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Design and synthesis of 2-amino-6-(1*H*,3*H*-benzo[*de*]isochromen-6-yl)-1,3,5-triazines as novel Hsp90 inhibitors ☆

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ABSTRACT

A novel series of 2-amino-1,3,5-triazines bearing a tricyclic moiety as heat shock protein 90 (Hsp90) inhibitors is described. Molecular design was performed using X-ray cocrystal structures of the lead compound CH5015765 and natural Hsp90 inhibitor geldanamycin with Hsp90. We optimized affinity to Hsp90, in vitro cell growth inhibitory activity, water solubility, and liver microsomal stability of inhibitors and identified CH5138303. This compound showed high binding affinity for N-terminal Hsp90α (K_d = 0.52 nM) and strong in vitro cell growth inhibition against human cancer cell lines (HCT116 IC_{50} = 0.098 μM, NCI-N87 IC_{50} = 0.066 μM) and also displayed high oral bioavailability in mice (F = 44.0%) and potent antitumor efficacy in a human NCI-N87 gastric cancer xenograft model (tumor growth inhibition = 136%).

1. Introduction

Heat shock protein 90 (Hsp90) is an ATP-dependent molecular chaperone that is abundantly expressed in eukaryotic cells and essential for their survival. Hsp90 participates in the maturation and stability of many regulatory and signaling proteins that control cell proliferation and survival by facilitating normal protein folding and guarding protein from misfolding and aggregation.[1–3] In many tumors Hsp90 is overexpressed, because cancer cells use Hsp90 to maintain cellular homeostasis in a hostile tumor microenvironment, such as hypoxia, low nutrition and acidosis.[4] Because Hsp90 becomes activated in cancer cells after forming a complex with a series of co-chaperones,[5] inhibitors that are selective to activated Hsp90 could lead to an effective suppression of cancer cell proliferation and survival, and Hsp90 is considered an attractive molecular target for cancer therapies.[4,6–9]

In humans, Hsp90 has two cytosolic isoforms, Hsp90α (inducible form) and Hsp90β (constitutive form), and the functional differences between these isoforms are poorly understood. Recent studies showed that cancer cells need secretion of Hsp90α into the extracellular matrix for invasion and metastasis.[10] Hsp90 exists predominantly as a homodimer in the cytoplasm and consists of three main domains, namely, the N-terminal, middle, and C-terminal domains. The N-terminal domain contains a binding site to adenosine triphosphate (ATP), which needs to be hydrolyzed for chaperone activity to occur. The middle domain, as well as having a key role in binding many client proteins to Hsp90, also modulates the ATP hydrolysis by interacting with the γ-phosphate of the ATP that is bound in the N-terminal pocket.[11] The C-terminal domain, which contains an additional ATP-binding site, is responsible for the inherent dimerization of Hsp90.[12]

The N-terminal ATP-binding site is also the binding site of many known Hsp90 inhibitors, such as the natural products geldanamycin (GM, **1a**)[13] and radicicol (**2**),[14] the semi-synthetic analog of GM known as 17-allylamino-17-demethoxygeldanamycin (17-AAG, **1b**),[15] and many synthetic small molecules like those shown in Figure 1 (**3**–**6**).[16–19] These Hsp90 inhibitors interact with the N-terminal ATP-binding site to prevent ATP binding, stop the chaperone cycle, and finally lead to degradation of multiple oncogenic client proteins involved in tumor progression. 17-AAG (**1b**) entered clinical trial as an intravenous (iv) formulation in 1999 and is currently in phase III trials. Several small molecule inhibitors are also

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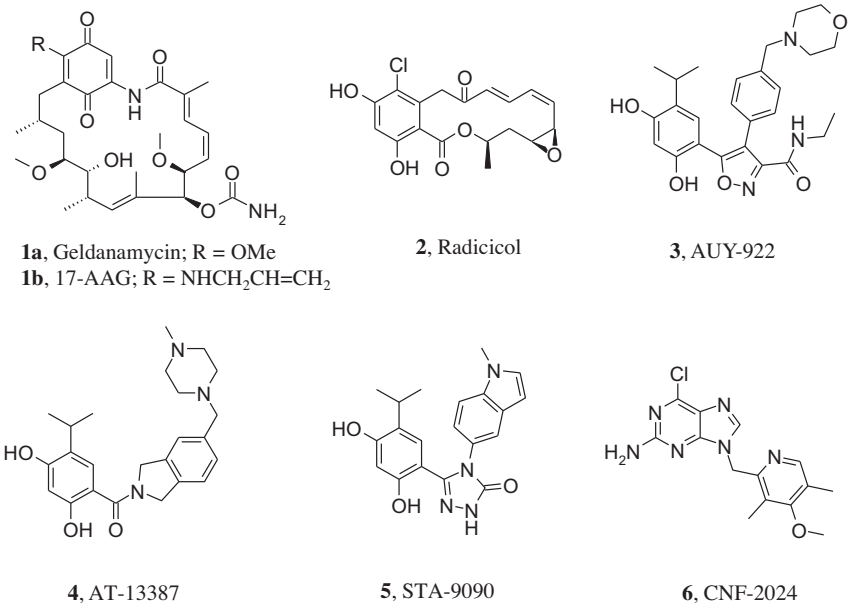


Figure 1. Known Hsp90 inhibitors.

undergoing clinical trials: iv formulations AUY-922 (**3**) and AT-13387 (**4**) in phase II, and STA-9090 (**5**) in phase III, which have the resorcinol component, and the oral (po) formulation CNF-2024 (**6**) in phase II.[6]

Although the majority of these small molecule inhibitors share a similar core structure, using a different core structure may result in a different profile with the potential for further improving efficacy, pharmacokinetics and safety. We therefore focused our effort on identifying novel orally bioavailable small molecules that would expand the structural diversity of the small molecule inhibitors presently available for this important pharmacological target.

We have already reported using a combination of fragment screening, virtual screening, and structure-based drug design to identify a new class of orally available Hsp90 inhibitor that binds to the N-terminal ATP binding site, CH5015765 (**7**, Fig. 2).[20] Compound **7** showed a high binding affinity for N-terminal Hsp90 (K_d = 3.4 nM) and in vitro cell growth inhibition against human cancer cell lines (HCT116, IC_{50} = 0.46 μM; NCI-N87, IC_{50} = 0.57 - μM). However, its poor water solubility (solubility in fasted state simulated intestinal fluid (FaSSIF) = 29 μM) limited the oral bioavailability (F = 4.5%) and produced only moderate tumor growth inhibition (TGI = 54%) against the xenografted NCI-N87 mouse model (po, 400 mg/kg, 11 qd), both of which aspects needed to be improved.

As we already reported, a tricyclic (1*H*,3*H*-benzo[*de*]isochromen-6-yl) moiety makes hydrophobic interactions with the side chains of Val150, Leu107, and Phe138, and an etheric oxygen at 3

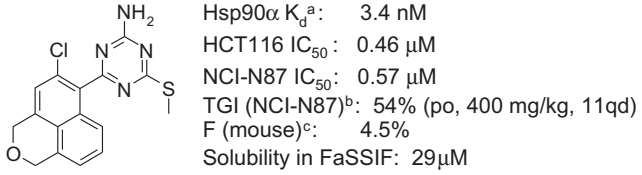


Figure 2. Chemical structure of CH5015765 (**7**) and its biological, physicochemical, and pharmacokinetic profiles. [a]Values were measured by surface plasmon resonance (SPR) using human N-terminal Hsp90α. [b]Growth inhibitory effect of compound **7** in a NCI-N87 tumor xenograft model. SCID mice bearing NCI-N87 cells were orally administered on a daily basis for 11 consecutive days (Days 11–21). Mean tumor volume is shown. [c]Oral bioavailability.

position forms a hydrogen bond (HB) with a water molecule that is hydrogen-bound to the backbone of Phe138 and the side chain of Asp51.[20] Herein we report a transformation of the methyl group on the sulfur atom at the triazine ring to other substituents to improve affinity and physicochemical properties while retaining the tricyclic moiety.

2. Results and discussion

2.1. Chemistry

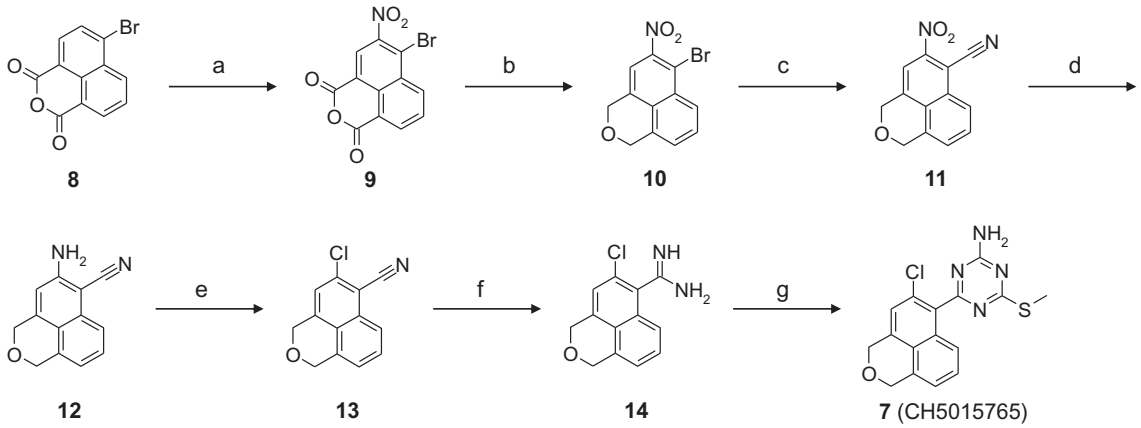
The preparation of compound **7** as the key intermediate is shown in Scheme 1. Nitration of commercial 4-bromo-1,8-naphthalic anhydride (**8**) gave compound **9** as a 4:1 mixture of regioisomers (3-nitro/6-nitro derivatives). Since the undesired regioisomer (6-nitro derivative) was more soluble than the desired regioisomer (3-nitro derivative) in acetonitrile, most of the byproduct was removed by filtering the product suspension in acetonitrile (82%, 3-nitro/6-nitro derivative = 13:1). After reducing anhydride with lithium aluminum hydride (LAH) (**10**, 99%), substitution of bromo to cyano group gave compound **11** (85%). Reduction of nitro group (**12**, 86%) followed by introduction of chloro group by Sandmeyer reaction gave compound **13** (70%). Transformation of cyano to amidino group (**14**, 95%) and the consequent 2-amino-1,3,5-triazine ring formation gave compound **7** (88%).

The preparation of compounds **16a–l** is shown in Scheme 2. Sulfoxide **15** was prepared from compound **7** by *m*-chloroperbenzoic acid (mCPBA) oxidation (78%). This sulfoxide **15** was coupled with the corresponding alkylthiols and the following amidation gave compounds **16a–l**.

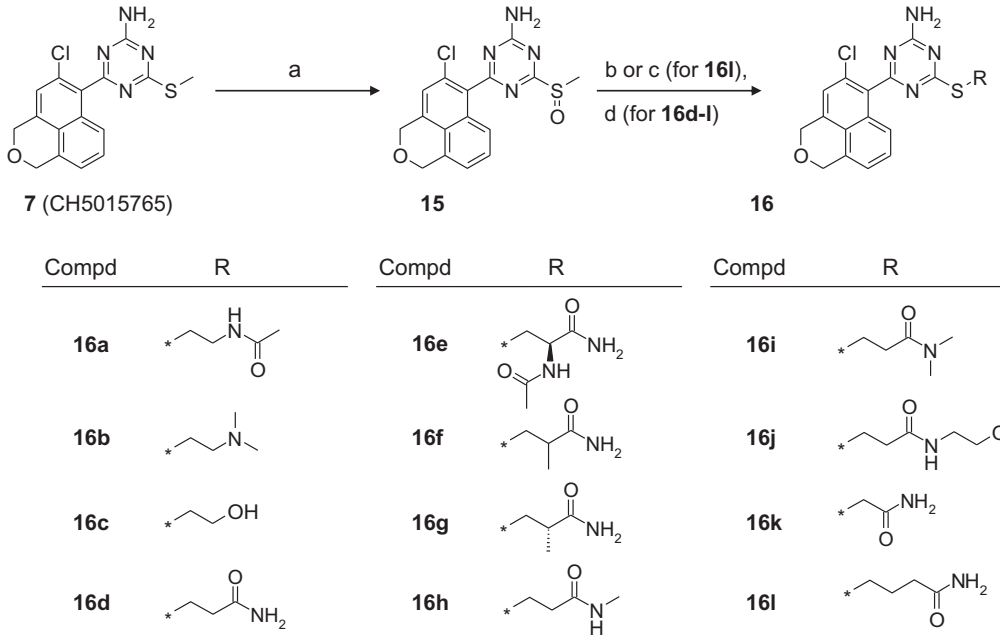
The preparation of compounds **18a–f** is shown in Scheme 3. Thiol **17** was prepared from compound **15** by treatment with potassium thioacetate, followed by hydrolysis (92%). This thiol **17** was coupled with the corresponding alkyl halides and the following amidation gave compounds **18a–f**.

2.2. Molecular design

Our strategy is depicted in Figure 3. Since GM (**1a**) forms a HB to the side chain of Lys58 (PDB code: 1YET), we attempted to incorporate this interaction into our molecular design. We expected



Scheme 1. Synthesis of compound **7**. Reagents and conditions: (a) HNO_3, H_2SO_4, 4 °C, 2.5 h, 82%; (b) $LiBH_4$, $BF_3{\cdot}OEt_2$, THF, 40 to 50 °C, 2 h, 99%; (c) CuCN, DMF, 120 °C, 1 h, 85%; (d) $Na_2S_2O_4$, THF-water, reflux, 30 min, then HCl aq. (5 M), rt, 1 h, 86%; (e); $CuCl_2$, *t*-BuONO, CH_3CN, rt, 1 h, 70%; (f) NH_4Cl, $Al(CH_3)_3$, toluene, reflux, 21 h, 95%; (g) dimethyl cyanodithioiminocarbonate, DIPEA, THF, reflux, 3 h, 88%. *Abbreviations:* aq, aqueous solution; DIPEA, *N*,*N*-diisopropylehylamine; DMF, *N*,*N*-dimethylformamide; rt, room temperature; THF, tetrahydrofuran.



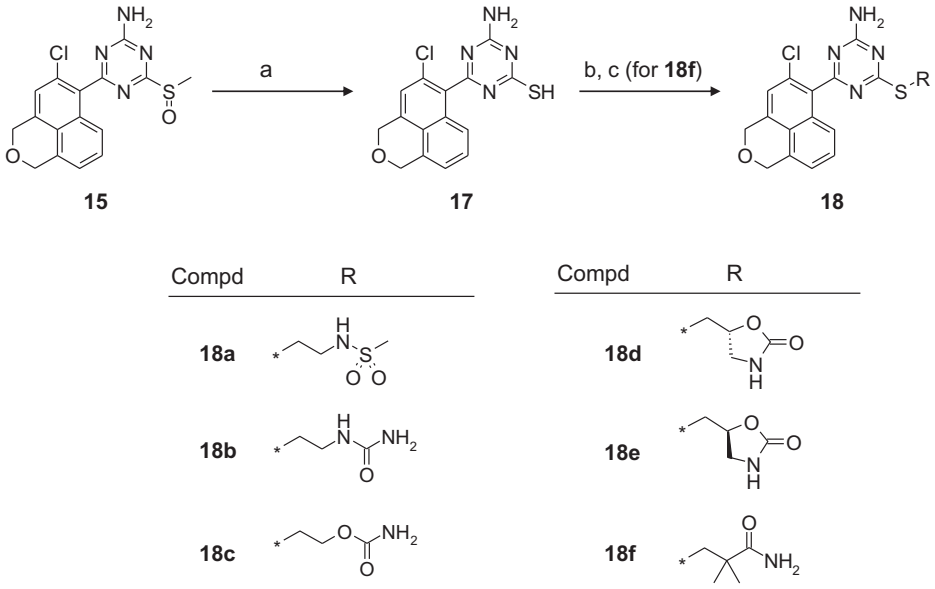
Scheme 2. Synthesis of compound **16a**–**l**. Reagents and conditions: (a) mCPBA, CH_2Cl_2, rt, 3 h, 78%; (b) RSH, base (TEA, DIPEA or K_2CO_3), solvent (THF, THF-DMF, or DMF), rt; (c) 4,4-dithiobutyric acid, tributylphosphine, DIPEA, DMF-water, rt; (d) amine (NH_4Cl, $CH_3NH_2{\cdot}HCl$, $(CH_3)_2NH{\cdot}HCl$, or 2-aminoethan-1-ol), HOBT, EDC·HCl, DIPEA, DMF, rt. *Abbreviations:* mCPBA, *m*-chloroperbenzoic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBT, 1-hydroxybenzotriazole; TEA, triethylamine.

affinity and physicochemical properties to be improved by introducing a HB acceptor via methylene carbons on the sulfur atom at the triazine ring. As an option to improve the physicochemical properties, introducing a solubilizing group on a HB acceptor was also attempted.

We analyzed the superimposed structures of **1a** and compound **7** in Hsp90α (Fig. 4) and found that two to three methylene carbons could be acceptable because the side chain of Lys58, located near the solvent-exposed region, is flexible. First we synthesized derivatives having various HB acceptors with two methylene carbons.

2.3. Structure–activity relationships

Tables 1–3 summarize the affinity to Hsp90α, the in vitro cell growth inhibitory activity against an HCT116 colorectal cancer cell line (a KRAS mutant cell line) and an NCI-N87 gastric cancer cell line (a HER2-positive cell line), the physicochemical properties, and the in vitro metabolic stability. As described in an introduction, Hsp90α is important for invasion and metastasis of cancer cells. We thought that the utilization of Hsp90α as an index for lead optimization was important for identification of a potent antitumor agent. We first examined acetylamino (**16a**) and mesylamino (**18a**) as HB acceptors aiming to Lys58. As expected, introducing these HB acceptors increased affinity to Hsp90 (K_d = 0.82 and 0.43 nM, respectively) by 4- to 8-fold in comparison with that of compound **7**. With regards to water solubility, **16a** increased it by 30-fold (856 μM) but **18a** did not show any improvement (<31 μM). Water solubility can be described by some physicochemical properties such as pK_a, polar surface area (PSA), and the general solubility equation using Log*P* (the molar octanol–water partition coefficient).[21] In this case, we expected the weak acidic property of the mesylamino group (calculated pK_a = 9.08) to affect water solubility negatively in FaSSIF at pH 6.5. In order to improve water solubility with sub-nanomolar



Scheme 3. Synthesis of compound **18a–f**. Reagents and conditions: (a) potassium thioacetate, DMF, rt, 19 h, and then NaOH aq (2 M), rt, 1.5 h, 92%; (b) RBr/Cl, DIPEA, DMF, 70–80 °C, (c) NH_4Cl, HOBT, EDC·HCl, DIPEA, DMF, rt.

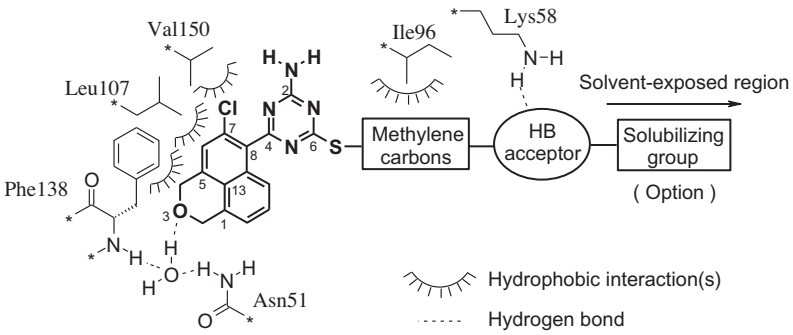


Figure 3. Design of derivatives having an aliphatic side chain on the S atom.

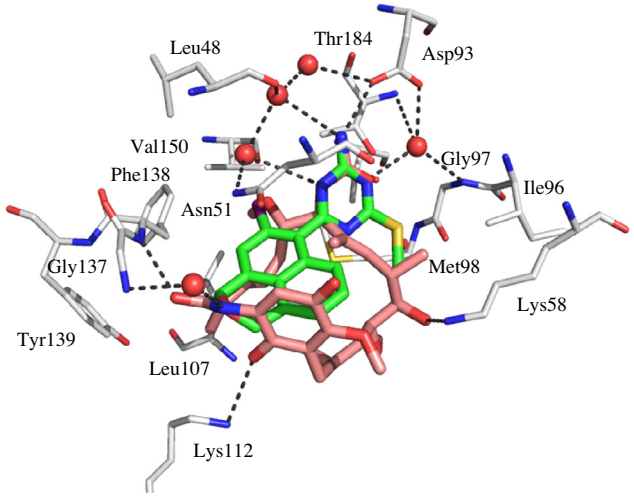
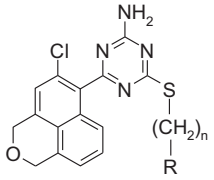


Figure 4. The superimposed structures of GM (**1a**) and CH5015765 (**7**) in Hsp90α. Atom types were distinguished as separate colors: gray for $C_{Hsp90\alpha}$, pink for $C_{\mathbf{1a}}$, green for C_7, red for O, blue for N, yellow for S, and purple for Cl.

level affinity, ureido (**18b**), dimethylamino (**16b**), and hydroxyl (**16c**) were prepared. Although the calculated Log*P* (*c*Log*P*) of **18b** is lower than that of **16a**, **18b** showed similar solubility (773 μM) to **16a**. Even though its K_d values were not improved (K_d = 1.4 and 1.5 nM, respectively), **16b** had significantly improved water solubility (3320 μM). This basic functional group is assumed to be ionized in FaSSIF. Human liver microsomal clearance (LM CL) of **16b** (30 μL/min/mg protein) was higher than that of acetylamino **16a** (19 μL/min/mg protein). Although sub-nanomolar level affinity was retained (K_d = 0.54 nM), the hydroxyl **16c** showed poor water solubility (<32 μM). Carbamoyloxy (**18c**) and carboxamide (**16d**) showed acceptable water solubility (362 and 113 μM, respectively) with high binding affinity (K_d = 0.46 and 0.66 nM, respectively). **18c** and **16d** also showed potent in vitro cell growth inhibition (NCI-N87 IC_{50} = 0.036 and 0.056 μM, respectively) and sufficient stability (human LM CL = 20 and 7.0 μL/min/mg protein). After judging all the differences, we used these two acceptors for further modification.

Introduction of the acetylamino group (**16e**) to compound **16a** decreased affinity (K_d = 4.4 nM) and cellular IC_{50} (NCI-N87 IC_{50} = 1.0 μM) by 10-fold. Introduction of the methyl group (**16f**) slightly improved affinity (K_d = 0.60 nM) and cellular IC_{50} (NCI-N87 IC_{50} = 0.032 μM), but water solubility decreased (53 μM). The compound with a chiral methyl group (**16g**) showed almost the same activity as the racemate (**16f**). These data suggested hydrophobic subsitituents are favorable on a methylene carbon, but introducing the geminal dimethyl group (**18f**) as a hydrophobic substituent caused a significant decrease in affinity (K_d = 41 nM)

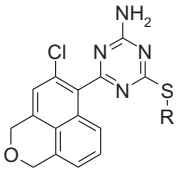
Table 1
Binding affinity, in vitro cell growth inhibitory activity, water solubility and in vivo antitumor activity of derivatives having various HB acceptors attached on an aliphatic side chain

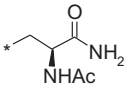
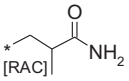
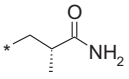
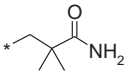
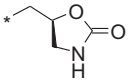
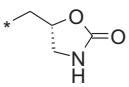


Compd	n	R	K_d[a] (nM)	IC_{50} (μM)		Solubility[b] (μM)	*c*Log*P*[c]	PSA[d] (Å2)	LM CL (μL/min/mg protein)	
				HCT116	NCI-N87				Human	Mouse
16a	2	NHAc	0.82	0.13	0.13	856	2.25	85.1	19	32
18a	2	$NHSO_2Me$	0.43	0.34	0.90	<31	2.34	101.3	26	44
18b	2	$NHCONH_2$	1.4	0.23	0.23	773	1.85	107.5	13	8.8
16b	2	NMe_2	1.5	0.30	0.29	3320	2.98	64.1	30	82
16c	2	OH	0.54	0.14	0.094	<32	2.31	75.8	22	18
18c	2	$OCONH_2$	0.46	0.076	0.036	362	2.43	104.4	24	20
16d	2	$CONH_2$	0.66	0.12	0.056	113	1.78	95.7	13	7.0

[a] Values were measured by SPR using human N-terminal Hsp90α.
[b] Solubility in FaSSIF. (The use of FaSSIF may over-predict the solubility in physiological in vivo conditions).
[c] *c*Log*P* was calculated by the PCModels software.
[d] Molecular PSA was calculated by the TPSA software.

Table 2
Binding affinity, in vitro cell growth inhibitory activity, and water solubility of carboxamide derivatives and carbamoyloxy derivatives



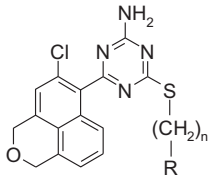
Compd	R	K_d[a] (nM)	Cell IC_{50} (μM)		Solubility[b] (μM)	*c*Log*P*[c]	PSA[d] (Å^2)	LM CL (μL/min/mg protein)	
			HCT116	NCI-N87				Human	Mouse
16e		4.4	1.2	1.0	ND	0.88	119.0	ND	ND
16f		0.60	0.048	0.032	53	2.20	95.3	11	15
16g		0.33	0.034	0.026	34	2.20	95.4	53	49
18f		41	6.4	7.3	ND	2.65	95.0	ND	ND
18d		1.8	0.15	0.14	249	2.71	96.1	40	37
18e		1.6	0.15	0.12	ND	2.71	95.8	ND	ND

[a] Values were measured by SPR using human N-terminal Hsp90α.
[b] Solubility in FaSSIF.
[c] *c*Log*P* was calculated by the PCModels software.
[d] Molecular PSA was calculated by the TPSA software.

and cellular IC_{50} (NCI-N87 IC_{50} = 7.3 μM). The geminal dimethyl group was considered to cause a conformation that is unfavorable for binding to the protein and to expose one of the methyl groups to the solvent-exposed region.

In the case of cyclic carbamoyl derivatives (**18d** and **e**), affinity (K_d = 1.8 and 1.6 nM, respectively) and cellular IC_{50} (NCI-N87 IC_{50} = 0.14 and 0.12 μM, respectively) were decreased in comparison to those of compound **18c**. These results suggested that there is

Table 3
Binding affinity, in vitro cell growth inhibitory activity, and water solubility of carboxamide derivatives



Compd	*n*	R	K_d[a] (nM)	Cell IC_{50} (μM)		Solubility[b] (μM)	*c*Log*P*[c]	PSA[d] (Å2)	LM CL (μL/min/mg protein)	
				HCT116	NCI-N87				Human	Mouse
16h	2	CONHMe	0.66	0.086	0.069	1370	2.25	84.9	11	24
16i	2	$CONMe_2$	2.5	0.31	0.31	35	2.46	76.3	30	18
16j	2	$CONH(CH_2)_2OH$	1.3	0.29	0.16	2310	1.27	101.0	8.7	10
16k	1	$CONH_2$	1.3	0.27	0.16	86	1.29	95.5	11	7.6
16l	3	$CONH_2$	0.48	0.098	0.066	646	2.27	95.8	14	14

[a] Values were measured by SPR using human N-terminal Hsp90α.
[b] Solubility in FaSSIF.
[c] *c*Log*P* was calculated by the PCModels software.
[d] Molecular PSA was calculated by the TPSA software.

no advantage to introducing substituents to a methylene carbon or to forming cyclic carbamoyl groups.

The effect that different substituents had on the amide nitrogen of compound **16d** was examined. Methylamide (**16h**) significantly improved water solubility by 12-fold (1370 μM) in comparison to compound **16d** while retaining affinity (K_d = 0.66 nM). However, dimethylamide (**16i**) decreased affinity (K_d = 2.5 nM) and water solubility (35 μM). 2-Hydroxyethylamide (**16j**) decreased affinity (K_d = 1.3 nM), although water solubility was improved (2310 μM). From the above examples, various HB acceptors are acceptable from the viewpoint of efficacy, and unsubstituted and *N*-methyl-substituted carboxamide groups have an advantage in terms of water solubility and liver microsomal stability. Finally, the number of methylene carbons was optimized. As expected from the analysis of the X-ray cocrystal structure, compound **16k** with one methylene carbon showed a slight decrease in affinity (K_d = 1.3 nM), and compound **16l** with three methylene carbons, showed improved water solubility (646 μM) and retained affinity (K_d = 0.48 nM). In these three compounds (**16d**, **16k**, and **16l**), the numbers of rotatable bonds correlate to water solubility.[22] In conclusion, compound **16l** excels in the series of compounds by its in vitro efficacy and physicochemical properties. In addition, compound **16l** reduced the phosphorylation and protein level of multiple Hsp90 client proteins[9] such as EGFR, HER2, Raf1, and AKT (Fig. 5). This result indicates that our compound inhibits proliferation of cancer cells by inducing degradation of multiple Hsp90 client proteins.

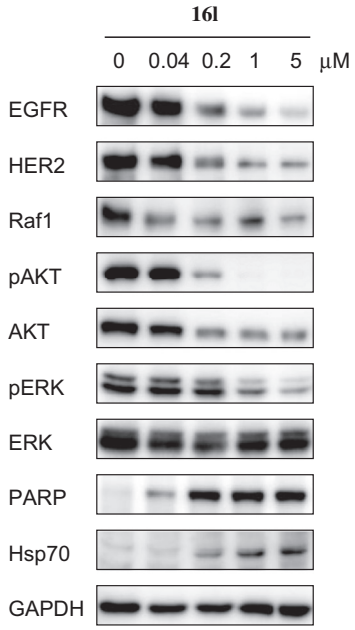


Figure 5. Degradation of multiple Hsp90 client proteins induced by compound **16l** in NCI-N87 cells. NCI-N87 cells were treated with the indicated concentration of compound **16l** for 24 h before lysing and analysis by Western blotting.

2.4. X-ray cocrystal structure

The X-ray cocrystal structure of compound **16l** and Hsp90 (PDB: 3WHA) reveals that the butyramide side chain contributes to the improved affinity to Hsp90. The methylene groups at 4- and 2-position of the butyramide side chain (C-4 and C-2, respectively) is located at a distance (*d*) of lipophilic interactions with the methylene group at γ-position (C-γ) in the side chain of Met98 (d(C-4···C-γ_{Met98}) = 3.5 Å) and with the methyl group on β-carbon (C-βCH_3) in the side chain of Ile96 (d(C-2···C-βCH_3 $_{Ile96}$) = 3.5 Å), respectively. The amide group of the butyramide

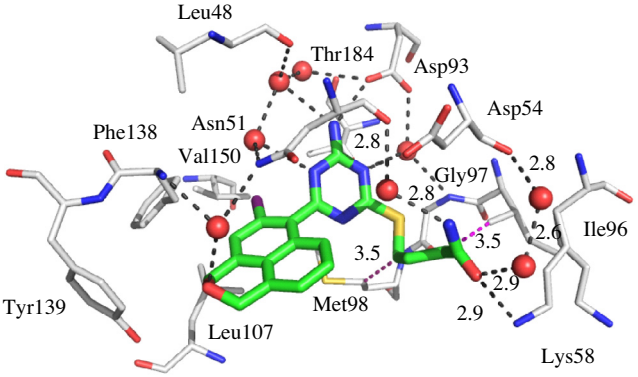


Figure 6. An X-ray structure of N-terminal Hsp90α in complex with **16l**. The numbering of the residues corresponds to those in the X-ray crystal structure (gray $C_{Hsp90\alpha}$, green C_{ligand}, red O, blue N, yellow S, Purple Cl). Hydrogen bonds are indicated by black dashes and lipophilic interactions are indicated by purple dashes. Detailed binding mode of compound **16l** (PDB code: 3WHA).

Table 4
Pharmacokinetic parameters of **7** and **16l** in nude mice

	Administration	Dose (mg/kg)	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (μg/mL)	AUC_{inf} (μg·h/mL)	CL or CL/F (mL/h/kg)	F^a (%)
7	iv	10	0.250	–	–	8.55	1170	–
(CH5015765)	po	200	2.62	0.5	3.92	7.65	26100	4.5
16l	iv	10	0.963	–	–	24.9	403	–
(CH5138303)	po	50	0.944	0.75	36.7	54.8	938	44.0

[a] Oral bioavailability.

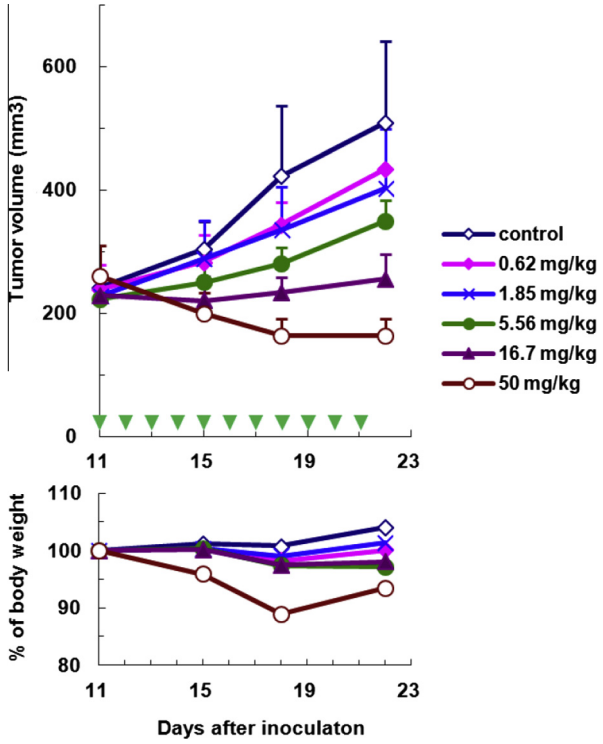


Figure 7. Growth inhibitory effect of compound **16l** on NCI-N87 tumor xenograft model. SCID mice bearing NCI-N87 cells were treated orally with the indicated doses of compound **16l** on a daily basis for 11 consecutive days (days 11–21) at 0.62 mg/kg (TGI = 27%), 1.85 mg/kg (TGI = 34%), 5.56 mg/kg (TGI = 53%), 16.7 mg/kg (TGI = 90%), and 50 mg/kg (TGI = 136%). Mean tumor volume and mean body weight change of mice are shown.

side chain does not interact with the amino group of Lys58 strongly. As seen in the cocrystal structure, there are two alternative conformations of Lys58, depending on whether the amino group at ε-position (N-ε) in the side chain of Lys58 is located at a distance of one HB from the amide oxygen (d(N-ε $_{Lys58}$···O) = 2.9 Å) or not. This means the HB between Lys58 and the amide is partial. On the other hand, the amide group forms HBs to the protein via water molecules. Namely, the amide nitrogen forms a HB to the carbonyl oxygen in the main chain of Asn51 via a water molecule and the amide oxygen forms HBs to the carbonyl oxygen in the main chain of Asp54 via two water molecules. In the X-ray cocrystal structure of GM and Hsp90, the water molecules are excluded by GM (PDB code: 1YET). Hence, introducing the polar substituents improves the affinity by forming a HB network of water molecules (Fig. 6).

2.5. Pharmacokinetics

Since compound **16l** showed high binding affinity, strong in vitro cell growth inhibition, and sufficient in vitro metabolic stability, we conducted in vivo profiling. The pharmacokinetic study in mice demonstrated that compound **16l** had improved PK profiles of total systemic exposure (AUC), plasma clearance (CL), and oral bioavailability (F) in comparison to those of compound **7** (Table 4).

2.6. Efficacy in tumor xenograft studies

Compound **16l** was further evaluated in a human NCI-N87 gastric cancer xenograft mouse model (Fig. 7). Mice bearing NCI-N87 were orally treated with compound **16l** once daily for 11 days at 0.62, 1.85, 5.56, 16.7, and 50 mg/kg, and their body weight and tumor volume were measured twice a week. Compound **16l** showed potent antitumor efficacy with TGI of 136% and a median effective dose (ED_{50}) of 3.9 mg/kg without significant loss of body weight.

3. Conclusion

In summary, we identified novel 2-amino-6-(1*H*,3*H*-benzo[*de*]isochromen-6-yl)-1,3,5-triazines as Hsp90 inhibitors by modifying the lead compound **7** (CH5015765). We improved the binding affinity together with the physicochemical properties of water solubility, liver microsomal stability and in vitro cell growth inhibition. Of the resulting compounds, compound **16l** (CH5138303) demonstrated high oral bioavailability in mice and potent antitumor efficacy in a human NCI-N87 gastric cancer xenograft mouse model.

4. Experiments

4.1. Materials and measurements

Organic synthesis reactions were conducted using commercially available reagents without further purification. Room temperature refers to the range of 20–25 °C. All moisture-sensitive reactions were performed under a nitrogen or argon atmosphere. Concentration or solvent removal under reduced pressure was carried out using a rotary evaporator, unless otherwise stated. Thin layer chromatography (TLC) analytical separations were conducted with silica gel 60 F254 precoated TLC plates or NH TLC plates, each manufactured by E. Merck. Standard silica gel column chromatography was employed as a method of purification using the indicated solvent mixture.

NMR analyses were carried out using a JNM-EX270 (270 MHz), a JNM-GSX400 (400 MHz), a JNM-A500 (500 MHz) (all manufactured by JEOL), or an ARX-300 (300 MHz) manufactured by Bruker. Chemical shifts are reported in parts per million (δ). The deuterium lock signal of the sample solvent was used as a reference, and coupling constants (J) are given in hertz (Hz). The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; br, broadened; and m, unresolved multiplet due to the field strength of the instrument. High performance liquid chromatography-mass spectrometric data were obtained using a Micromass ZMD coupled with a Waters 996–600E gradient high performance liquid chromatography system or a Micromass ZQ

coupled with a Waters 2525 high performance liquid chromatography system using the positive electrospray ionization technique (ESI^+), both using a mobile phase of CH_3CN/water with 0.05% TFA. The purity of all tested compounds is ⩾95% unless explicitly stated otherwise. High resolution mass spectra (HRMS) of ESI^+ were obtained using an LTQ Orbitrap XL (Thermofisher scientific) with an ACQUITY ultra performance liquid chromatography system (Waters), or a QSTAR XL (AB Sciex) with Agilent 1100 (Agilent). Fast atom bombardment (FAB) and electron impact (EI) mass spectra were obtained using a JMC-GCmate II (JEOL). The abbreviations of mass spectrometry are as follows: *m*/*z* is the mass-to-charge ratio; M is the molecular weight of the molecule itself; and M^+ is the molecular ion.

4.2. Synthetic procedures

4.2.1. 8-Bromo-7-nitro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaene-2,4-dione (9)

8-Bromo-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaene-2,4-dione (**8**, 126.2 g, 456 mmol) was dissolved in sulfuric acid (620 mL). The reaction vessel was cooled in an ice bath, and a mixed solution of fuming nitric acid (65.5 mL) and sulfuric acid (84.8 mL) was added dropwise while keeping the internal temperature below 4 °C. Then, the reaction mixture was stirred for 2.5 h while keeping the internal temperature below 4 °C. Ice water (1.5 L) was slowly added to the reaction mixture, and stirred at room temperature for 30 min. Water (1.5 L) was added to the aqueous mixture, and the resulting precipitates were filtered. The precipitates were suspended in acetonitrile (1.0 L). The suspension was stirred at room temperature overnight and filtered (3-nitro/6-nitro derivative = 6:1 from ^1H NMR analysis). After washing with acetonitrile, the resulting precipitates were again suspended in acetonitrile (1.0 L). The same treatment was repeated, and the precipitates were dried under reduced pressure to yield the title compound as a pale yellow solid (120 g, 82%, 3-nitro/6-nitro derivative = 13:1 from ^1H NMR analysis). ^1H NMR (300 MHz, DMSO-d_6) δ: 8.19 (1H, dd, J = 7.2, 8.8 Hz), 8.74 (1H, d, J = 8.8 Hz), 8.83 (1H, d, J = 7.2 Hz), 8.92 (1H, s). HRMS (EI^+) *m*/*z*: $[M]^+$ calcd for $C_{12}H_4{}^{79}BrNO_5$, 320.9273; found, 320.9260, $[M]^+$ calcd for $C_{12}H_4{}^{81}BrNO_5$, 322.9252; found, 322.9250.

4.2.2. 8-Bromo-7-nitro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaene (10)

Tetrahydrofuran (2.3 L) was added to 8-bromo-7-nitro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaene-2,4-dione (**9**, 120 g, 372.6 mmol), and stirred thoroughly to prepare a suspension. The suspension was cooled in an ice-water bath. Lithium borohydride (16.3 g, 745.2 mmol) and trifluoroborane ether complex (141.6 mL, 1118 mmol) were added to the suspension successively while keeping the internal temperature below 4 °C. The resulting reaction mixture was stirred at 40–50 °C for 2 h. The mixture was cooled to room temperature, and then water (500 mL) was added and extracted twice with ethyl acetate (1.5 L). The organic layer was washed with brine (500 mL), dried over anhydrous magnesium sulfate, and then concentrated under reduced pressure. The crude product was suspended in a mixed solvent of n-hexane and ethyl acetate (5:1, 1.2 L). The mixture was stirred at room temperature for 2 h, and filtered. After washing with *n*-hexane–ethyl acetate (5:1), the mixture was dried under reduced pressure to yield the title compound as a pale yellow solid (108 g, 99%). ^1H NMR (300 MHz, DMSO-d_6) δ: 5.05 (2H, s), 5.08 (2H, s), 7.59 (1H, d, J = 7.0 Hz), 7.81–7.86 (2H, m), 8.24 (1H, d, J = 8.3 Hz). HRMS (ESI^-) *m*/*z*: $[M-H]^-$ calcd for $C_{12}H_7{}^{79}BrNO_3$, 291.96148; found, 291.96067, $[M-H]^-$ calcd for $C_{12}H_7{}^{81}BrNO_3$, 293.94058; found, 293.93925.

4.2.3. 7-Nitro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaene-8-carbonitrile (11)

8-Bromo-7-nitro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaene (**10**, 50.0 g, 170 mmol) and copper(I) cyanide (18.3 g, 204 mmol) were dissolved in anhydrous *N*,*N*-dimethylformamide (500 mL). Then, the flask was purged with nitrogen to replace air. The mixture was stirred at 120 °C for 1 h, and then cooled to room temperature. The resulting material was added to 10% aqueous ammonia (1.4 L) and stirred for 30 min. The resulting mixture was diluted with water (1.2 L) and the precipitates were collected by filtration. The precipitates were washed with 5% aqueous ammonia (1.0 L) and water successively. The precipitates were suspended in acetonitrile (200 mL) with stirring at room temperature overnight, collected by filtration, washed with acetonitrile (20 mL, twice) to yield the title compound as a yellow solid (34.7 g, 85%). ^1H NMR (300 MHz, $CDCl_3$) δ: 5.13 (2H, s), 5.16 (2H, s), 7.53 (1H, d, J = 7.4 Hz), 7.84 (1H, dd, J = 8.3, 7.4 Hz), 8.15 (1H, s), 8.42 (1H, d, J = 8.3 Hz). HRMS (ESI^-) *m*/*z*: $[M-H]^-$ calcd for $C_{13}H_7N_2O_3$, 239.04622; found, 239.04526.

4.2.4. 7-Amino-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaene-8-carbonitrile (12)

7-Nitro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaene-8-carbonitrile (**11**, 50.0 g, 208 mmol) was suspended in tetrahydrofuran (680 mL). The suspension was heated to reflux and sodium dithionite (109 g, 624 mmol) in water (500 mL) was added to the solution under refluxing for over 1 h. After addition, the mixture was stirred under refluxing for 30 min. Insoluble materials were removed by filtration when the resulting mixture was cooled to around 60 °C. 5 N hydrochloric acid (510 mL) was added to the filtrate and the resulting mixture was stirred at room temperature for 1 h. The pH was adjusted to approximately 10 by dropwise addition of 5 N aqueous sodium hydroxide solution (650 mL) over 1.5 h in a water bath. The mixture was diluted with water (800 mL) and stirred for 30 min. The precipitates were collected by filtration, washed with water until washings became neutral, and dried under reduced pressure. The title compound was obtained as a pale yellow solid (37.5 g, 86%). ^1H NMR (300 MHz, $CDCl_3$) δ: 4.75 (2H, br s), 4.93 (2H, s), 4.99 (2H, s), 6.60 (1H, s), 7.02 (1H, d, J = 7.2 Hz), 7.50 (1H, dd, J = 8.4, 7.2 Hz), 7.79 (1H, d, J = 8.4 Hz). HRMS (ESI^+) *m*/*z*: $[M+H]^+$ calcd for $C_{13}H_{11}N_2O$, 211.0865; found, 211.0871.

4.2.5. 7-Chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaene-8-carbonitrile (13)

To a suspension of copper(II) chloride (21.5 g, 160 mmol) in anhydrous acetonitrile (1.1 mL) was added *tert*-butyl nitrite (24 mL, 200 mmol) at room temperature under nitrogen atmosphere. 7-Amino-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaene-8-carbonitrile (**12**, 28.0 g, 133 mmol) was added portionwise thereto over 30 min. The resulting mixture was stirred at room temperature for 1 h. 1 N hydrochloric acid (1.1 L) and water (1.1 L) were added slowly to the mixture successively, and the resulting mixture was stirred for 20 min. The precipitates were collected by filtration, washed with water, and dried under reduced pressure. The resulting residue was roughly purified by passing through silica gel (100 g) using dichloromethane (1.5 L). The eluent containing the desired product was concentrated to give the crude product (26.4 g). The crude product was purified by crystallization from acetonitrile (1.0 L). The crystals were collected by filtration, washed with cold acetonitrile (50 mL, twice), and dried under reduced pressure. The title compound was obtained as a yellow solid (21.3 g, 70%). ^1H NMR (300 MHz, $CDCl_3$) δ: 5.05 (2H, s), 5.07 (2H, s), 7.27 (1H, s), 7.32 (1H, d, J = 7.2 Hz), 7.68 (1H, dd, J = 8.7, 7.2 Hz), 8.07 (1H, d, J = 8.7 Hz). HRMS (ESI^+) *m*/*z*: $[M+H]^+$ calcd for $C_{13}H_9{}^{35}ClNO$, 230.0367; found, 230.0362, $[M+H]^+$ calcd for $C_{13}H_9{}^{37}ClNO$,

232.0337; found, 232.0342, $[M+NH_4]^+$ calcd for $C_{13}H_{12}{}^{35}ClN_2O$, 247.0632; found, 247.0640.

4.2.6. 7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaene-8-carboximidamide (14)

To a suspension of ammonium chloride (31.4 g, 699 mmol) in toluene (70 mL) was added dropwise a solution of 2 N trimethylaluminum in toluene (350 mL, 699 mmol) over 1 h at room temperature. The resulting solution was stirred for 30 min and 7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaene-8-carbonitrile (**13**, 8.03 g, 35.0 mmol) was added thereto. The mixture was stirred while heating under reflux for 21 h, and then cooled to room temperature. A saturated aqueous potassium sodium tartrate solution (1 L) and 3 N aqueous sodium hydroxide solution (500 mL) were added to the reaction mixture successively, and then extracted with ethyl acetate (2.0 L, twice). The organic layers were combined, washed with water (500 mL, twice) and brine (500 mL) successively, and dried over anhydrous magnesium sulfate. The inorganic salts were removed by filtration, and the filtrate was concentrated under reduced pressure. The title compound was obtained as a yellow solid (8.15 g, 95%). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 5.01 (4H, s), 6.65–6.90 (3H, br s), 7.32 (1H, d, J = 7.4 Hz), 7.36 (1H, s), 7.57 (1H, dd, J = 7.4, 8.3 Hz), 7.79 (1H, d, J = 8.3 Hz). HRMS (ESI$^+$) m/z: $[M+H]^+$ calcd for $C_{13}H_{12}{}^{35}ClN_2O$, 247.06327; found, 247.06268, $[M+H]^+$ calcd for $C_{13}H_{12}{}^{37}ClN_2O$, 249.06032; found, 249.05964.

4.2.7. 4-{7-Chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-6-(methylsulfanyl)-1,3,5-triazin-2-amine (7)

To a solution of 7-chloro-3-oxatricyclo[7.3.1.^{05,13}]trideca-1(13),5,7,9,11-pentaene-8-carboximidamide (**14**, 5.8 g, 23.9 mmol) in ethanol (60 mL) were added dimethyl cyanodithioiminocarbonate (3.8 g, 26.3 mmol) and *N*-ethyldiisopropylamine (8.3 mL, 47.8 mmol) at room temperature. The resulting mixture was stirred under refluxing ethanol for 3 h and then cooled in an ice-water bath. The precipitates were collected by filtration, washed with cold ethanol (100 mL), and dried under reduced pressure. The title compound was obtained as a yellow solid (7.1 g, 88%). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.45 (3H, s), 5.04 (2H, s), 5.05 (2H, s), 7.34 (1H, d, J = 6.9 Hz), 7.40 (1H, d, J = 8.4 Hz), 7.44 (1H, s), 7.52 (1H, dd, J = 6.9, 8.4 Hz), 7.79 (2H, br s). MS (ESI$^+$) m/z: 345, 347 $[M+H]^+$. HRMS (ESI$^+$) m/z: $[M+H]^+$ calcd for $C_{16}H_{14}ON_4{}^{35}ClS$, 345.0571; found, 345.0573, $[M+H]^+$ calcd for $C_{16}H_{14}ON_4{}^{37}ClS$, 347.0542; found, 347.0542.

4.2.8. 4-{7-Chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-6-methanesulfinyl-1,3,5-triazin-2-amine (15)

To a solution of 4-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-6-(methylsulfanyl)-1,3,5-triazin-2-amine (**7**, 7.1 g, 20.6 mmol) in dichloromethane (70 mL) was added 3-chloroperbenzoic acid (5.7 g, 32.9 mmol) at room temperature. After the reaction mixture was stirred at room temperature for 3 h, the precipitate was collected by filtration and washed with dichloromethane (100 mL). The resulting precipitate was suspended in dichloromethane (1.5 L), and stirred at room temperature for 1 h. The precipitates were collected by filtration, washed with dichloromethane, and dried under reduced pressure. The title compound was obtained as a light brown solid (5.8 g, 78%). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.88 (3H, s), 5.05 (2H, s), 5.06 (2H, s), 7.32–7.40 (1H, m), 7.49–7.58 (3H, m), 8.38 (1H, br s), 8.53 (1H, br s). MS (ESI$^+$) m/z: 361, 363 $[M+H]^+$. HRMS (ESI$^+$) m/z: $[M+H]^+$ calcd for $C_{16}H_{14}{}^{35}ClN_4O_2S$, 361.05205; found, 361.05145, $[M+H]^+$ calcd for $C_{16}H_{14}{}^{37}ClN_4O_2S$, 363.04910; found, 363.04838.

4.2.9. *N*-{2-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]ethyl}acetamide (16a)

4-{7-Chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-6-methanesulfinyl-1,3,5-triazin-2-amine (**15**, 5.8 g, 16.1 mmol) was dissolved in a mixture of tetrahydrofuran (400 mL) and *N*,*N*-dimethylformamide (30 mL). *N*-(2-Sulfanylethyl)acetamide (3.4 mL, 32.1 mmol) and triethylamine (6.7 mL, 48.2 mmol) were added to the solution at room temperature. The resulting mixture was stirred for 15 h at room temperature and then the solvent was removed by evaporation. The resulting residue was dissolved in ethyl acetate (2.0 L), washed with water (1.0 L, twice) and brine (1.0 L) successively, dried over anhydrous magnesium sulfate, and filtered. The filtrate was concentrated by evaporation and the resulting residue was purified by recrystallization from ethanol (80 mL). The solids were dissolved with a mixture of ethyl acetate (2.0 L) and methanol (100 mL) washed with water (1.0 L, 3 times). The organic layer was diluted with ethanol (1.0 L) and concentrated by evaporation. The resulting solids were triturated in ethanol (50 mL), collected by filtration, and dried under reduced pressure. The title compound was obtained as a white solid (4.8 g, 72%). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.77 (3H, s), 3.10 (2H, t, J = 6.6 Hz), 3.31 (2H, m), 5.04 (2H, s), 5.05 (2H, s), 7.34 (1H, d, J = 6.5 Hz), 7.41 (1H, d, J = 8.4 Hz), 7.45 (1H, s), 7.52 (1H, dd, J = 8.4, 6.5 Hz), 7.81 (1H, br s), 7.83 (1H, br s), 8.07 (1H, t, J = 6.2 Hz). MS (ESI$^+$) m/z: 416, 418 $[M+H]^+$. HRMS (ESI$^+$) m/z: $[M+H]^+$ calcd for $C_{19}H_{19}{}^{35}ClN_5O_2S$, 416.09425; found, 416.09423, $[M+H]^+$ calcd for $C_{19}H_{19}{}^{37}ClN_5O_2S$, 418.09130; found, 418.09126.

4.2.10. 4-{7-Chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-6-{[2-(dimethylamino)ethyl]sulfanyl}-1,3,5-triazin-2-amine (16b)

4-{7-Chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-6-methanesulfinyl-1,3,5-triazin-2-amine (**15**, 50.0 mg, 0.139 mmol) and 2-(dimethylamino)ethanethiol hydrochloride (39.3 mg, 0.277 mmol) was dissolved in *N*,*N*-dimethylformamide (3.5 mL). Potassium carbonate (57.5 mg, 0.416 mmol) was added to the solution and the resulting mixture was stirred for 1 h at room temperature. The reaction mixture was diluted with ethylacetate and the solution was washed with water and brine successively. The organic layer was dried over anhydrous magnesium sulfate. The inorganic salts were removed by filtration and the filtrate was concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (dichloromethane/methanol = 10:1 to 4:1). The obtained product was triturated in a mixture of dichloromethane and n-hexane (1:2, 10 mL), washed with n-hexane, and dried in a vacuum. The title compound was obtained as a light brown solid (29.2 mg, 52%). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.12 (6H, s), 2.50 (2H, t, J = 7.2 Hz), 3.15 (2H, t, J = 7.2 Hz), 5.05 (4H, s), 7.34 (1H, d, J = 6.9 Hz), 7.40 (1H, d, J = 8.4 Hz), 7.45 (1H, s), 7.52 (1H, dd, J = 8.4, 6.9 Hz), 7.79 (2H, br s). MS (ESI$^+$) m/z: 402, 404 $[M+H]^+$. HRMS (ESI$^+$) m/z: $[M+H]^+$ calcd for $C_{19}H_{21}{}^{35}ClN_5OS$, 402.11499; found, 402.11427, $[M+H]^+$ calcd for $C_{19}H_{21}{}^{37}ClN_5OS$, 404.11204; found, 404.11119.

4.2.11. 2-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]ethan-1-ol (16c)

4-{7-Chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-6-methanesulfinyl-1,3,5-triazin-2-amine (**15**, 50.0 mg, 0.139 mmol) was dissolved in tetrahydrofuran (5.0 mL). 2-Hydroxyethanethiol (19.5 μL, 0.278 mmol) and triethylamine (97.0 μL, 0.700 mmol) was added successively to the solution at 0 °C and the resulting mixture was stirred for 16 h at 15 °C. The solvent was removed by evaporation and the resulting residue was purified by silica gel column chromatography (dichloromethane/

ethylacetate = 10:1 to 1:1). The title compound was obtained as a light brown solid (31.0 mg, 59%). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.14 (2H, d, J = 6.5 Hz), 3.62 (2H, dt, J = 5.5, 6.5 Hz), 4.91 (1H, t, J = 5.5 Hz), 5.04 (2H, s), 5.05 (2H, s), 7.34 (1H, d, J = 7.8 Hz), 7.40 (1H, d, J = 8.0 Hz), 7.44 (1H, s), 7.52 (1H, dd, J = 8.0, 7.8 Hz), 7.78 (2H, br s). MS (ESI$^+$) m/z: 375, 377 [M+H]$^+$. HRMS (ESI$^+$) m/z: [M+H]$^+$ calcd for $C_{17}H_{16}{}^{35}ClN_4O_2S$, 375.06770; found, 375.06695, [M+H]$^+$ calcd for $C_{17}H_{16}{}^{37}ClN_4O_2S$, 377.06475; found, 377.06383.

4.2.12. 3-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]propanamide (16d)

To the solution of 4-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-6-methanesulfinyl-1,3,5-triazin-2-amine (**15**, 8.07 g, 22.4 mmol) in *N*,*N*-dimethylformamide (81.0 mL) was added 3-sulfanylpropanoic acid (4.9 mL, 55.9 mmol) and *N*-ethyldiisopropylamine (11.7 mL, 67.1 mmol) at room temperature successively. The resulting mixture was stirred at room temperature for 1 h. The reaction mixture was poured into a mixture of 0.5 N aqueous potassium hydrogen sulfate solution (800 mL) and water (400 mL) and the resulting aqueous solution was stirred at room temperature for 0.5 h. The precipitates were collected by filtration, washed with water (400 mL), dried under reduced pressure. 3-[(4-amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]propanoic acid was obtained as a white solid (8.86 g, 98%). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.68 (2H, t, J = 6.9 Hz), 3.18 (2H, t, J = 6.9 Hz), 5.04 (2H, s), 5.05 (2H, s), 7.34 (1H, d, J = 6.9 Hz), 7.42 (1H, d, J = 8.0 Hz), 7.45 (1H, s), 7.52 (1H, dd, J = 8.0, 6.9 Hz), 7.87 (2H, br s), 12.40 (1H, br s). MS (ESI$^+$) m/z: 403, 405 [M+H]$^+$.

To a solution of 3-[(4-amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]propanoic acid (6.85 g, 17.0 mmol), ammonium chloride (2.73 g, 51.0 mmol), 1-hydroxybenzotirazole (6.89 g, 51.0 mmol), and 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (9.78 g, 51.0 mmol) in *N*,*N*-dimethylformamide (68.5 mL) was added *N*-ethyldiisopropylamine (11.9 mL, 68.0 mmol) at room temperature. The resulting mixture was stirred at room temperature for 20 h. The reaction mixture was diluted with ethyl acetate (1.2 L), washed with a saturated sodium hydrogen carbonate aqueous solution (200 mL, twice), water (200 mL, twice) and brine (200 mL) successively, dried over anhydrous magnesium sulfate, and filtered. The filtrate was concentrated and the resulting residue was purified by silica gel column chromatography (dichromethane/methanol = 20:1) to yield the title compound as a white solid (5.63 g, 82%). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.49 (2H, t, J = 6.9 Hz), 3.21 (2H, t, J = 6.9 Hz), 5.05 (4H, s), 6.96 (1H, br s), 7.35 (1H, d, J = 6.9 Hz), 7.38 (1H, br s), 7.42 (1H, d, J = 7.7 Hz), 7.46 (1H, s), 7.52 (1H, dd, J = 7.7, 6.9 Hz), 7.86 (2H, br s). MS (ESI$^+$) m/z: 402, 404 [M+H]$^+$. HRMS (ESI$^+$) m/z: [M+H]$^+$ calcd for $C_{18}H_{17}{}^{35}ClN_5O_2S$, 402.07860; found, 402.07776, [M+H]$^+$ calcd for $C_{18}H_{17}{}^{37}ClN_5O_2S$, 404.07565; found, 404.07475.

4.2.13. (2*R*)-3-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]-2-acetamidopropanamide (16e)

Compound **16e** was prepared from compound **15** and (2R)-2-acetamido-3-sulfanylpropanoic acid instead of 3-sulfanylpropanoic acid in the same manner as that described for the preparation of compound **16d** (31%, a white solid). ^{1}H NMR (270 MHz, CD_3OD) δ: 1.97 (3H, s), 3.27 (1H, dd, J = 14.0, 9.2 Hz), 3.79 (1H, dd, J = 14.0, 4.6 Hz), 4.78 (1H, dd, J = 9.2, 4.6 Hz), 5.06 (4H, s), 7.28 (1H, d, J = 6.4 Hz), 7.35 (1H, s), 7.42–7.53 (2H, m). MS (ESI$^+$) m/z: 459, 461 [M+H]$^+$. HRMS (ESI$^+$) m/z: [M+H]$^+$ calcd for $C_{20}H_{20}{}^{35}ClN_6O_3S$, 459.10006; found, 459.09941, [M+H]$^+$ calcd for $C_{20}H_{20}{}^{37}ClN_6O_3S$, 461.09711; found, 461.09648.

4.2.14. 3-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]-2-methylpropanamide (16f)

Compound **16f** was prepared from compound **15** and 2-methyl-3-sulfanylpropanoic acid instead of 3-sulfanylpropanoic acid in the same manner as that described for the preparation of compound **16d** (94%, a white solid).

^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.09 (3H, d, J = 6.5 Hz), 2.51–2.67 (1H, m), 3.10–3.19 (2H, m), 5.05 (4H, s), 6.89 (1H, br s), 7.34 (1H, d, J = 7.3 Hz), 7.36 (1H, s), 7.42 (1H, d, J = 8.9 Hz), 7.45 (1H, s), 7.51 (1H, dd, J = 8.9, 7.3 Hz), 7.82 (2H, br s). MS (ESI$^+$) m/z: 416, 418 [M+H]$^+$. HRMS (ESI$^+$) m/z: [M+H]$^+$ calcd for $C_{19}H_{19}{}^{35}ClN_5O_2S$, 416.09425; found, 416.09409, [M+H]$^+$ calcd for $C_{19}H_{19}{}^{37}ClN_5O_2S$, 418.09130; found, 418.09110.

4.2.15. (2*S*)-3-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]-2-methylpropanamide (16g)

Compound **16g** was prepared from compound **15** and (2*S*)-2-methyl-3-sulfanylpropanoic acid instead of 3-sulfanylpropanoic acid in the same manner as that described for the preparation of compound **16d** (78%, a white solid). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.10 (3H, d, J = 7.1 Hz), 2.54–2.67 (1H, m), 3.10–3.19 (2H, m), 5.05 (2H, s), 5.05 (2H, s), 6.88 (1H, br s), 7.34 (1H, d, J = 7.1 Hz), 7.36 (1H, br s), 7.42 (1H, d, J = 8.2 Hz), 7.45 (1H, s), 7.53 (1H, dd, J = 8.2, 7.1 Hz), 7.81 (2H, br s). MS (ESI$^+$) m/z: 416, 418 [M+H]$^+$.

4.2.16. 3-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]-*N*-methylpropanamide (16h)

Compound **16h** was prepared from compound **15** and methylamine hydrochloride instead of ammonium chloride in the same manner as that described for the preparation of compound **16d** (75%, a white solid). ^{1}H NMR (270 MHz, CD_3OD) δ: 2.67 (2H, t, J = 7.1 Hz), 2.71 (3H, s), 3.39 (2H, t, J = 7.1 Hz), 5.06 (4H, s), 7.28 (1H, d, J = 6.8 Hz), 7.35 (1H, s), 7.41 (1H, d, J = 8.6 Hz), 7.49 (1H, dd, J = 8.6, 6.8 Hz). MS (ESI$^+$) m/z: 416, 418 [M+H]$^+$. HRMS (ESI$^+$) m/z: [M+H]$^+$ calcd for $C_{19}H_{19}{}^{35}ClN_5O_2S$, 416.09425; found, 416.09359, [M+H]$^+$ calcd for $C_{19}H_{19}{}^{37}ClN_5O_2S$, 418.09130; found, 418.09054.

4.2.17. 3-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]-*N*,*N*-dimethylpropanamide (16i)

Compound **16i** was prepared from compound **15** and dimethylamine hydrochloride instead of ammonium chloride in the same manner as that described for the preparation of compound **16d** (33%, a white solid). ^{1}H NMR (270 MHz, $CDCl_3$) δ: 2.80 (2H, t, J = 7.3 Hz), 2.90 (3H, s), 2.92 (3H, s), 3.37 (2H, t, J = 7.3 Hz), 5.06 (2H, s), 5.07 (2H, s), 5.60 (2H, br s), 7.20 (1H, dd, J = 4.9, 3.1 Hz), 7.24 (1H, s), 7.45 (1H, d, J = 4.9 Hz), 7.45 (1H, d, J = 3.1 Hz). MS (ESI$^+$) m/z: 430, 432 [M+H]$^+$. HRMS (ESI$^+$) m/z: [M+H]$^+$ calcd for $C_{20}H_{21}{}^{35}ClN_5O_2S$, 430.10990; found, 430.10919, [M+H]$^+$ calcd for $C_{20}H_{21}{}^{37}ClN_5O_2S$, 432.10695; found, 432.10626.

4.2.18. 3-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]-*N*-(2-hydroxyethyl)propanamide (16j)

Compound **16j** was prepared from compound **15** and 2-aminoethanol instead of ammonium chloride in the same manner as that described for the preparation of compound **16d** (90%, a white solid).

^{1}H NMR (270 MHz, $CDCl_3$) δ: 2.66 (2H, t, J = 7.2 Hz), 3.26–3.32 (2H, m), 3.35 (2H, t, J = 7.2 Hz), 3.61 (2H, t, J = 4.6 Hz), 5.06 (2H, s), 5.07 (2H, s), 5.82 (1H, br s), 6.03 (1H, br s), 6.31 (1H, br t), 7.21 (1H,

dd, J = 4.6, 3.6 Hz), 7.25 (1H, s), 7.46 (1H, d, J = 4.6 Hz), 7.46 (1H, d, J = 3.6 Hz). MS (ESI^+) m/z: 446, 448 $[M+H]^+$. HRMS (ESI^+) m/z: $[M+H]^+$ calcd for $C_{20}H_{21}{}^{35}ClN_5O_3S$; 446.10481; found, 446.10466, $[M+H]^+$ calcd for $C_{20}H_{21}{}^{37}ClN_5O_3S$; 448.10186; found, 448.10172.

4.2.19. 2-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]acetamide (16k)

Compound **16k** was prepared from compound **15** and 2-sulfanylacetic acid instead of 3-sulfanylpropanoic acid in the same manner as that described for the preparation of compound **16d** (87%, a white solid). ^1H NMR (300 MHz, DMSO-d_6) δ: 3.78 (2H, s), 5.04 (2H, s), 5.05 (2H, s), 7.12 (1H, br s), 7.34 (1H, d, J = 7.7 Hz), 7.41 (1H, d, J = 8.5 Hz), 7.44 (1H, s), 7.44 (1H, br s), 7.52 (1H, dd, J = 8.5, 7.7 Hz), 7.81 (1H, br s), 7.83 (1H, br s). MS (ESI^+) m/z: 388, 390 $[M+H]^+$. HRMS (ESI^+) m/z: $[M+H]^+$ calcd for $C_{17}H_{15}{}^{35}ClN_5O_2S$, 388.06295; found, 388.06216, $[M+H]^+$. HRMS (ESI^+) m/z: $[M+H]^+$ calcd for $C_{17}H_{15}{}^{37}ClN_5O_2S$, 390.06000; found, 390.05913.

4.2.20. 4-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]butanamide (16l)

4, 4-dithiobutyric acid (2.75 g, 7.62 mmol) was dissolved in *N*,*N*-dimethylformamide (25 mL) and water (2.7 mL) under nitrogen atmosphere. Tri-n-butylphosphine (2.7 mL, 10.8 mmol) was added to the solution and the resulting mixture was stirred for 1.5 h at room temperature. To the obtained mixture were added *N*-ethyldiisopropylamine (4.0 mL, 23.2 mmol) and 4-(5-chloro-1H, 3H-benzo[de]isochromen-6-yl)-6-methanesulfinyl-1,3,5-triazin-2-ylamine (2.77 g, 10.6 mmol) in *N*,*N*-dimethylformamide (14 mL) successively, and stirring was continued for 2 h. The mixture was diluted with water (100 mL) and 1 N HCl (20 mL), and extracted with ethyl acetate (100 mL, three times). The 0rganic layers were combined and washed with water (100 mL) and brine (100 mL) successively, and then dried over anhydrous sodium sulfate. After removal of inorganic salts by filtration, the filtrate was concentrated in vacuo giving crude 4-[4-amino-6-(5-chloro-1H,3H-benzo[de]isochromen-6-yl)-1,3,5-triazin-2-ylsulfanyl]-butyric acid as a crude yellow oil (9.77 g). MS (ESI^+) m/z: 417, 419$[M+H]^+$.

The obtained crude product was dissolved in *N*,*N*-dimethylformamide (50 mL). To the solution were added ammonium chloride (1.24 g, 23.1 mmol), 1-ethyl-3-(3-dimethylamino propyl)carbodiimide (4.43 g, 23.1 mmol), 1-hydroxybenzotriazole monohydrate (3.54 g, 23.1 mmol), and *N*-ethyldiisopropylamine (6.6 mL, 38.5 mmol) successively at room temperature. The mixture was stirred overnight at the same temperature. The reaction mixture was poured into water (450 mL) and extracted with ethyl acetate (300 mL, three times). The organic layers were combined and washed with water (100 mL) and brine (100 mL) successively, and then dried over anhydrous sodium sulfate. After removal of inorganic salts by filtration, the filtrate was concentrated in vacuo and the resulting residue was purified by silica gel column chromatography (dichromethane/methanol = 30:1 to 15:1) to yield the title compound as a white solid (1.52 g, 81% in 2 steps). ^1H NMR (400 MHz, DMSO-d_6) δ: 1.81–1.89 (2H, m), 2.16 (2H, t, J = 7.6 Hz), 3.05 (2H, t, J = 7.0 Hz), 5.05 (2H, s), 5.06 (2H, s), 6.75 (1H, br s), 7.28 (1H, br s), 7.34 (1H, d, J = 6.7 Hz), 7.41 (1H, d, J = 8.5 Hz), 7.45 (1H, s), 7.52 (1H, dd, J = 8.5, 6.7 Hz), 7.79 (1H, s), 7.80 (1H, s). MS (ESI^+) m/z: 416, 418 $[M+H]^+$. HRMS (ESI^+) m/z: $[M+H]^+$ calcd for $C_{19}H_{19}O_2N_5{}^{35}ClS$, 416.0942; found, 416.0942, $[M+H]^+$ calcd for $C_{19}H_{19}O_2N_5{}^{37}ClS$, 418.0913; found, 418.0913.

4.2.21. 4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazine-2-thiol (17)

To the suspension of 4-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-6-methanesulfinyl-1,3,5-triazin-2-amine (**15**, 4.55 g, 11.1 mmol) in *N*,*N*-dimethylformamide (100 mL) was added potassium thioacetate (2.54 g, 22.3 mmol) at room temperature. The resulting mixture was stirred at room temperature for 19 h and then the reaction mixture was cooled in an ice-water bath. To the cold solution was added 2 N aqueous sodium hydroxide solution (100 mL) and the resulting mixture was stirred at room temperature for 1.5 h. The reaction mixture was diluted with water (100 mL) and the pH of the solution was adjusted to approximately 5 to 6 with 1 N aqueous hydrochloric acid (185 mL). The aqueous mixture was diluted with water (400 mL) and the resulting mixture was stirred at room temperature for 1 h. The precipitates were collected by filtration, washed with water (50 mL, three times), and dried under reduced pressure. The title compound was obtained as a brown solid (3.75 g, 92%). ^1H NMR (300 MHz, DMSO-d_6) δ: 5.03 (2H, s), 5.06 (2H, s), 7.39 (1H, d, J = 5.8 Hz), 7.50 (1H, s), 7.55–7.64 (2H, m), 8.11 (1H, br s), 8.23 (1H, br s), 13.43 (1H, br s). MS (ESI^+) m/z: 331, 333 $[M+H]^+$. HRMS (ESI^+) m/z: $[M+H]^+$ calcd for $C_{15}H_{12}{}^{35}ClN_4OS$, 331.04149; found, 331.04138, $[M+H]^+$ calcd for $C_{15}H_{12}{}^{37}ClN_4OS$, 333.03854; found, 333.03854.

4.2.22. N-{2-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.05,13]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]ethyl}methanesulfonamide (18a)

To the solution of 4-amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazine-2-thiol (**17**, 399 mg, 1.21 mmol) and *N*-(2-chloroethyl)methanesulfonamide (381 mg, 2.41 mmol) in *N*,*N*-dimethylformamide (20 mL) was added *N*-ethyldiisopropylamine (631 μL, 3.14 mmol) at room temperature. The resulting mixture was stirred for 2.5 h at 70 °C. After cooling to room temperature, the mixture was extracted between ethylacetate and water. The organic layer was washed with water and brine successively, dried over anhydrous magnesium sulfate, and filtered. The filtrate was concentrated by evaporation and the resulting residue was purified by preparative HPLC to give the title compound (383 mg, 86%) as a white solid. ^1H NMR (270 MHz, DMSO-d_6) δ: 2.84 (3H, s), 3.16 (2H, t, J = 6.6 Hz), 3.24–3.28 (2H, m), 5.04 (2H, br s), 5.05 (2H, br s), 7.21 (1H, br s), 7.34 (1H, d, J = 6.8 Hz), 7.42 (1H, d, J = 8.3 Hz), 7.45 (1H, s), 7.52 (1H, dd, J = 8.3, 6.8 Hz), 7.78 (1H, br s), 7.83 (1H, br s). MS (ESI^+) m/z: 452, 454 $[M+H]^+$. HRMS (ESI^+) m/z: $[M+H]^+$ calcd for $C_{18}H_{19}{}^{35}ClN_5O_3S_2$, 452.06124; found, 452.06032, $[M+H]^+$ calcd for $C_{18}H_{19}{}^{37}ClN_5O_3S_2$, 454.05828; found, 454.05725.

4.2.23. {2-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]ethyl}urea (18b)

Compound **18b** was prepared from compound **17** and 2-chloroethylurea instead of *N*-(2-chloroethyl)methanesulfonamide in the same manner as that described for the preparation of compound **18a** (55%, a white solid). ^1H NMR (400 MHz, DMSO-d_6) δ: 3.10 (2H, t, J = 6.4 Hz), 3.25 (2H, m), 5.04 (2H, br s), 5.05 (2H, br s), 5.48 (2H, br s), 6.18 (1H, t, J = 5.5 Hz), 7.34 (1H, d, J = 6.9 Hz), 7.42 (1H, d, J = 8.2 Hz), 7.45 (1H, s), 7.52 (1H, dd, J = 8.2, 6.9 Hz), 7.82 (2H, br s). MS (ESI^+) m/z: 417, 419 $[M+H]^+$. HRMS (ESI^+) m/z: $[M+H]^+$ calcd for $C_{18}H_{18}{}^{35}ClN_6O_2S$, 417.08950; found, 417.08949, $[M+H]^+$ calcd for $C_{18}H_{18}{}^{37}ClN_6O_2S$, 419.08655; found, 419.08637.

4.2.24. 2-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]ethyl carbamate (18c)

To a solution of 2-bromoethan-1-ol (10.0 mL, 141.1 mmol) in *N*,*N*-dimethylformamide (140 mL) was added 4-nitrophenyl chloroformate (32.0 g, 158.8 mmol) and *N*-ethyldiisopropylamine (34.4 mL, 197.5 mmol) at 0 °C successively. The resulting mixture was stirred at 0 °C for 1.5 h. To the mixture was added 28%

ammonia solution in water (17.5 mL) at 0 °C and the resulting mixture was stirred for 20 min. Water (300 mL) was added to the reaction mixture and the pH was adjusted to approximately 4 by addition of 1 N hydrochloric acid. The aqueous solution was extracted with ethyl acetate (200 mL, three times). The organic layers were combined, washed with 0.1 N hydrochloric acid (100 mL), water (100 mL), 0.5 N aqueous sodium hydroxide solution (100 mL, four times), water (100 mL, three times), and brine (100 mL) successively, dried over anhydrous sodium sulfate, and filtered. The filtrate was concentrated by evaporation and dried under reduced pressure to yield 2-bromoethyl carbamate along with 4-nitrophenol (5.42 g). The crude product was dissolved in *N*,*N*-dimethylformamide (130 mL), and to the solution was added 4-amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazine-2-thiol (**17**, 2.90 g, 8.77 mmol) and *N*-ethyldiisopropylamine (14 mL, 80.4 mmol) at room temperature. The resulting mixture was stirred at 70 °C for 2 h. Water (300 mL) was added to the reaction mixture and the pH was adjusted to approximately 4 by addition of 1 N hydrochloric acid. The aqueous solution was extracted with ethyl acetate (200 mL, three times). The organic layers were combined, washed with 0.2 N hydrochloric acid (100 mL), water (100 mL, twice), and brine (100 mL) successively, dried over anhydrous sodium sulfate, and filtered. The filtrate was concentrated by evaporation and the resulting residue was purified by preparative HPLC (0.05% trifluoroacetic acid in acetonitrile/0.05% trifluoroacetic acid in water = 35:65) to yield the title compound as a white solid (1.33 g, 36%). ^{1}H NMR (270 MHz, DMSO-d_6) δ: 3.27 (2H, t, J = 6.4 Hz), 4.13 (2H, t, J = 6.4 Hz), 5.05 (4H, br s), 6.55 (2H, br s), 7.33–7.55 (4H, m), 7.86 (2H, br s). MS (ESI$^+$) m/z: 418, 420 [M+H]$^+$. HRMS (ESI$^+$) m/z: [M+H]$^+$ calcd for $C_{18}H_{17}{}^{35}ClN_5O_3S$, 418.07351; found, 418.07325, [M+H]$^+$ calcd for $C_{18}H_{17}{}^{37}ClN_5O_3S$, 420.07056; found, 420.07023.

4.2.25. (5S)-5-{[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]tri deca-1(13),5,7,9,11-pentaen-8-yl-1,3,5-triazin-2-yl)sulfanyl]methyl}-1,3-oxazolidin-2-one (18d)

To a solution of (2S)-2-(chloromethyl)oxirane (0.813 g, 8.79 mmol) in water (70 mL) was added potassium cyanate (1.43 g, 17.63 mmol) at room temperature and the resulting mixture was refluxed with stirring for 19 h. The reaction mixture was allowed to cool to room temperature and extracted with ethyl acetate (50 mL, three times). The organic layers were combined, dried over anhydrous sodium sulfate, and filtered. The filtrate was concentrated by evaporation to yield (5S)-5-(chloromethyl)-1,3-oxazolidin-2-one along with ethyl acetate as colorless oil (87% in ethyl acetate, 0.242 g, 18%,). ^{1}H NMR (270 MHz, $CDCl_3$) δ: 3.52–3.58 (1H, m), 3.63–3.80 (3H, m), 4.81–4.91 (1H, m), 5.56 (1H, br s). MS (ESI$^+$) m/z: 136, 138 [M+H]$^+$.

Compound **18d** was prepared from compound **17** and (5S)-5-(chloromethyl)-1,3-oxazolidin-2-one instead of *N*-(2-chloroethyl)methanesulfonamide in the same manner as that described for the preparation of compound **18a** (62%, a light brown solid).^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.26 (1H, dd, J = 6.6, 8.8 Hz), 3.38 (1H, dd, J = 14.0, 6.0 Hz), 3.47 (1H, dd, J = 14.0, 6.0 Hz), 3.57 (1H, dd, J = 8.8, 8.8 Hz), 4.79–4. 87 (1H, m), 5.05 (2H, s), 5.06 (2H, s), 7.35 (1H, d, J = 7.1 Hz), 7.43 (1H, d, J = 8.8 Hz), 7.45 (1H, s), 7.53 (1H, dd, J = 8.8, 7.1 Hz), 7.57 (1H, s), 7.91 (2H, s). MS (ESI$^+$) m/z: 430, 432 [M+H]$^+$. HRMS (ESI+) m/z: [M+H]+ calcd for $C_{19}H_{17}{}^{35}ClN_5O_3S$, 430.07351; found, 430.07277, [M+H]+ calcd for $C_{19}H_{17}{}^{37}ClN_5O_3S$, 432.07056; found, 432.06954.

4.2.26. (5R)-5-{[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]methyl}-1,3-oxazolidin-2-one (18e)

(5*R*)-5-(Chloromethyl)-1,3-oxazolidin-2-one was prepared from (2*R*)-2-(chloromethyl)oxirane instead of (2*S*)-2-(chloromethyl)oxirane in the same manner as that described for the preparation of compound **18d** (79% in ethyl acetate, 15%, colorless oil). ^{1}H NMR (270 MHz, $CDCl_3$) δ: 3.52–3.58 (1H, m), 3.63–3.80 (3H, m), 4.81–4.91 (1H, m), 5.56 (1H, br s). MS (ESI$^+$) m/z: 136, 138 [M+H]$^+$.

Compound **18e** was prepared from compound **17** and (5*R*)-5-(chloromethyl)-1,3-oxazolidin-2-one instead of *N*-(2-chloroethyl)methanesulfonamide in the same manner as that described for the preparation of compound **18a** (60%, a light brown solid). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.26 (1H, dd, J = 8.8, 6.6 Hz), 3.38 (1H, dd, J = 14.0, 6.0 Hz), 3.47 (1H, dd, J = 14.0, 6.0 Hz), 3.57 (1H, dd, J = 8.8, 8.8 Hz), 4.79–4. 87 (1H, m), 5.05 (2H, s), 5.06 (2H, s), 7.35 (1H, d, J = 7.1 Hz), 7.43 (1H, d, J = 8.8 Hz), 7.45 (1H, s), 7.53 (1H, dd, J = 8.8, 7.1 Hz), 7.57 (1H, s), 7.91 (2H, s). MS (ESI$^+$) m/z: 430, 432 [M+H]$^+$. HRMS (ESI$^+$) m/z: [M+H]$^+$ calcd for $C_{19}H_{17}{}^{35}ClN_5O_3S$, 430.07351; found, 430.07340, [M+H]$^+$ calcd for $C_{19}H_{17}{}^{37}ClN_5O_3S$, 432.07056; found, 432.07043.

4.2.27. 3-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]tri deca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]-2,2-dimethylpropanamide (18f)

To the solution of 4-amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazine-2-thiol (**17**, 50 mg, 0.151 mmol) and 3-bromo-2,2-dimethylpropioic acid (55 mg, 0.302 mmol) in *N*,*N*-dimethylformamide (1.0 mL) was added *N*-ethyldiisopropylamine (79 μL, 0.453 mmol) at room temperature. The resulting mixture was stirred at 80 °C for 1 h. To the reaction mixture were added 0.5 N aqueous potassium hydrogen sulfate solution (2.5 mL) and water (3.0 mL) successively and the resulting aqueous solution was stirred at room temperature for 1 h. The precipitates were collected by filtration, washed with water (3.0 mL, three times), and dried under reduced pressure. 3-[(4-Amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]-2,2-dimethylpropanoic acid was obtained along with 2,2-dimethyl-3-sulfanylpropanoic acid as a brown solid (54 mg). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.18 (6H, s), 3.36 (2H, s), 5.05 (2H, s), 5.06 (2H, s), 7.32 (1H, d, J = 7.1 Hz), 7.37 (1H, d, J = 8.8 Hz), 7.45 (1H, s), 7.52 (1H, dd, J = 8.8, 7.1 Hz), 7.83 (1H, br s), 7.85 (1H, br s), 12.50 (1H, br s). MS (ESI$^+$) m/z: 431, 433 [M+H]$^+$.

To a solution of 3-[(4-amino-6-{7-chloro-3-oxatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-8-yl}-1,3,5-triazin-2-yl)sulfanyl]-2,2-dimethylpropanoic acid (25 mg, 0.0581 mmol), ammonium chloride (16 mg, 0.290 mmol), 1-hydroxybenzotirazole (49 mg, 0.290 mmol), and 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (56 mg, 0.292 mmol) in *N*,*N*-dimethylformamide (1.0 mL) was added *N*-ethyldiisopropylamine (60 μL, 0.348 mmol) at room temperature. The resulting mixture was stirred at room temperature for 1 h. The reaction mixture was diluted with ethyl acetate (10 mL), washed with water (5.0 mL, twice), saturated sodium hydrogen carbonate aqueous (5 mL, twice), and brine (5 mL) successively, dried over anhydrous magnesium sulfate, and filtered. The filtrate was concentrated and the resulting residue was purified by silica gel column chromatography (dichloromethane/methanol = 99:1 to 9:1) to yield the title compound as a white solid (15 mg, 60%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.16 (6H, s), 3.31 (2H, s), 5.05 (2H, s), 5.06 (2H, s), 6.95 (1H, s), 7.19 (1H, s), 7.35 (1H, d, J = 7.1 Hz), 7.36 (1H, d, J = 8.2 Hz), 7.45 (1H, s), 7.52 (1H, dd, J = 8.2, 7.1 Hz), 7.79 (1H, s), 7.81 (1H, s). MS (ESI$^+$) m/z: 430, 432 [M+H]$^+$. HRMS (ESI$^+$) m/z: [M+H]$^+$ calcd for $C_{20}H_{21}{}^{35}ClN_5O_2S$, 430.10990; found, 430.10928, [M+H]$^+$ calcd for $C_{20}H_{21}{}^{37}ClN_5O_2S$, 432.10695; found, 432.10637.

4.3. Surface plasmon resonance direct binding assay for Hsp90α

Measurements were performed on a Biacore2000 at a flow rate of 30 μL/min, 25 °C in 50 mM Tris-based saline, pH 7.6, 0.005%

Tween20 and 1% DMSO. Biotinylated N-terminal Hsp90α (9–236) was coupled on a streptavidin-coated surface of a sensorchip (Type SA, GE healthcare) to a density of ca. 2000 RU.

4.4. In vitro cell growth assay

HCT116 cell line (CCL-247, a colorectal cancer cell line) and NCI-N87 cell line (CRL-5822, a gastric cancer cell line) were purchased from ATCC. All cell lines were cultured according to the supplier's instructions. Cells suspended in a medium were added to solutions containing various concentrations of the test substance, and the cells were cultured at 37 °C in 5% CO_2. Four days later, Cell Counting Kit-8 solution (Dojindo Laboratories) was added and absorbance at 450 nm was measured with Microplate-Reader iMark (Bio-Rad Laboratories). Antiproliferative activity was calculated by the formula $(1-T/C) \times 100$ (%), where T represents the absorbance of drug-treated cells and C that of untreated control cells at 450 nm. The 50% inhibition concentration (IC_{50}) values were calculated using Microsoft Excel 2007.

4.5. Lyophilized solubility assay

Samples were prepared in triplicate from 10 mM DMSO stock solutions. After evaporation (1 h) of DMSO with a centrifugal vacuum evaporator, the compounds were dissolved in FaSSIF, and shaken for 2 h. The solutions were filtered using a microtiter filter plate (Whatman UNIFILTER) and the filtrate was analyzed by UV measurement or HPLC-UV. In addition, a four point calibration curve was prepared from the 10 mM stock solutions and used to determine the solubility of the compounds. The results are expressed in μM.

4.6. Liver microsomal stability assay

Mouse or human microsome incubations were conducted by an automated procedure implemented on a Biomek FX (Beckman Coulter). Compounds (5 μM) were incubated in liver microsomes (1.0 mg protein/mL) in a 50 mM potassium phosphate buffer (pH 7.4) at 37 °C. NADPH as a cofactor was produced by a generating system (glucose 6-phosphate, 3.2 mM; β-NADP, 2.6 mM; $MgCl_2$, 6.5 mM). NADPH from the generating system was added to the pre-warmed liver microsomes containing the test compound. Aliquots (50 μL) were taken at four defined time points within 60 min and transferred into 100 μL of methanol containing an internal standard. Concentration of each compound was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an ODS-3 RP 18 column (GL Sciences). Quantitative detection was achieved on an API 365 (AB SCIEX) using ESI. Concentrations were determined by ratio of test compound and internal standard peaks and given as a percentage of the concentration measured at the first time point (substrate depletion). Intrinsic clearance (CL, in μL/min/mg microsomal protein) is the rate constant of the first-order decay of the test compound, normalized for the protein concentration in the incubation.

4.7. Western blotting

NCI-N87 cells were treated with various concentrations (0, 0.04, 0.2, 1, 5 μM) of the test substance for 24 h and lysed with cell lysis buffer (Cell Signaling Technology) supplemented with protease inhibitor cocktail (Roche Diagnostics) and phosphatase inhibitors (Sigma–Aldrich). Equivalent amounts of cell lysate were resolved by SDS–PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked with Blocking One (Nacalai Tesque) incubated overnight with primary antibodies (pS473-Akt, Akt, pT202/Y204-ERK, ERK, and Cleaved-PARP [Cell Signaling], EGFR, HER2, Raf1 and GAPDH [Santa Cruz Biotechnology], and Hsp70 [Stressgen]) at 4 °C, and then washed with 0.1% Tween20 in TBS buffer (TBS-T buffer; Nacalai Tesque). The membranes were incubated for 1 h at room temperature with secondary antibodies (Cell Signaling Technology or Invitrogen), washed with TBS-T buffer, and then the bands were detected with ECL Plus (GE Healthcare) followed by LAS-4000 (Fujifilm).

4.8. Xenograft model and in vivo antitumor study

All animal studies were approved by the Chugai Institutional Animal Care and Use Committee. Cancer cells ($0.5–1 \times 10^7$) were implanted subcutaneously into the right flank of athymic nude (BALB/c nu/nu) mice (CAnN.Cg-Foxn1<nu>/CrlCrlj nu/nu, Charles River Laboratories). Tumor volume was calculated using the formula $TV = ab^2/2$, where a and b represent tumor length and width, respectively. Once the TV had reached approximately 200–300 mm^3, animals were randomized into each group ($n = 4$ or 5), and treatment was initiated. CH5164840 (**16**) was dissolved in a vehicle of 10% DMSO/10% Cremophor EL/0.02 N HCl in water and then orally administered to mice. TGI was calculated using the formula $TGI = [1-(T_t-T_0)/(C_t-C_0)] \times 100$ (%), where T (tumor volume of treated group, T_0 on day 0 or T_t on day t) and C (tumor volume of control group, C_0 on day 0 or C_t on day t) represent mean tumor volume.

4.9. Pharmacokinetics

Pharmacokinetic studies of test compounds were performed in female athymic *nu*/*nu* mice following an oral (po) or intravenous (iv) route. The test compounds were dissolved in vehicles (CH5015765 (**7**): 10% DMSO/10% Cremophor EL/4% glucose in water, CH5164840 (**16**): 10% DMSO/10% Cremophor EL/0.025 N HCl in water). Blood samples were collected from the orbital cavity using a heparinized capillary or by heart puncture using a disposable syringe with a 25G needle under isoflurane anesthesia at the scheduled time points of 2, 15 min, 1, 2, 4, 7 and 24 h following iv dosing and at those of 30 min, 1, 2, 4, 7 and 24 h following po dosing. Blood samples were immediately centrifuged by a hematocrit centrifuge (Model 3220, Kubota) at 12,000 rpm for 3 min or by a centrifuge at 3000 rpm for 10 min at 4 °C (LX-130, Tomy Seiko) to separate the plasma. Plasma samples were immediately frozen on dry ice and stored at −80 °C until analysis. The plasma sample (5 μL) was added with 35 μL of CH_3CN/MeOH/water (15:15:5 v/v) including an internal standard, and then thoroughly mixed to precipitate plasma protein. The suspensions were transferred to 96-well MultiScreen filtration plates (0.45 μm) and filtered by centrifugation at 1000*g* for 5 min at 4 °C (LX-130, Tomy Seiko). The filtrate was applied to an LC-MS/MS system (API3000, AB SCIEX) to measure concentrations of the compounds. The lower limit of quantification (LLOQ) was 1 ng/mL. The pharmacokinetic parameters were calculated by non-compartmental analysis using Watson ver. 6.3 (Thermo Fisher Scientific).

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