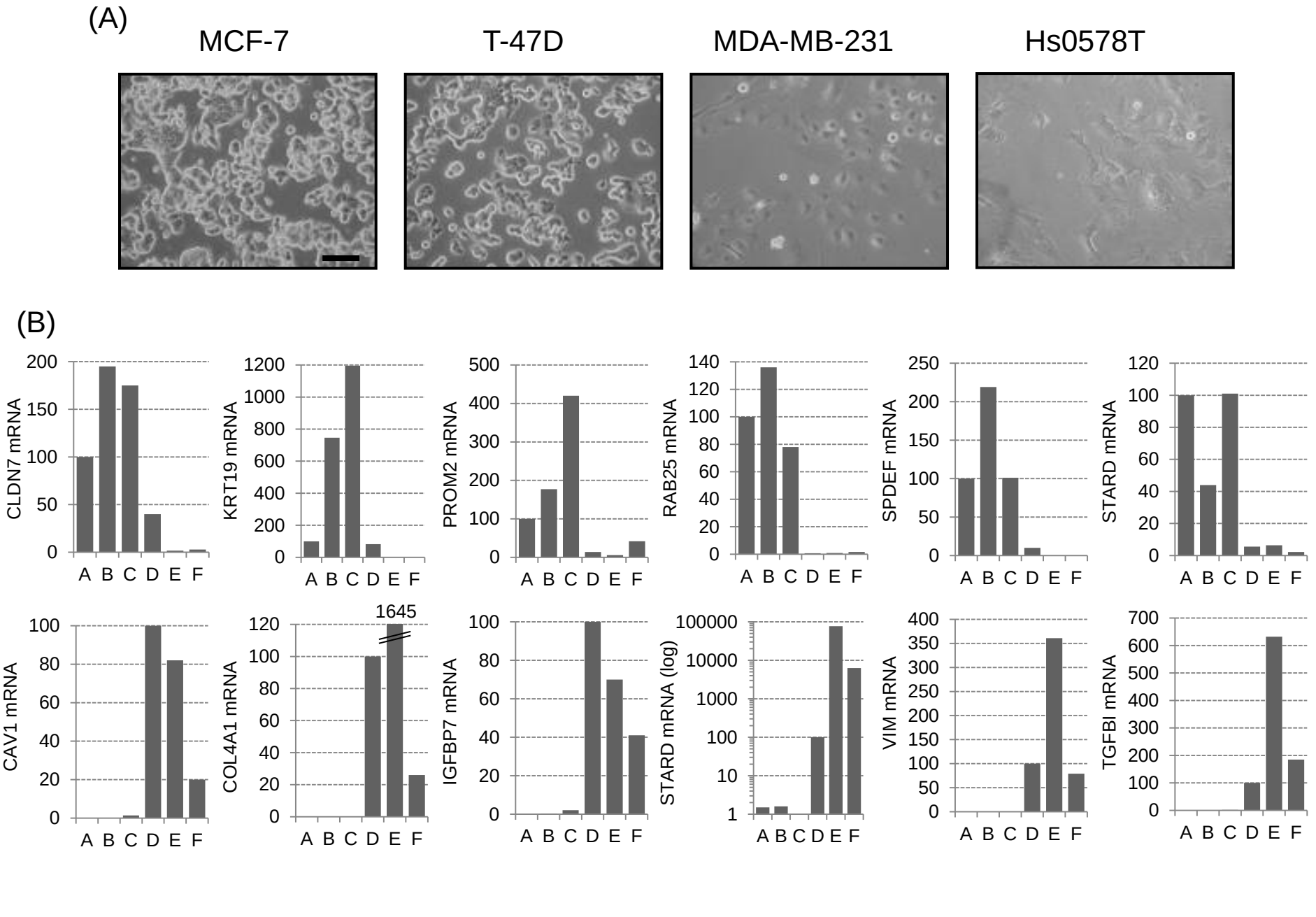


Figure 6


(A)
MCF-7
T-47D
MDA-MB-231
Hs0578T
(B)
CLDN7 mRNA
KRT19 mRNA
PROM2 mRNA
RAB25 mRNA
SPDEF mRNA
STARD mRNA
CAV1 mRNA
COL4A1 mRNA
1645
IGFBP7 mRNA
STARD mRNA (log)
VIM mRNA
TGFBI mRNA
A B C D E F