TAS-116, a Highly Selective Inhibitor of Heat Shock Protein 90α and β, Demonstrates Potent Antitumor Activity and Minimal Ocular Toxicity in Preclinical Models

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Abstract

The molecular chaperone heat shock protein 90 (HSP90) plays a crucial role in cancer cell growth and survival by stabilizing cancer-related proteins. A number of HSP90 inhibitors have been developed clinically for cancer therapy; however, potential off-target and/or HSP90-related toxicities have proved problematic. The 4-(1H-pyrazolo[3,4-b]pyridine-1-y)benzamide TAS-116 is a selective inhibitor of cytosolic HSP90α and β that does not inhibit HSP90 paralogs such as endoplasmic reticulum GRP94 or mitochondrial TRAP1. Oral administration of TAS-116 led to tumor shrinkage in human tumor xenograft mouse models accompanied by depletion of multiple HSP90 clients, demonstrating that the inhibition of HSP90α and β alone was sufficient to exert antitumor activity in certain tumor models. One of the most notable HSP90-related adverse events universally observed to differing degrees in the clinical setting is visual disturbance. A two-week administration of the isoxazole resorcinol NVP-AUY922, an HSP90 inhibitor, caused marked degeneration and disarrangement of the outer nuclear layer of the retina and induced photoreceptor cell death in rats. In contrast, TAS-116 did not produce detectable photoreceptor injury in rats, probably due to its lower distribution in retinal tissue. Importantly, in a rat model, the antitumor activity of TAS-116 was accompanied by a higher distribution of the compound in subcutaneously xenografted NCI-H1975 non–small cell lung carcinoma tumors than in retina. Moreover, TAS-116 showed activity against orthotopically transplanted NCI-H1975 lung tumors. Together, these data suggest that TAS-116 has a potential to maximize antitumor activity while minimizing adverse effects such as visual disturbances that are observed with other compounds of this class. Mol Cancer Ther; 14(1). 14–22. ©2014 AACR.

Introduction

The molecular chaperone heat shock protein 90 (HSP90) is a highly conserved protein that regulates the activity and stability of a diverse range of “client” proteins (1, 2). HSP90 clients include many of the cancer-related proteins necessary for tumor development, including receptor tyrosine kinases, signal transducers, cell-cycle regulators, and transcriptional factors (1-3). HSP90 is reported to be specifically overexpressed (4, 5) and to exist as activated multi-chaperone complexes in cancer cells and cancer tissues (6, 7); indeed, it has been proposed that cancers are highly “addicted” to HSP90 for their survival and proliferation (1). HSP90 has therefore emerged as an attractive target for cancer interventions, and many HSP90 inhibitors have been developed (8).

Though HSP90 inhibitors have been shown in different preclinical models to exert their potent antitumor activities by destabilizing HSP90 clients (9-13), they have also been shown to have limited single-agent activity in the clinical setting (3, 8, 14). One explanation for this discrepancy may be that the levels of drug exposure reached in patients is insufficient to effectively inhibit tumor cell HSP90 because of undesirable off-target and/or HSP90-related adverse events. For example, liver toxicity was a major limitation in the clinical development of the first-generation ansamycin derivatives tanespimycin (17-AAG) and alvespimycin (17-DMAG; ref. 15). It is possible that this excess liver toxicity is related to the quinone structure of ansamycin (16). The second generation of HSP90 inhibitors are based on a diverse variety of chemical scaffolds and exhibit less liver toxicity (11, 13). However, these compounds cause other dose-limiting toxicities, some of which are difficult to manage. For example, neurological toxicities such as syncope and dizziness were seen in a phase I trial of the purine analogue BIIB021 (17), although there is no evidence to support this to be target or purine-scaffold related. In clinical trials of other HSP90 inhibitors, the most commonly observed HSP90-related adverse events have been visual disorders such as night blindness, photopsia, blurred vision, and visual impairment, though the frequency and degree varied among the compounds (18-21). For example, 43% of...
patients suffered visual disturbances in a phase I trial of NVP-ALIY922 (21). Though HSP90-related visual disorders, which have included grade 3 events, are reversible (21), these adverse events interfere with obtaining sufficient drug exposure because of drug interruption or discontinuation, or dose reduction.

In this report, we describe our preclinical characterization of TAS-116, a novel, selective HSP90α and β inhibitor, and show that, due to limited exposure of nontarget eye tissues and greater exposure of target tumor tissues to the compound, TAS-116 has a favorable therapeutic index.

Materials and Methods

Chemical compounds

The HSP90 inhibitors TAS-116 (Fig. 1A), NVP-ALIY922, BIIB021, SNX-2112, and ganetespib were synthesized at Taiho Pharmaceutical, Co. Ltd., and 17-AAG and 17-DMAG were purchased from Biotrend Chemikalien GmbH and LC Laboratories, respectively. Fluorescein isothiocyanate labeled geldanamycin-FITC (22, 23) was produced and geldanamycin-FITC (IC50) was determined and Ki values were calculated from the IC50 values with the Cheng–Prusoff equation (23, 24).

Western blot analysis

Cells were lysed in lysis buffer [M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific Inc.) supplemented with cOmplete, Mini, Protease Inhibitor Cocktail (Roche Applied Science) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche Applied Science)]. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc.). Membranes were then incubated with blocking One P blocking reagent (Nacalai Tesque, Inc.) and probed with the appropriate primary antibodies. The membranes were then incubated with horseradish peroxidase–linked secondary antibodies (Cell Signaling Technology, Inc.), and proteins were visualized by means of luminol-based enhanced chemiluminescence (Thermo Fisher Scientific Inc.). Luminescent images were captured with an LAS-3000 imaging system (Fuji Photo Film Co., Ltd.).

Proximity ligation assay

Cells were grown on Lab-Tek II Chambered Coverglass (Thermo Fisher Scientific Inc.), and then treated with TAS-116 or 17-AAG for 8 hours. The cells were then fixed in 4% paraformaldehyde and blocked in Duolink II solution (Sigma-Aldrich Co. LLC.). The slides were incubated with anti-LRP6 rabbit (ab134146; Abcam plc) and anti-GRP94 mouse (AM09037PU-L) antibodies, followed by incubation with

HSP90 affinity determination

Recombinant human HSP90AA1 (HSP90α), HSP90AB1 (HSP90β), and TRAP1 were purchased from Enzo Life Sciences International, Inc., and HSP90B1 (GRP94) was purchased from ATGen Co., Ltd. The interactions of TAS-116 with proteins from the HSP90 protein family were determined by means of a fluorescence polarization competitive binding assay with geldanamycin-FITC (22, 23). Each compound was incubated with recombinant HSP90α, HSP90β, GRP94, or TRAP1 for 2 hours. Geldanamycin-FITC was added and the mixture was incubated for another 5 hours. The concentration of each compound required to reduce by 50% the amount of recombinant proteins bound to geldanamycin-FITC (IC50) was determined and Ki values were calculated from the IC50 values with the Cheng–Prusoff equation (23, 24).

Cell lines and cell culture

All cell lines were purchased from American Type Culture Collection in 2008 and 2009, and reauthenticated by means of short tandem repeat–based DNA profiling in 2012. Cells were cultured in McCoy’s 5A medium (HCT116), RPMI 1640 medium (NCI-N87 and NCI-H1975), or Iscove’s modified Dulbecco’s medium (MV-4-11) supplemented with 10% FBS.

Figure 1

Effects of TAS-116 on the levels of HSP90-related proteins and GRP94/LRP6 complex formation in HCT116 cells. A, chemical structure of TAS-116, 3-ethyl-4-[(3-(1-methylpyrazol-4-yl)-4-H-imidazo-[1,2]-1H-pyrazolo[3,4-b]pyridin-1-yl)benzamide. B, dose-dependent change of HSP90-related proteins in HCT116 cells treated with TAS-116 or 17-AAG for 8 hours. Western blotting was performed by using 10 µg of cell lysate. C, in situ proximity ligation assay in HCT116 cells treated with 3 µmol/L of TAS-116 or 1 µmol/L of 17-AAG for 8 hours. Primary mouse and rabbit antibodies against GRP94 and LRP6 were combined with secondary proximity ligation assay probes (Sigma-Aldrich Co., LLC.). Interaction events are visible as red dots. Nuclei were stained blue. Scale bar, 20 µm.
Duolink PLA Rabbit MINUS and PLA Mouse PLUS proximity probes (Sigma-Aldrich Co. LLC). The proximity ligation assay was performed by using a Duolink detection reagent kit (Sigma-Aldrich Co. LLC) according to the manufacturer's instructions. Nuclei were visualized by means of Hoechst 33342 (Nacalai Tesque, Inc.) staining. Stained cells were imaged by using an LSM 510 confocal microscope with a Plan-Apochromat 20×/0.8 objective (Carl Zeiss AG) and the LSM Image Browser software.

In vivo efficacy studies

Cancer cells were subcutaneously implanted into six-week-old male BALB/c nude mice (Clea Japan, Inc.) or six-week-old male F344 nude rats (Clea Japan, Inc.) and allowed to grow. Five or six animals were assigned to each group for each experiment, and TAS-116 formulated in 0.5% (w/v) hydroxypropyl methylcellulose (Metolose 60SH-50; Shin-Etsu Chemical Co., Ltd.) in H2O was administrated orally every day at 0.9–720.2 mg/kg/day. Tumor volume was calculated with the formula \[ \text{Volume} = \frac{1}{2} \times \text{length} \times \text{width}^2 \].

Quantitation of plasma, tumor, and retin tissue exposure to the test compounds

Test compounds were administered orally or intravenously to SD, LE, or tumor-bearing F344 nude rats. Plasma, retina, and/or tumor samples were collected according to the sampling schedule after dosing. Retina or tumor samples were homogenized by means of liquid chromatography with tandem mass spectrometry or high-performance liquid chromatography. The pharmacokinetic parameters of TAS-116 in mice, rats, and dogs were calculated by using noncompartmental methods.

Results

TAS-116 selectively inhibits HSP90α and β

TAS-116 is a novel, small-molecule HSP90 inhibitor that was discovered during a multiparameter lead optimization campaign to have high target-selectivity for certain HSP90 proteins (26). In the present study, TAS-116 inhibited geldanamycin–FITC binding to HSP90 proteins with Ki values of 34.7 nmol/L, 21.3 nmol/L, >50,000 nmol/L, and >50,000 nmol/L for HSP90α, HSP90β, GRP94, and TRAP1, respectively (Table 1). Other HSP90 inhibitors, including 17-AAG, 17-DMAG, NVP-AUY922, BIIB021, and SNX-2112, also inhibited GRP94 and TRAP1 in addition to HSP90α and β, though generally these compounds inhibited GRP94 and TRAP1 less than they did HSP90α and β. Furthermore, TAS-116 did not inhibit other ATPases such as HSP70 (IC50 >200 μmol/L, data not shown) or any of 48 different protein kinases tested (IC50 all >30 μmol/L, Supplementary Table S1).

The HSP90 selectivity of TAS-116 was further evaluated at the cellular level. Treatment of HCT116 cells with either 0.1 μmol/L of 17-AAG or 0.3 μmol/L of TAS-116 for 8 hours resulted in reduced levels of DDR1, which interacts with HSP90α (27), and induction of HSP70, which is a surrogate marker of cytosolic HSP90 inhibition (3, 28), at comparable levels for both compounds (Fig. 1B), thereby demonstrating the compounds’ cytosolic HSP90 inhibitory effects. GRP94 interacts with low-density lipoprotein receptor-related protein 6 (LRP6) and has a role in chaperoning LRP6 (29). Therefore, LRP6/GRP94 complex formation was evaluated in HCT116 cells in situ by means of a proximity ligation assay in which PLA-probe–labeled LRP6 or GRP94 molecules in close proximity were detected as red dots (30). LRP6/GRP complex formation with TAS-116 was inhibited by 50% at 14.7 μmol/L.

Table 1. Binding affinity of HSP90 inhibitors to proteins in the HSP90 protein family

<table>
<thead>
<tr>
<th>Compound</th>
<th>HSP90α Ki (nmol/L)</th>
<th>HSP90β Ki (nmol/L)</th>
<th>GRP94 Ki (nmol/L)</th>
<th>TRAP1 Ki (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAS-116</td>
<td>34.7 ± 8.4</td>
<td>213 ± 3.0</td>
<td>&gt;50,000</td>
<td>&gt;50,000</td>
</tr>
<tr>
<td>17-AAG</td>
<td>10.0 ± 1.3</td>
<td>6.6 ± 0.3</td>
<td>19.9 ± 4.2</td>
<td>968 ± 405.0</td>
</tr>
<tr>
<td>17-DMAG</td>
<td>5.8 ± 0.5</td>
<td>4.8 ± 0.4</td>
<td>12.6 ± 1.0</td>
<td>107.7 ± 14.7</td>
</tr>
<tr>
<td>NVP-AUY922</td>
<td>4.5 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>27.0 ± 2.6</td>
<td>718 ± 48.8</td>
</tr>
<tr>
<td>BIIB021</td>
<td>6.1 ± 0.9</td>
<td>5.5 ± 0.3</td>
<td>117.6 ± 23.7</td>
<td>101.9 ± 5.1</td>
</tr>
<tr>
<td>SNX-2112</td>
<td>11.5 ± 1.8</td>
<td>9.0 ± 0.5</td>
<td>720.2 ± 22.1</td>
<td>533.1 ± 58.0</td>
</tr>
</tbody>
</table>

NOTE: Data are presented as mean ± SEM (n = 9).

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was observed in untreated cells; however, treatment with 1 μmol/L of 17-AAG for 8 hours caused a reduction in the amount of LRP6/GRP94 complex detected (Fig. 1C) and slightly reduced was observed in untreated cells; however, treatment with 1 μmol/L of TAS-116 did not markedly affect the LRP6–GRP94 interaction. However, TAS-116 was unable to be used at higher concentrations because HCT116 cells became detached from the glass slide at concentrations of TAS-116 above 3 μmol/L. Our data suggest that 3 μmol/L of TAS-116 does not inhibit GRP94, whereas 0.3 μmol/L of TAS-116 inhibits HSP90α and β in HCT116 cells.

TAS-116 shows favorable pharmacokinetics
Pharmacokinetic profiling of TAS-116 in rodent and nonrodent species showed that TAS-116 was orally absorbed and had a bioavailability of almost 100% in mice, 69.0% in rats, and 73.9% in dogs without special formulation (Table 2). In addition, the inhibitory activity of TAS-116 on cytochrome P450 (CYP) enzymes was minimal; only a limited number of isoforms were inhibited (e.g., CYP2C9; 21.6 μg/mL and 18.5 μg/mL for CYP2C8 and CYP2C9, respectively; Supplementary Table S2). The half-life of TAS-116 (Cmax of TAS-116 at a dose of 14.0 mg/kg in mice was 6.7 μg/mL; Fig. 2A). Thus, it may be possible to combine TAS-116 with other anticancer agents without inducing clinically relevant drug–drug interactions.

TAS-116 exhibits potent antitumor activity accompanied by depletion of HSP90 clients in human tumor xenograft models
The antitumor activity of TAS-116 was evaluated in a HER2-expressing NCI-N87 (31) human gastric cancer xenograft mouse model. The favorable pharmacokinetic profile of TAS-116 was reflected in its dose-dependent antitumor activity; the T/C (tumor volume of TAS-116–treated mice vs. vehicle-treated mice) was 47%, 21%, and 9% for doses of 3.6 mg/kg, 7.1 mg/kg, and 14.0 mg/kg, respectively (Fig. 2A). Chronic administration of TAS-116 was tolerable, with the average weight loss in mice not exceeding 10% during the treatment period (Fig. 2B). In the pharmacodynamic analysis, TAS-116 (14.0 mg/kg) downregulated HER2, HER3, and AKT protein levels, leading to inhibition of PI3K/AKT and MAPK/ERK signaling in xenografted tumors (Fig. 2C).

We then determined the antitumor activity of TAS-116 in an FLT3-ITD–expressing human acute myeloid leukemia (MV-4-11; ref. 32) xenograft mouse model. Oral administration of TAS-116 at a dose of 14.0 mg/kg/day for 14 days led to tumor shrinkage (Fig. 2D), accompanied by depletion of FLT3-ITD (Fig. 2E) in this model.

TAS-116 shows antitumor activity without inducing eye injury in rats
In clinical trials of HSP90 inhibitors, visual disturbances has been a frequently observed adverse event. Indeed, ocular toxicity has been seen even in preclinical models (33). It is possible that ocular toxicity is associated with the extent to which eye tissues are exposed to the drug (33). Therefore, we evaluated the tissue distribution profile and ocular toxicity of TAS-116 in rats bearing subcutaneous human tumors (NCI-H1975). NVP-AUY922 was used as the reference compound because visual impairment was frequently observed in a phase 1 study of this compound (21). After administration of NVP-AUY922 intravenously or TAS-116 orally, plasma, retina, and tumor samples were collected and the concentration of each compound in the tissues was determined. Whereas NVP-AUY922 was distributed more in retina than plasma and was detectable at 24 hours after administration, TAS-116 was distributed less in retina than plasma and was eliminated from the retina within 24 hours in tumor-bearing nude rats (Fig. 3A). TAS-116 was also distributed less in retina than in plasma after intravenous administration (data not shown), indicating the rapid establishment of a distribution equilibrium between plasma and retina.

In a histopathologic examination of eye tissues to evaluate the ocular toxicity of the compounds, NVP-AUY922 was administered intravenously at 10.0 mg/kg/day three times a week for two weeks and TAS-116 was administered orally at 12.0 mg/kg/day every day in albino SD rats. NVP-AUY922 caused marked degeneration and disarrangement of the outer nuclear layer of the retina, and the numbers of TUNEL-positive apoptotic cells increased according to the retinal distribution of the compound (Fig. 3B). In contrast, administration of TAS-116 resulted in no histologic changes, no abnormalities in the photoreceptor cell ratio, and no increases in the number of TUNEL-positive cells in the outer nuclear layer of the retina in SD rats. Although retinal pigment epithelium has numerous functions in the maintenance of the integrity and function of photoreceptors (34), TAS-116 was less distributed in retina than in plasma and did not cause photoreceptor injury in pigmented LE rats (data not shown).

TAS-116 showed a much higher distribution in tumor than in retina in tumor-bearing rats (Fig. 3A). Importantly, a two-week administration of TAS-116 at a dose of 12.0 mg/kg/day resulted in antitumor activity accompanied by depletion of HSP90 client, mutated-EGFR, leading to inhibition of PI3K/AKT and MAPK/ERK signaling in the NCI-H1975 rat xenograft model (Fig. 3C and D). Again, TAS-116 did not produce detectable photoreceptor injury in this model (data not shown). These data suggested that TAS-116 did not cause ocular toxicity at the effective dose in rats.

To further evaluate the tissue distribution profiles of other structurally unrelated HSP90 inhibitors, we also measured plasma, retina, and tumor exposure to 17-DMAG, ganetespib, and SNX-5422 in a subcutaneous xenograft rat model. Though all the HSP90 inhibitors tested, including NVP-AUY922 and TAS-116, were highly distributed and retained in

Table 2. Pharmacokinetic profiles of TAS-116 in rodent and nonrodent species

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Dose (mg/kg)</th>
<th>tmax (h)</th>
<th>Cmax (μg/mL)</th>
<th>t1/2 (h)</th>
<th>AUC0–24 (μg h/mL)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>3</td>
<td>3.6</td>
<td>1.0</td>
<td>1.8</td>
<td>8.2</td>
<td>16.4</td>
<td>~100</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.1</td>
<td>2.0</td>
<td>3.1</td>
<td>2.5</td>
<td>28.3</td>
<td>~100</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14.0</td>
<td>1.0</td>
<td>6.7</td>
<td>4.4</td>
<td>60.2</td>
<td>~100</td>
</tr>
<tr>
<td>Rat</td>
<td>4</td>
<td>4.0</td>
<td>1.4</td>
<td>1.4</td>
<td>2.2</td>
<td>8.4</td>
<td>69.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.0</td>
<td>2.7</td>
<td>0.6</td>
<td>2.9</td>
<td>5.3</td>
<td>75.9</td>
</tr>
</tbody>
</table>

Abbreviations: AUC, area under the concentration–versus-time curve; Cmax, maximum plasma concentration; F, bioavailability; tmax, time of maximum concentration; t1/2, elimination half-life.
tumor (Fig. 3A and Supplementary Fig. S1), as previously reported (9, 11, 35, 36), TAS-116 was more rapidly eliminated from retina ($t_{1/2} = 3.4$ hours) than the other HSP90 inhibitors ($t_{1/2} = 7.1–19.1$ hours), thereby demonstrating its lower retinal distribution profile (Fig. 3A and Supplementary Table S3). Furthermore, the drug exposure ratio between tumor and retina of each compound ($T/R$ ratio) was calculated to assess which compound was distributed more in tumor than in retina; TAS-116 showed the highest $T/R$ ratio ($T/R = 5.7$) from among the tested HSP90 inhibitors ($T/R = 0.7–2.3$), indicating its unique tissue distribution profile (Fig. 3E).

**TAS-116 significantly suppresses tumor growth in an orthotopic lung tumor model expressing EGFR (L858R/T790M)**

TAS-116 demonstrated antitumor activity without ocular toxicity in a subcutaneous xenograft model. However, it is possible that the favorable tissue distribution profile of TAS-116 is only observed under such subcutaneous conditions. To rule out this possibility, we evaluated the antitumor activity of TAS-116 by using an orthotopic human lung cancer model in mice. Luciferase-transduced NCI-H1975 cells harboring EGFR (L858R/T790M; ref. 37) were directly transplanted into the lungs of mice, and TAS-116 was administered six weeks after transplantation. Tumor volume was assessed by means of bioluminescence imaging (Fig. 4). Although the intensity of luminescence was increased in the vehicle-treated group, TAS-116 administration resulted in significantly decreased luminescence. These results suggested that the tumor growth in the mouse lung was suppressed by treatment with TAS-116.

**Discussion**

HSP90 inhibitors simultaneously affect multiple cancer-related proteins, and are therefore potentially useful for treating targeted therapy–resistant tumors that have arisen from mutations within the target itself or from activation of alternative signaling pathways. For example, NVP-AUY922 is active against ALK-TKI– or EGFR-TKI–resistant non–small cell lung carcinoma (8, 38). However, because HSP90 also plays a role in normal cells, it is essential to balance efficacy and toxicity to maximize the antitumor potential of HSP90 inhibitors. Here, we characterize a novel HSP90 inhibitor that showed superior druggability with respect to target selectivity and its pharmacologic, pharmacokinetic, and safety profiles.

TAS-116 represents one of the most selective HSP90α and β inhibitors developed to date; it shows greater specific binding to HSP90α and β than to the highly homologous HSP90 family members GRP94 and TRAP1. The relative importance of the four HSP90 paralogs with respect to cancer treatment remains poorly understood. Although inhibition or downregulation of GRP94 inhibits growth and induces apoptosis in human multiple myeloma and HER2–expressing cell lines (39, 40), the role of GRP94 in liver tumorigenesis remains controversial, with recent studies indicating the potential for GRP94 to act as both a tumor suppressor and an oncogene (41, 42). Inhibition of TRAP1 by a mitochondria-directed variant of 17-AAG leads to specific apoptosis in cancer cells (43); however, TRAP1 may also play, in a context-dependent manner, a tumor suppressor or an oncogene role (44). Our present finding that the highly selective HSP90α...
Figure 3.
Comparative pharmacokinetic and retinal toxicity analysis of TAS-116 and NVP-AUY922. A, biodistribution of TAS-116 or NVP-AUY922 in athymic nude rats with established NCI-H1975 xenografts after oral or intravenous administration of TAS-116 at 12.0 mg/kg or NVP-AUY922 at 20.0 mg/kg, respectively. Data are presented as mean ± SD (n = 3). po, oral administration; iv, intravenous administration. B, effects of TAS-116 or NVP-AUY922 on retinal morphology and photoreceptor cell death in SD rats. TAS-116 was administered orally every day for two weeks. NVP-AUY922 was administered intravenously three times a week for two weeks. Eye sections were subjected to HE staining and TUNEL. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; po qd, daily oral administration; w, week. Scale bars, 50 μm. Effect on HSP90 clients (C) and antitumor activity of TAS-116 (D) in an NCI-H1975 rat xenograft model. For pharmacodynamic analysis, rats with established NCI-H1975 xenografts were treated with an oral dose of 12.0 mg/kg of TAS-116. Tumors (n = 3 per group) were harvested at 8 hours after administration. The levels of the HSP90 clients were evaluated by means of Western blotting. To evaluate the antitumor activity, NCI-H1975 tumor-bearing rats (n = 5 rats per group) were orally treated with TAS-116 at 12.0 mg/kg daily for two weeks. **, P < 0.01 versus untreated control. E, comparison of the drug exposure ratio between tumor and retina (AUC0–24, tumor/AUC0–24, retina) of HSP90 inhibitors in an NCI-H1975 rat xenograft model. HSP90 inhibitors were administered in tumor-bearing rats with the routes and doses described in Supplementary Table S3 and then subjected to pharmacokinetic analysis.
and β inhibitor TAS-116 has strong antitumor activity against several xenografted tumors indicates that inhibition of the HSP90α and β members of the HSP90 family is sufficient to exert antitumor activity.

The impact of paralog selectivity on the therapeutic index and toxic effects of HSP90 inhibitors is yet to be sufficiently explored (3). Recently, it was discovered that GRP94, a critical chaperone for the Wnt coreceptor LRP6, has a functional role in gut homeostasis via regulation of the canonical Wnt signaling pathway (29). Conditional deletion of grp94 results in rapid weight loss, diarrhea, and hematochezia in mice that are characterized by a gut-intrinsic defect in the proliferation of intestinal crypts, as well as compromised nuclear β-catenin translocation, loss of crypt–villus structure, and impaired barrier function (29). It however remains unclear whether pharmacologic inhibition of GRP94 would mimic such effect. Gastrointestinal toxicity is a major clinical adverse event associated with HSP90 inhibitors (17–19, 21, 45) that is considered an on-target effect of HSP90 inhibition. However, because TAS-116 did not inhibit the interaction between GRP94 and LRP6 in the present study, it is conceivable that a selective inhibitor such as TAS-116 may not cause GRP94-related gastrointestinal toxicity. This however remains to be validated.

One approach to balancing the efficacy and toxicity of antitumor agents is optimization of the dosing schedule. In this regard, oral rather than intravenous administration is preferable as it provides greater dose and schedule flexibility, and TAS-116 has been shown to have high oral availability in several species without the need for special formulation or prodrg development.

Sustained tumoral exposure with minimal exposure of other organs is another approach to achieve a maximal therapeutic window for antitumor agents. TAS-116 addresses the issues that have hampered the clinical development of HSP90 inhibitors because it shows a favorable tissue distribution profile. One of the most notable HSP90-related adverse events universally observed but to differing degrees in the clinic is visual disturbance (18–21, 45–48). The observed symptoms include night blindness, photopsia, blurred vision, and visual impairment, which are likely associated with retinal dysfunction (20, 21). Two independent reports of rodent models suggest that HSP90 plays a critical role in retinal function and that sustained HSP90

Figure 4. Effects of TAS-116 in a lung cancer mouse model based on orthotopically xenografted luciferase-transduced NCI-H1975 cells. A, representative photographs of photon emissions from individual mice with an orthotopic lung tumor with or without TAS-116 administration. Color gradation scale indicates the number of live tumor cells (purple, low bioluminescence; red, high bioluminescence). V, ventral image; D, dorsal image; ROI, region of interest. B, emitted bioluminescence (photons/s) at the indicated time points (n = 8 mice per group). Data are presented as mean ± SD. **P < 0.01 versus vehicle-treated control.
inhibition might adversely affect visual function (34, 49). Therefore, ocular effects may be minimized by reducing retinal exposure. In the present study, TAS-116 was distributed less in retina than in plasma in rats; consequently, TAS-116 did not produce any detectable photoreceptor injury. It has been proposed that the retina/plasma drug-exposure ratio and retinal elimination profile might be useful indicators of the ocular toxic potential of HSP90 inhibitors (33). Indeed, our results are consistent with previous results showing that ganetespib does not cause the same degree of visual symptoms and is eliminated from retina more rapidly than other HSP90 inhibitors (33). However, there is the possibility that the lower retinal distribution of some HSP90 inhibitors is merely correlated with lower tissue penetration of the compounds. If so, such compounds weaken the risk of ocular toxicities; however, this is at the cost of less antitumor potential. Importantly, in addition to the lower retinal distribution, TAS-116 showed a much higher distribution in tumor and antitumor activity in an NCI-H1975 subcutaneous xenograft rat model. To assess the risk-to-benefit ratio of HSP90 inhibitors, we calculated the tumor/retina (T/R) drug-exposure ratio for several HSP90 inhibitors in this xenograft setting. Except for TAS-116, all the tested compounds showed comparable drug exposure between tumor and retina (T/R = 0.9–2.3). However, TAS-116 demonstrated a higher T/R ratio (T/R = 5.7), indicating that it has a unique, favorable tissue distribution profile. Though the molecular mechanisms by which TAS-116 achieves this higher T/R exposure ratio remain to be elucidated, we expect TAS-116 to show a higher therapeutic window in the clinic than the other HSP90 inhibitors with respect to ocular toxicity. The drug T/R exposure ratio may also be useful in future preclinical screenings of candidate HSP90 inhibitor compounds that show an improved therapeutic index.

In conclusion, TAS-116 is an orally available HSP90 inhibitor that selectively binds to HSP90α and β. TAS-116 showed antitumor activity without detectable ocular toxicities in a rodent model, probably due to its favorable tissue distribution. Likewise, TAS-116 did not inhibit CYP450 within the effective concentration range, indicating a low drug–drug interaction potential that might allow combination administration with other drugs. These tractable yet specific properties of TAS-116 support the selection of this compound as a candidate for clinical evaluation as an antitumor agent characterized by maximal antitumor activity and minimal HSP90-related toxicities such as ocular toxicity.

Disclosure of Potential Conflicts of Interest
S. Ohkubo, C. Yoshimura, and M. Kitade have ownership interest in a patent, WO2011004610 A1. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
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