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Ethyl 2-(benzylidene)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate analogues as a new scaffold for protein kinase casein kinase 2 inhibitor

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ABSTRACT

Protein kinase casein kinase 2 (PKCK2) is a constitutively active, growth factor-independent serine/threonine kinase, and changes in PKCK2 expression or its activity are reported in many cancer cells. To develop a novel PKCK2 inhibitor(s), we first performed cell-based phenotypic screening using 4000 chemicals purchased from ChemDiv chemical libraries (2000: randomly selected; 2000: kinase-biased) and performed in vitro kinase assay-based screening using hits found from the first screening. We identified compound **24** (C**24**)[(*Z*)-ethyl 5-(4-chlorophenyl)-2-(3,4-dihydroxybenzylidene)-7-methyl-3-oxo-3,5-dihydro-2*H*-thiazolo[3,2-*a*] pyrimidine-6-carboxylate] as a novel inhibitor of PKCK2 that is more potent and selective than 4,5,6,7-tetrabromobenzotriazole (TBB). In particular, compound **24** [half maximal inhibitory concentration (IC_{50}) = 0.56 μM] inhibited PKCK2 2.2-fold more efficiently than did TBB (IC_{50} = 1.24 μM), which is quite specific toward PKCK2 with respect to ATP binding, in a panel of 31 human protein kinases. The K_i values of compound **24** and TBB for PKCK2 were 0.78 μM and 2.70 μM, respectively. Treatment of cells with compound **24** inhibited endogenous PKCK2 activity and showed anti-proliferative and pro-apoptotic effects against stomach and hepatocellular cancer cell lines more efficiently than did TBB. As expected, compound **24** also enabled tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-resistant cancer cells to be sensitive toward TRAIL. In comparing the molecular docking of compound **24** bound to PKCK2α versus previously reported complexes of PKCK2 with other inhibitors, our findings suggest a new scaffold for specific PKCK2α inhibitors. Thus, compound **24** appears to be a selective, cell-permeable, potent, and novel PKCK2 inhibitor worthy of further characterization.

1. Introduction

Protein kinase casein kinase 2 (PKCK2) is a highly pleiotropic serine/threonine-specific kinase ubiquitously expressed in all types of eukaryotic cells.[1] There are more than 300 protein substrates already identified so far, and their number is increasing rapidly.[2] Unlike the majority of the protein kinases family, which are turned on only in response to specific stimuli, the two catalytic (α and/or α′) subunits of PKCK2 are in fact constitutively active either alone or in combination with the two regulatory (β) subunits (to give a heterotetrameric holoenzyme), which appear to play roles in targeting and substrate recruiting rather than controlling catalytic activity.[3,4] PKCK2 plays a central role in controlling nearly all cellular functions, with special reference to signal transduction, gene expression, DNA repair, RNA and protein synthesis,[2,4] and participates in a wide variety of cellular processes including cell cycle control, cell differentiation, proliferation, survival, protection of cells from apoptosis, and tumorigenesis.[4–8] Abnormally elevated PKCK2 activity has been documented in a number of cancers including that of kidney,[9] lung,[10] head and neck,[11] prostate,[12] and mammary gland,[13] and in a number of experimental models.[14] Coincidental arguments support the notion that PKCK2 promotes cell survival through the regulation of oncogenes and plays a global anti-apoptotic role.[14] Those actions of PKCK2 in tumorigenesis are through the regulation of key oncogenes and tumor suppressor

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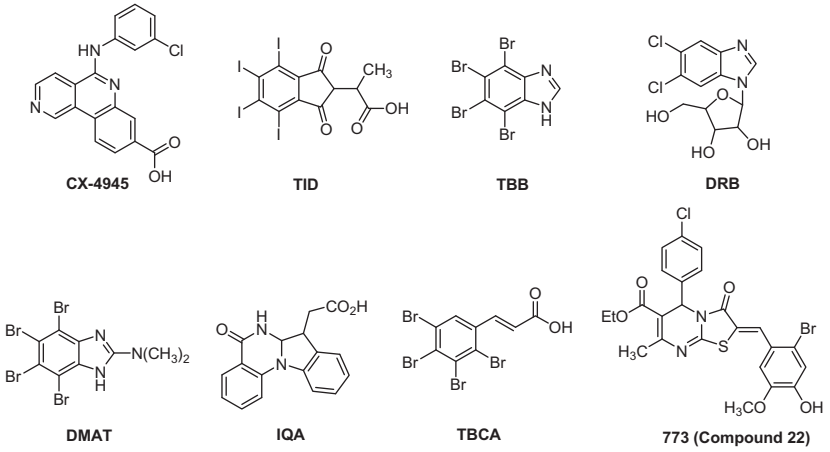
0968-0896/

proteins within various prosurvival pathways, including the Wnt[15] and nuclear factor-κB networks.[16] The feasibility of targeting PKCK2 for cancer therapy[6,17–20] has been shown by experiments where successful disruption of PKCK2 activity in vitro with antisense PKCK2 resulted in the induction of cell apoptosis. This concept has been validated in vivo in prostate cancer xenograft.[20] Furthermore, the reports that many viral proteins are PKCK2 substrates indicate that this kinase may play a role in viral infections as well.[2] For these reasons, there is interest in PKCK2 as a potential target for anti-neoplastic and anti-infectious compounds, with the obvious caveat that its activity cannot be fully, permanently, and ubiquitously inhibited without lethal consequences.

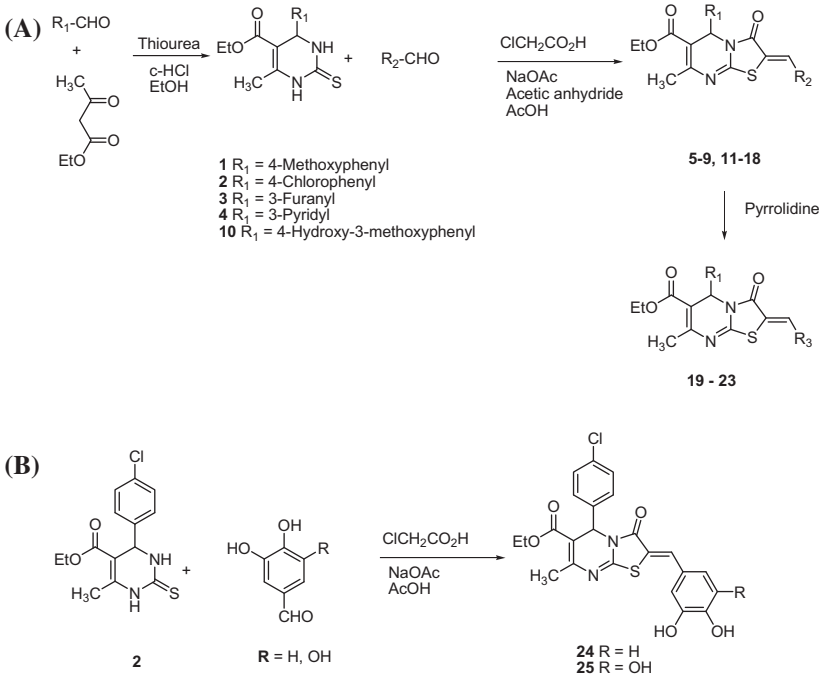


Currently, a number of PKCK2 inhibitors are known, including CX-4945,[21] 4,5,6,7-tetrahalogeno-1*H*-isoindole-1,3(2*H*)-diones (TID),[22] tetrabromobenzotriazole (TBB),[23] 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB),[24] 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole (DMAT),[9] emodin,[25] apigenin,[26] [5-oxo-5,6-dihydroindolo-(1,2-*a*)quinazolin-7-yl]acetic acid (IQA),[27] and tetrabromocinnamic acid (TBCA).[28] However, except for CX-4945, currently available PKCK2 inhibitors lack the potency and the pharmacological properties necessary to be suitable and successful in clinical applications. Accordingly, the purpose of this study was to develop efficient, selective, and cell-permeable inhibitors of PKCK2.

2. Results and discussion

2.1. Screening compounds library for PKCK2 inhibitors

Previously, we reported that PKCK2 inhibition could sensitize tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-resistant cancer cells to TRAIL.[8] Therefore, we first conducted cell-based phenotypic screening using 4000 chemicals (2000: randomly selected; 2000: kinase-biased) purchased from ChemDiv to identify potential inhibitors of PKCK2. Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using TRAIL-resistant HCE4 cells. The cells were incubated with each chemical (10 μM) for 24 h and subsequently



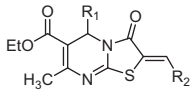
Scheme 1. Synthetic methods for benzylidenethiazolopyrimidones **5**–**9** and **11**–**25**.

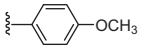
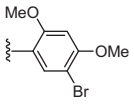
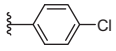
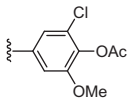
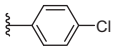
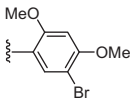
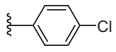
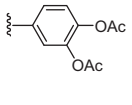
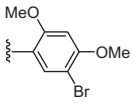
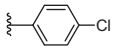
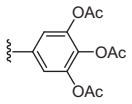
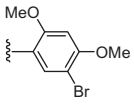
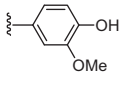
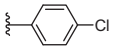
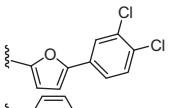
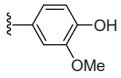
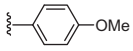
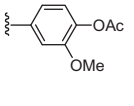
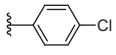
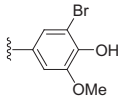
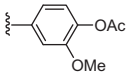
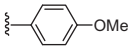
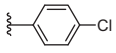
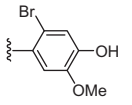
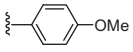
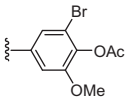
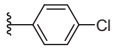
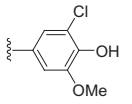
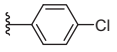
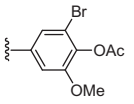
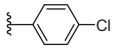
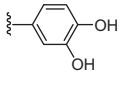
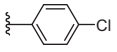
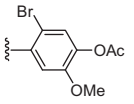
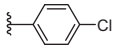
treated or untreated with TRAIL (200 ng/mL) for 2 h. A 'hit' was defined as a chemical that showed >90% of cell viability in the presence of the chemical alone and <50% of cell viability in the presence of both TRAIL and each chemical (Fig. S1). We performed in vitro kinase assays against human recombinant PKCK2α (rhCK2α) using the positive 'hit' chemicals. As shown in Figure S2, among the hits, one lead chemical (773) exhibited a remarkable inhibitory activity against PKCK2α.

2.2. Chemistry

To optimize the inhibitory activity of 773 against PKCK2, we synthesized 19 derivatives that have structural similarity. Synthesis for the benzylidenethiazolopyrimidine derivatives was accomplished by the reported method.[29] First, according to the Biginelli method, the key starting arylthiazolopyrimidine compounds **1**–**4** were prepared from ethylacetoacetate, thiourea, and the corresponding aldehyde, using c-HCl as catalyst in ethanol solvent.[30,31] Compounds **2**, however, showed a better reaction yield with chloroacetic acid as catalyst without solvent instead of the c-HCl.[32] All the intermediate compounds were confirmed by the spectroscopic methods and compared with reported information.[29–32] Prepared compounds **1**–**4** were condensed with 2,4-dimethoxy-5-bromobenzaldehyde (or 5-(3,4-dichlorophenyl)furfural) and chloroacetic acid in acetic anhydride and AcOH to afford the desired bicyclic compounds **5**–**9**. In the ^{1}H NMR spectra, compounds **5**–**9** showed a singlet peak around δ 6.14–6.24 corresponding to the methine proton of the thiazolopyrimidone skeleton, and the alkenyl proton of benzylidene appeared at around δ 7.60 as singlet. Synthetic methods for compounds **1**–**9** were described in Scheme 1. But the aldehyde or arylthiazolopyrimidine compounds possessing a hydroxyl group, generated O-acetylated benzylidenethiazolopyrimidines compounds **11**–**18**. ^{1}H NMR spectra showed a methyl peak of an acetyl group at around δ 2.30 as a singlet, and in the

Table 1
Structures of synthesized compounds



Compd	R_1	R_2	Compd	R_1	R_2
5			**16**		
6			**17**		
7			**18**		
8			**19**		
9			**20**		
11			**21**		
12			**22**		
13			**23**		
14			**24**		
15			**25**		

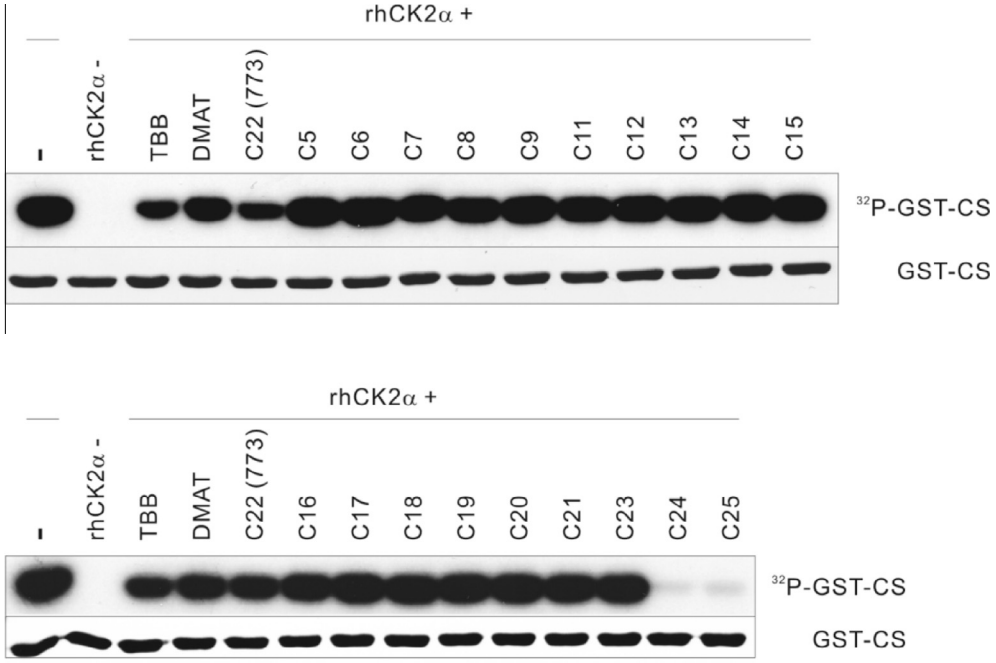


Figure 1. PKCK2 inhibitory efficacy of derivatives of lead compound 773. In vitro kinase assay was performed in the presence of 773 derivatives (10 μM) as described in Section 4. After incubation, samples were subjected to SDS–PAGE followed by staining with Coomassie brilliant blue and autoradiography. ^{32}P-GST-CS represents phosphorylated GST-CS and GST-CS represents Coomassie blue stained input GST-CS.

^{13}C NMR spectra, carbon signals of an acetyl group appeared at around 20 ppm and 168 ppm. All other analytical data confirmed the introduction of acetyl group to the phenolic OH group. Deprotection of the acetyl group was achieved by treatment with pyrrolidine instead of with strong bases (i.e., KOH or NaOH). The acetyl groups were successfully removed in good reaction yields, 80–100%, under this condition. Synthetic methods for the reaction were shown in Scheme 1A. Compounds **17** and **18** have provided a complicated product mixture under the pyrrolidine deprotection condition. To overcome this problem, an alternative method was employed for compounds **24** and **25**. To prepare **24** and **25**, the reaction was conducted with the same methods for compounds **11**–**18** without acetic anhydride. In the ^{1}H and ^{13}C NMR spectra, the signals of acetyl groups did not appear and other peaks corresponding to the desired structures were observed (Scheme 1B). Structures of the synthesized compounds are described in Table 1.

2.3. Inhibitory effect of new PKCK2 inhibitors

To evaluate the inhibitory effect of new PKCK2 inhibitors, in vitro kinase assays were performed. As shown in Figure 1, in vitro kinase assays were conducted in the presence or absence of rhCK2α. TBB and DMAT (reference PKCK2 inhibitors) are commercially available, selective, cell-permeable, and fairly potent PKCK2 inhibitors. The phosphorylation of GST-CS (GST-tagged recombinant CK2 Substrate) was partially inhibited by 10 μM of TBB, DMAT, or 773 (compound **22**) treatments. Among exploited PKCK2 inhibitors, compounds **24** and **25** strongly inhibited phosphorylation of GST-CS, and their PKCK2 inhibitory activity was greater than that of **22**, TBB, or DMAT.

2.4. Selectivity of new PKCK2 inhibitors

Protein kinases are central components of signal transduction cascades often dysregulated in cancer, and they represent some of the most promising drug targets. However, target selectivity is a major concern because most characterized kinase inhibitors target the highly conserved ATP-binding pocket. To determine the selectivity of the new inhibitors against PKCK2, TBB,

Table 2
Kinase selectivity profiles of TBB, compounds **24**, and **25**

Number	Kinase	TBB (10 μM)	**C24** (10 μM)	**C25** (10 μM)
1	AMPKα1	75 ± 2	82 ± 8	64 ± 6
2	AMPKα2	83 ± 3	65 ± 6	48 ± 1
3	CDK2/cyclinA	49 ± 0	69 ± 7	65 ± 3
4	CHK1	149 ± 12	117 ± 0	127 ± 0
5	CK1	51 ± 7	50 ± 1	77 ± 2
6	CK2	6 ± 1	0 ± 2	54 ± 1
7	CSK	116 ± 4	82 ± 8	54 ± 5
8	DYRK2	2 ± 4	14 ± 2	118 ± 9
9	Fgr	102 ± 2	98 ± 8	75 ± 1
10	GCK	92 ± 5	87 ± 2	87 ± 4
11	GSK3β	42 ± 2	52 ± 10	51 ± 9
12	JNK1α1	51 ± 1	101 ± 7	92 ± 0
13	JNK2α2	50 ± 5	91 ± 3	92 ± 6
14	Lck	78 ± 6	57 ± 2	61 ± 2
15	Lyn	113 ± 1	20 ± 5	5 ± 1
16	MAPK2	104 ± 4	85 ± 3	87 ± 4
17	MAPKAP-K2	78 ± 2	98 ± 7	86 ± 7
18	MAPKAP-K3	95 ± 2	117 ± 2	122 ± 4
19	MSK1	66 ± 5	127 ± 1	124 ± 5
20	PDK1	93 ± 8	78 ± 3	69 ± 1
21	PKA	109 ± 14	130 ± 1	92 ± 6
22	PKBα	61 ± 1	90 ± 11	50 ± 11
23	PKCα	106 ± 5	91 ± 7	91 ± 3
24	PRAK	60 ± 2	36 ± 3	28 ± 3
25	ROCK-II	73 ± 0	89 ± 12	108 ± 5
26	SAPK2a	99 ± 0	72 ± 5	59 ± 6
27	SAPK2b	112 ± 6	114 ± 3	101 ± 11
28	SAPK3	29 ± 3	69 ± 0	63 ± 1
29	SAPK4	106 ± 4	79 ± 0	76 ± 3
30	SGK	65 ± 4	85 ± 5	61 ± 1
31	Syk	87 ± 5	77 ± 6	28 ± 3

*Residual kinase activities were determined in the presence of the indicated concentrations of inhibitors and are expressed as a percentage of the control without inhibitor.

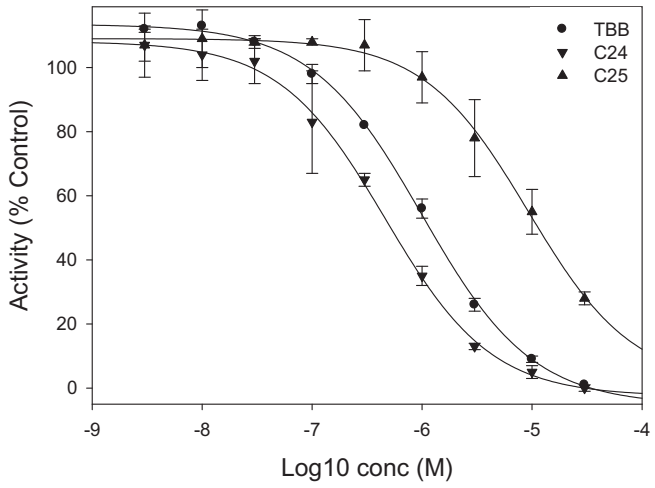


Figure 2. IC_{50} of TBB, compounds **24**, and **25**. PKCK2 activities were determined, as described in Section 4, in the presence of the indicated concentration of inhibitors. The data represent the means of experiments run in triplicate. Standard deviation (SD) did not exceed 10%.

compounds **24**, and **25** were tested at 10 μM concentrations for their ability to inhibit a panel of 31 protein kinases. As shown in Table 2, TBB was sufficient to inhibit PKCK2 activity more than 90% (residual activity was approximately 6 ± 1%). However, there were a handful of kinases that were sensitive to the inhibitor, including DYRK2, which was inhibited to nearly the same extent as PKCK2 (residual activity was approximately 2 ± 4%), whereas SAPK3 was inhibited to a lesser extent (residual activity was approximately 29 ± 3%). On the other hand, compound **24** exhibited higher selectivity for PKCK2 than did TBB, and PKCK2 activity was completely suppressed (residual activity was approximately 0 ± 2%). All the other kinases were either unaffected or partially inhibited by compound **24**, except DYRK2 (residual activity was approximately 14 ± 2%) and Lyn (residual activity was approximately 20 ± 5%). With the observation that TBB also inhibits DYRK2 as efficiently as it does PKCK2, DYRK2 appears to be susceptible to PKCK2 inhibitors. We found that DYRK2 was much less susceptible to compound **24** than was PKCK2, which suggests that compound **24** can discriminate between PKCK2 and DYRK2. Collectively taken, the data indicate that compound **24** selectivity over 31 kinases is almost the same or better than TBB. Unfortunately, compound **25** was less selective than TBB or compound **24**, having lower PKCK2 inhibitory activity (residual activity was approximately 54 ± 1%), and it also drastically reduced the activities of Lyn (residual activity was approximately 5 ± 1%), PRAK (residual activity was approximately 28 ± 3%), and Syk (residual activity was approximately 28 ± 3%; Table 2).

As shown in Figure 2, compound **24** exhibited remarkable inhibition against PKCK2. While the IC_{50} value of compound **24** against PKCK2 calculated under comparable conditions (15 μM of ATP concentration) was 0.56 μM, the IC_{50} values of TBB and compound **25** were 1.24 μM and 8.79 μM, respectively. The K_i values of these compounds against PKCK2 were also calculated (Fig. 3). TBB displayed an ATP-competitive pattern with moderate K_i value (approximately 2.70 μM) against PKCK2 and K_i value of compound **24** was 0.78 μM. These results indicate that compound **24** may be a promising candidate for developing highly specific, selective, and potent inhibitor targeting PKCK2.

2.4.1. Cellular inhibitory activity of new PKCK2 inhibitors

The usefulness of a protein kinase inhibitor is also highly dependent on its cell permeability; the greater the permeability the better the likelihood of dissecting cellular functions affected by the kinase of interest. Firstly, to prove the in vitro efficacy of TBB and compound **24** for direct inhibition of PKCK2, in vitro kinase assays were performed. Compound **24** drastically inhibited PKCK2 in a dose-dependent manner, which decreased phosphorylation of GST-CS more effectively than did TBB (Fig. 4A).

To examine whether compound **24** effectively inhibits PKCK2 in vivo, TE2-CK2α (PKCK2α catalytic subunit over-expressing TE2 cells)[33] was treated with compound **24** or TBB. We found that compound **24** more efficiently inhibited intracellular PKCK2 activity than did TBB in a dose-dependent manner (Fig. 4B). Thus, the reasonable potency and the efficient inhibition of endogenous PKCK2 activity in a cancer cell line make compound **24** a promising candidate for targeting PKCK2. In addition, this observation, in conjunction with the remarkable selectivity of compound **24**, paved the road toward the widespread usage of this compound for studies aimed at unraveling the functional implications of PKCK2 activity.

2.4.2. Pro-apoptotic efficacy of new PKCK2 inhibitors in human stomach and liver cancer cell lines

Cytotoxicity of compound **24** was examined by the MTT assay, using cancer cells including eight human stomach cancer cells (AGS, MKN-1, MKN-28, MKN-45, MKN-74, NCI-N87, SNU484, and SNU668) and six liver cancer cells (HepG2, Hep3B, SK-HEP-1, SNU739, SNU761, and SNU878). The inhibition of PKCK2 by TBB

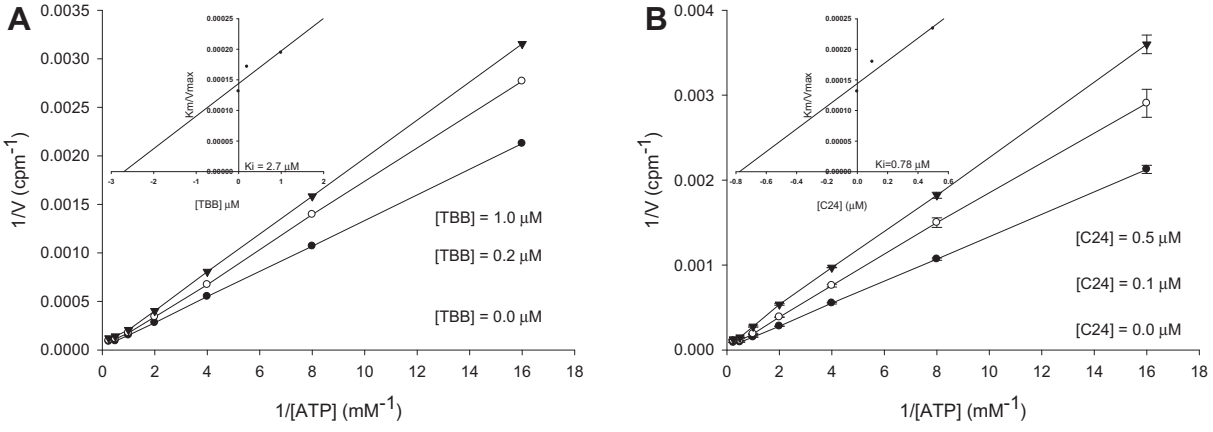


Figure 3. Kinetic analysis of PKCK2 inhibition by compound **24**. Competitive inhibition of PKCK2 by TBB (A) and **24** (B). Kinetic analysis was performed, as described in Section 4, in the presence of the indicated concentration of inhibitors. K_i was calculated by linear regression analysis of K_m/V_{max} against inhibitor concentration plots. The data represent the means of experiments run in triplicate. Standard deviation (SD) did not exceed 10%.

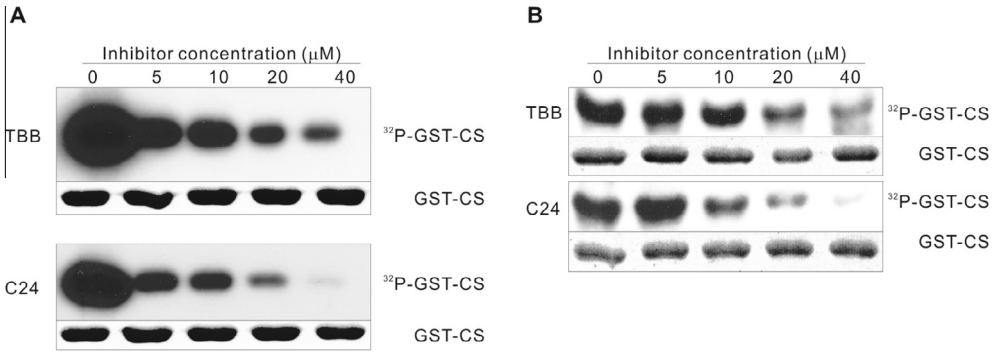


Figure 4. In vitro and in vivo inhibition of PKCK2 by C24. PKCK2 inhibitory activity of TBB and compound **24** in vitro (A) and in vivo (B). For in vivo experiments, TE2-CK2α cells were treated with the indicated concentration of TBB or compound **24** for 24 h followed by in vitro kinase assay.

or compound **24** showed cytotoxicity toward all the cancer cell lines examined, and compound **24** was more cytotoxic than TBB (Fig. 5A and B). The increase in cytotoxicity was attributed to increase in cellular apoptosis (Fig. 5C). The same results were obtained from TE2-GFP and TE2-CK2α cells (Supplemental Fig. 3).

2.4.3. Compound 24 sensitize TRAIL resistant cancer cells to TRAIL

Previously, we reported a novel mechanism by which PKCK2 inhibition sensitizes TRAIL-resistant cancer cells to TRAIL.[8] We evaluated TRAIL sensitivities and endogenous PKCK2 activities of eight human cancer cell lines. We found that six cancer cell lines (AGS, MKN-1, MKN-28, Hep3B, SK-HEP-1, and SNU739) showed higher PKCK2 activity as compared with two cancer cell lines (MKN45 and SNU886), and the cell lines with greater PKCK2 activity showed TRAIL resistance (Supplemental Fig. 4). To further evaluate whether compound **24** could sensitize TRAIL-resistant cancer cell lines, the cells were treated or untreated with TRAIL in the presence or absence of compound **24** pre-treatment. As shown in Figure 6, the cells were TRAIL resistant but underwent apoptosis in the presence of compound **24**, and cell death increased synergistically by the combination of compound **24** and TRAIL. These results suggest that compound **24** can be used alone or in combination with TRAIL for inducing cancer cell apoptosis.

2.5. Compound 24 potentially binds to the ATP binding site in PKCK2α

Docking studies were carried out for compound **24** with PKCK2α to examine the mode of action. An analysis of the docked compound **24** to the ATP binding site of PKCK2α showed that **24** fits into the ATP binding site. Compound **24** has hydrophobic

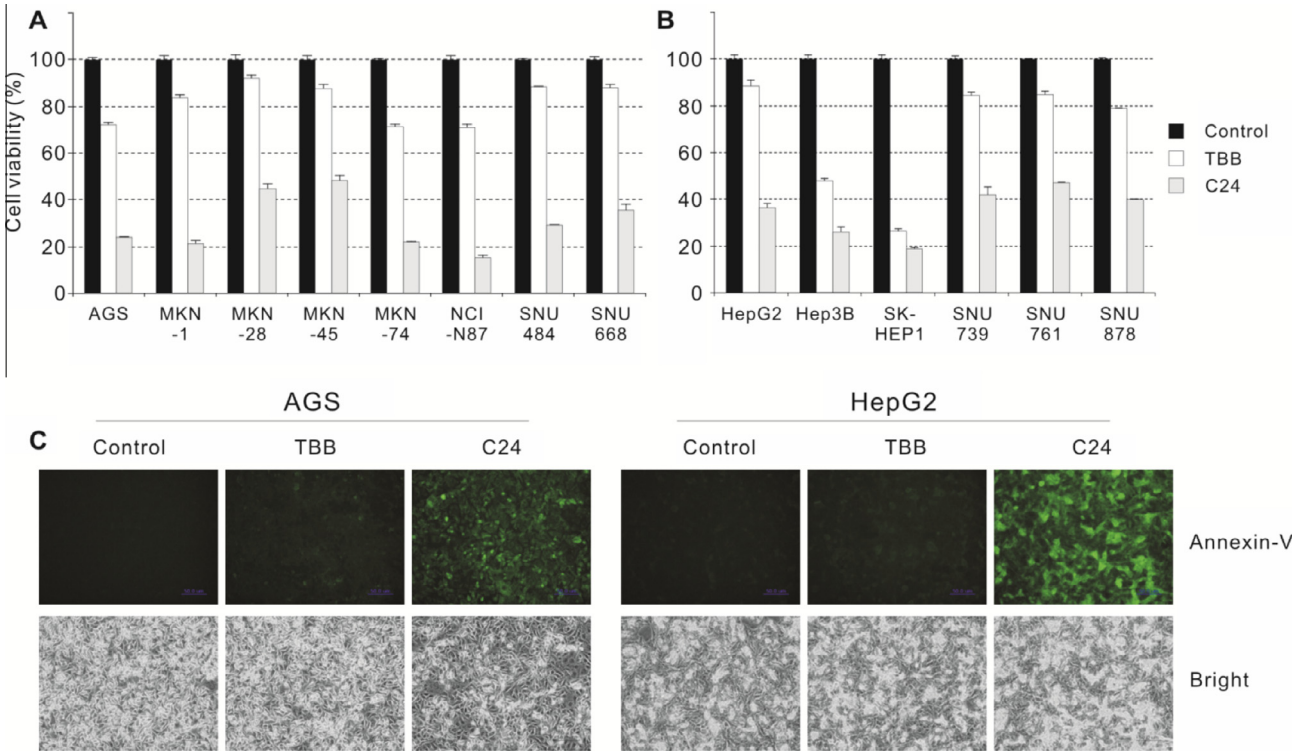


Figure 5. Anti-proliferative and pro-apoptotic efficacy of compound **24** on stomach- and liver-cancer cells. Anti-proliferative efficacy of compound **24** on stomach cancer cells (A) and on liver cancer cells (B). (C) Pro-apoptotic efficacy of TBB or compound **24** on AGS and HepG2 cancer cell lines. Cells were treated with TBB (10 μM) or compound **24** (10 μM) for 24 h. Cell viability and apoptosis were examined using MTT assay and Annexin-V-FLUOS staining, respectively. The data are expressed as mean ± SD. Experiments run in quadruplicate.

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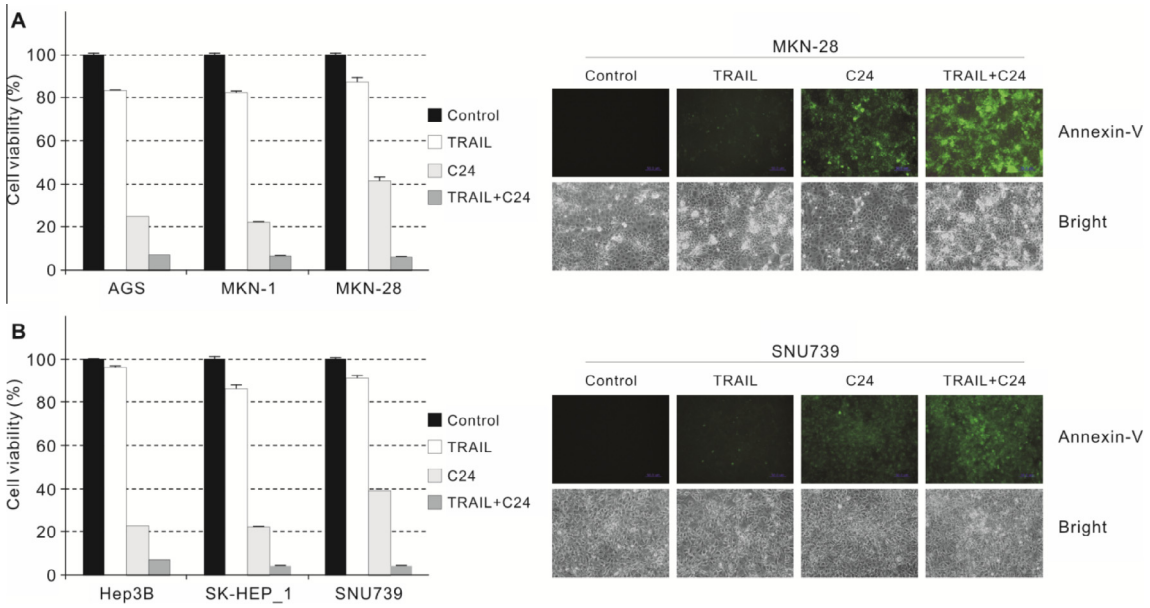

Figure 6. C24 sensitizes TRAIL-resistant cancer cells to TRAIL. TRAIL cytotoxicity toward stomach (A) or liver (B) cancer cells. Cell viability (left panel) was evaluated by the MTT assay and apoptosis (right panel) was examined by Annexin-V-FLUOS staining using cancer cells incubated with or without compound **24** (10 μM) for 24 h and subsequently treated with TRAIL (200 ng/mL) for 2 h. The data are expressed as mean ± SD.

interactions with Ser51, Val53, Val66, Ile95, Phe113, Val116, Asn118, Asp120, Ile174, and Asp175. The oxygen of the carboxylate group has hydrogen bonding interaction with the conserved water molecule. This water molecule forms a bridge between the inhibitors and residue Asp175. The hydroxyl group on the benzylidene ring on position 4 of compound **24** also forms a hydrogen bond with residue Asn118, which constitutes the protein kinase hinge region (Fig. 7). There are 22 structures of ATP-competitive PKCK2 inhibitors in complexes with PKCK2, reported in PDB,[34] including ellagic acid, benzimidazole derivatives, carboxylic acid derivatives, anthraquinone, xanthenone, fluorenone, and pyrazolotriazines. These compounds are mostly planar and fill the ATP binding pocket with hydrophobic interactions with residues Val53, Val66, Lys68, Ile95, Phe113, Val116, Met163, and Ile174. Some of the inhibitors have additional hydrogen bonds with the residues (from Glu114 to Val117) comprising the hinge region of PKCK2α. These interactions may improve the activity of the inhibitors but they are not essential because some only exhibit hydrophobic interactions between ligand and PKCK2α. Based on the comparison of compound **24** bound to PKCK2α with previously reported PKCK2

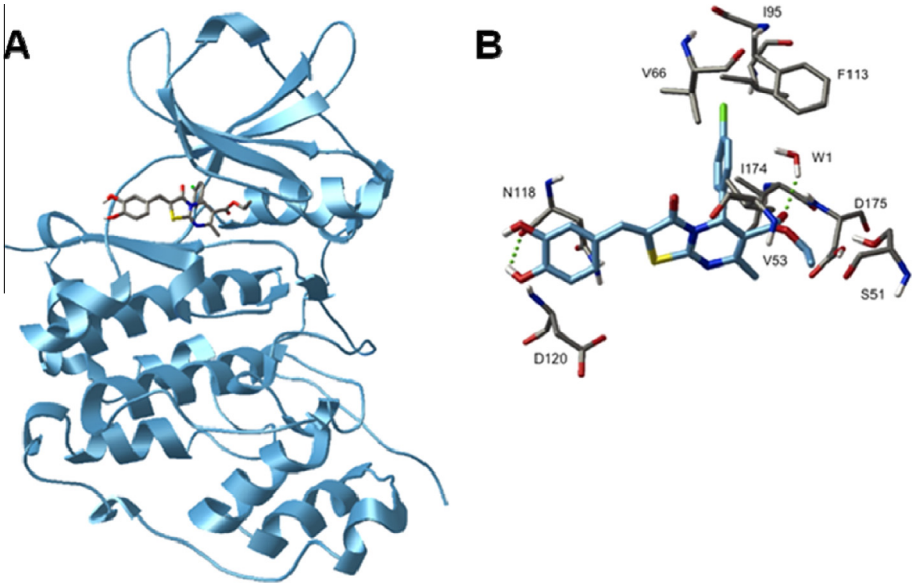

Figure 7. Molecular docking of compound **24** with PKCK2. (A) The graphical representation of compound **24** docked to the active site of CK2α. The protein is represented as a ribbon diagram and the compound is shown in stick representation, colored by atom type (carbon, gray; oxygen, red; nitrogen, blue; sulfur, yellow; hydrogen, white; halides, green). (B) The close up view of the docked complex. The residues of the active site of CK2 that have van der Waals contacts with compound **24** are shown. The stick representation of the complex is shown. The carbon atoms of the protein and complex are colored in gray and cyan, respectively. The rest of the atoms are colored as follows (oxygen, red; nitrogen, blue; hydrogen, cyan; and halides, green). Dotted green lines indicate hydrogen bonding interactions.

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inhibitor complexes, our compounds suggest a novel scaffold for specific PKCK2α inhibitors. The compound **24**-bound complex showed some similarities to known PKCK2 inhibitor complexes in terms of the residues: in the ATP binding pocket involved in hydrophobic interactions and in hydrogen bonding interactions involving the hinge region and the conserved water molecule. However, complexes with compound **24** are unique because the orientation of the chlorophenyl ring, which is almost perpendicular to the thiazolopyrimidine ring, leads to a different binding plane with respect to the adenine ring of ATP, whereas the planar ring parts of other known PKCK2 inhibitors are superimposed to the purine binding plane.[35]

3. Conclusion

In summary, screening of the compound library in an in vitro kinase assay permitted the discovery of two leads for PKCK2 inhibitors, 773 and 1062. Structural modification of the initial 773 further allowed us to identify two candidates for PKCK2 inhibitors (compounds **24** and **25**). Of particular interest is compound **24**, which is able to inhibit PKCK2 activity with remarkable selectivity and fairly high efficiency. Compound **24** is cell permeable, down-regulates endogenous PKCK2 activity, and displays remarkable pro-apoptotic efficacy when tested on a variety of cancer cell lines. In addition, compound **24** can sensitize TRAIL-resistant cancer cells to TRAIL. Molecular docking study of the active compounds in PKCK2 revealed possible binding modes, suggesting a novel scaffold for specific PKCK2α inhibitors. These docking studies may provide valuable information for the rational design of new derivatives with improved inhibitory properties. Based on our observations, compound **24** may be a candidate for the development and exploitation of anti-cancer drug targeting PKCK2.

4. Experimental methods

4.1. Chemistry general

Most of the chemicals and reagents used were obtained from Aldrich Chemical Co. and others were from companies like Junsei, Acros Organics, and Tokyo Chemicals. Melting points were measured without correction in open capillaries with Barnstead Electrothermal melting point apparatus, Manual MEL-TEMP (Model No: 1202D). Chromatographic separations were monitored by thin-layer chromatography using a commercially available pre-coated Merck Kieselgel 60 F_{254} plate (0.25 mm) and detected by visualizing under UV at 254 and 365 nm. Silica gel column chromatography was carried out with Merck Kiesel gel 60 (0.040–0.063 mm). All solvents used for chromatography were directly used without distillation. The purity was assessed by HPLC (Shimadzu LC-6AD) analysis under the following conditions; column, Synergi 4u Fusion-RP 80A (Phenomenex, 4.6 mm i.d. × 250 mm, 4 micron); mobile phase, isocratic elution of 80% acetonitrile for compounds **5**–**9**, **20**, and **21**, 60% acetonitrile for compounds **19** and **23** and 40% acetonitrile for compounds **22, 24,** and **25**; flow rate, 1.0 mL/min; detection, UV detector (Shimadzu SPD-M20A diode array detector, 400 nm). The purity of compound is described as percent (%). NMR spectra were recorded on Varian AS 400 (^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz) with tetramethyl silane as an internal standard. Chemical shift (δ) values are expressed in ppm and coupling constant (J) values in hertz (Hz). Mass spectral investigations were run on electron ionization method using GC:7890AMS:5975C MSD (Agilent) or liquid chromatography–electrospray ionization-time of flight mass spectrometry (Agilent) in a positive mode.

4.2. General synthetic methods for benzylidenethiazolopyrimidone compounds 5–9

A reaction mixture of thiopyrimidone (1 equiv), 2,4-dimethoxy-5-bromobenzaldehyde (1 equiv), chloroacetic acid (1.025 equiv) and NaOAc (1 equiv) in acetic anhydride and acetic acid was refluxed for 6–16 h. The reaction mixture was cooled to room temperature and poured into ice water and, subsequently extracted with CH_2Cl_2. Combined organic solvent was washed with $NaHCO_3$ and dried over Na_2SO_4. Solvent was removed under reduced pressure, and residue was purified by silica gel column chromatography or treatment with proper solvents.

4.2.1. (*Z*)-Ethyl 2-(5-bromo-2,4-dimethoxybenzylidene)-5-(4-methoxyphenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (5)

A reaction mixture of ethyl 4-(4-methoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (0.62 g, 2 mmol), 2,4-dimethoxy-5-bromobenzaldehyde (**1**) (0.49 g, 2 mmol), chloroacetic acid (0.19 g, 2.05 mmol) and NaOAc (0.16 g, 2 mmol) in acetic anhydride (3 mL) and acetic acid (4 mL) was refluxed for 10 h. The residue was purified by silica gel column chromatography (eluent ethyl acetate/CH_2Cl_2 = 2:3) to give compound **5** as a brown solid (0.90 g, 78.3%). mp: 216–218 °C; R_f 0.33 (ethyl acetate/*n*-hexane = 1:1); HPLC: R_T 14.76 min (purity; 96%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.19 (t, J = 7.2 Hz, 3H), 2.51 (s, 3H), 3.76 (s, 3H), 3.88 (s, 3H), 3.95 (s, 3H), 4.10 (dq, J = 2.8, 7.2 Hz, 2H), 6.14 (s, 1H), 6.44 (s, 1H), 6.81 (d, J = 8.2 Hz, 2H), 7.33 (d, J = 8.8 Hz, 1H), 7.56 (s, 1H), 7.93 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 23.0, 55.0, 55.4, 56.0, 56.6, 60.6, 96.1, 103.0, 109.1, 114.1, 116.6, 118.9, 127.8, 129.6, 132.8, 133.7, 152.6, 156.7, 159.0, 159.5, 159.8, 165.7, 165.9 ppm; HRMS (ESI) $[M+H]^+$ $C_{26}H_{26}BrN_2O_6S$ calcd 573.0689, found 573.0687.

4.2.2. (*Z*)-Ethyl 2-(5-bromo-2,4-dimethoxybenzylidene)-5-(4-chlorophenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (6)

A reaction mixture of ethyl 4-(4-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**2**) (0.59 g, 2 mmol), 2,4-dimethoxy-5-bromobenzaldehyde (0.49 g, 2 mmol), chloroacetic acid (0.19 g, 2.05 mmol) and NaOAc (0.16 g, 2 mmol) in acetic anhydride (3 mL) and acetic acid (4 mL) was refluxed for 16 h. The residue was triturated with ethyl acetate/CH_2Cl_2 and solid was collected and washed with same solvent. After vacuum drying, compound **6** was obtained as a brown solid (0.60 g, 52.0%). Mp: 222–224 °C; R_f 0.56 (ethyl acetate/*n*-hexane = 1:1); HPLC: R_T 20.66 min (purity; 90%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.19 (t, J = 7.2 Hz, 3H), 2.51 (s, 3H), 3.89 (s, 3H), 3.95 (s, 3H), 4.10 (dq, J = 2.0, 7.2 Hz, 2H), 6.15 (s, 1H), 6.44 (s, 1H), 7.27 (d, J = 8.2 Hz, 2H), 7.34 (d, J = 8.2 Hz, 2H), 7.56 (s, 1H), 7.93 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 23.1, 54.9, 56.0, 56.6, 60.8, 96.1, 103.0, 108.5, 116.4, 118.4, 128.3, 129.0, 129.7, 133.8, 134.7, 138.9, 153.2, 156.8, 159.2, 159.6, 165.5, 165.6 ppm; HRMS (ESI) $[M+H]^+$ $C_{25}H_{23}BrClN_2O_5S$ calcd 577.0194, found 577.0196.

4.2.3. (*Z*)-Ethyl 2-(5-bromo-2,4-dimethoxybenzylidene)-5-(furan-3-yl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (7)

A reaction mixture of ethyl 4-(furan-3-yl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**3**) (0.27 g, 1 mmol), 2,4-dimethoxy-5-bromobenzaldehyde (0.25 g, 1 mmol), chloroacetic acid (0.10 g, 1.03 mmol) and NaOAc (0.08 g, 1 mmol) in acetic anhydride (1.5 mL) and acetic acid (2.0 mL) was refluxed for 6 h. The residue was triturated with ethyl acetate/*n*-hexane and the solid was collected and washed with the same solvent. After vacuum drying, compound **7** was obtained as a brown solid (0.38 g,

71.2%). mp: 194–196 °C; R_f 0.43 (ethyl acetate/*n*-hexane = 1:1); HPLC: R_T 13.19 min (purity; 97%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.25 (t, J = 7.2 Hz, 3H), 2.49 (s, 3H), 3.92 (s, 3H), 3.96 (s, 3H), 4.18 (dq, J = 4.0, 7.2 Hz, 2H), 6.24 (s, 1H), 6.36 (s, 1H), 6.46 (s, 1H), 7.30 (s, 1H), 7.44 (s, 1H), 7.59 (s, 1H), 8.00 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.4, 23.0, 47.0, 56.0, 56.7, 60.7, 96.1, 103.1, 107.8, 109.6, 116.5, 118.9, 133.8, 141.3, 143.6, 153.5, 156.8, 159.1, 159.6, 165.7, 165.8 ppm. Two other peaks were not observed; HRMS (ESI) $[M+H]^+$ $C_{23}H_{22}BrN_2O_6S$ calcd 533.0376, found 533.0377.

4.2.4. (*Z*)-Ethyl 2-(5-bromo-2,4-dimethoxybenzylidene)-5-(pyridin-3-yl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (8)

A reaction mixture of ethyl 4-(pyridind-3-yl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**4**) (0.50 g, 1.8 mmol), 2,4-dimethoxy-5-bromobenzaldehyde (**1**) (0.44 g, 1.8 mmol), chloroacetic acid (0.17 g, 1.85 mmol) and NaOAc (0.15 g, 1.8 mmol) in acetic anhydride (3 mL) and acetic acid (4 mL) was refluxed for 9 h. The residue was purified by silica gel column chromatography (eluent: ethyl acetate/*n*-hexane = 1:5 → 1:3) to give compound **8** as an orange solid (0.05 g, 5.1%). mp: 188–190 °C; HPLC: R_T 8.83 min (purity; 97%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.19 (t, J = 7.2 Hz, 3H), 2.51 (s, 3H), 3.90 (s, 3H), 3.95 (s, 3H), 4.11 (dq, J = 1.2, 7.2 Hz, 2H), 6.20 (s, 1H), 6.45 (s, 1H), 7.25 (dd, J = 8.0, 5.6 Hz, 1H), 7.57 (s, 1H), 7.71 (ddd, J = 2.0, 2.0, 8.0 Hz, 1H), 7.94 (s, 1H), 8.52 (dd, J = 1.6, 5.6 Hz, 1H), 8.69 (d, J = 2.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3., 23.2, 53.5, 56.0, 56.7, 60.9, 96.1, 103.1, 107.8, 116.3, 118.3, 123.8, 128.6, 133.9, 135.8, 136.1, 149.8, 150.0, 153.9, 157.0, 159.3, 159.6, 165.4, 165.5 ppm; HRMS (ESI) $[M+H]^+$ $C_{24}H_{23}BrN_3O_5S$ calcd 544.0536, found 544.0538.

4.2.5. (*Z*)-Ethyl 5-(4-chlorophenyl)-2-((5-(3,4-dichlorophenyl)furan-2-yl)methylene)-7-methyl-3-oxo-3,5-dihydro-2*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (9)

A reaction mixture of ethyl 4-(4-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**2**) (0.5 g, 1.61 mmol), 5-(3,4-dichlorophenyl) furfural (0.39 g, 1.61 mmol), chloroacetic acid (0.17 g, 1.83 mmol), and NaOAc (0.14 g, 1.75 mmol) in acetic anhydride (8 mL) and acetic acid (12 mL) was refluxed for 16 h. Ethanol was added to the residue and the mixture was heated to around 60 °C. The solid was filtered and washed with EtOH to give compound **9** as a reddish brown solid (0.80 g, 86.7%). mp: 238–240 °C; R_f 0.54 (ethyl acetate/*n*-hexane = 1:3); HPLC: R_T 4.03 min (purity; 100%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.21 (t, J = 7.2 Hz, 3H), 2.54 (s, 3H), 4.12 (q, J = 7.2 Hz, 2H), 6.16 (s, 1H), 6.85 (s, 2H), 7.29 (d, J = 8.0 Hz, 2H), 7.35 (d, J = 8.0 Hz, 2H), 7.48 (s, 1H), 7.51 (d, J = 8.4 Hz, 1H), 7.58 (d, J = 8.4 Hz, 1H), 7.77 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) 14.3, 20.3, 55.1, 60.9, 108.7, 110.1, 118.3, 118.7, 120.3, 123.8, 126.2, 129.0, 129.1, 129.6, 131.4, 133.2, 133.7, 134.8, 138.7, 149.9, 152.8, 155.6, 157.1, 165.0, 165.4 ppm; HRMS (ESI) $[M+H]^+$ $C_{27}H_{20}Cl_3N_2O_4S$ calcd 573.0204, found 573.0204.

4.3. General synthetic method for O-acetylated benzylidenethiazolopyrimidone compounds 11–18

A reaction mixture of thiopyridmidone (1 equiv), corresponding aldehyde (1 equiv), chloroacetic acid (1.025 equiv), and NaOAc (1 equiv) in acetic anhydride and acetic acid was refluxed for 6–16 h. The reaction mixture was cooled to room temperature and poured into iced water, which was extracted with CH_2Cl_2. Combined organic solvent was washed with $NaHCO_3$ and dried over Na_2SO_4. Solvent was removed under reduced pressure and residue was purified by silica gel column chromatography or treatment with proper solvents.

4.3.1. (*Z*)-Ethyl 2-(4-Acetoxy-3-methoxybenzylidene)-5-(furan-2-yl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (11)

A reaction mixture of ethyl 4-(furan-2-yl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**3**)(0.27 g, 1.0 mmol), 4-hydroxy-3-methoxybenzaldehyde (0.15 g, 1.0 mmol), chloroacetic acid (0.10 g, 1.03 mmol), and NaOAc (0.08 g, 1.0 mmol) in acetic anhydride (3 mL) and acetic acid (4 mL) was refluxed for 12 h. The residue was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1:2 → 1:1) to afford compound **11** as an orange solid (0.33 g, 75.0%). mp: 166–168 °C; R_f 0.59 (ethyl acetate/*n*-hexane = 1:1); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.25 (t, J = 7.2 Hz, 3H), 2.33 (s, 3H), 2.51 (s, 3H), 3.88 (s, 3H), 4.11–4.23 (m, 2H), 6.29 (br s, 1H), 6.35 (d, J = 3.6 Hz, 1H), 6.36 (d, J = 2.4 Hz, 1H), 7.08 (s, 1H), 7.10 (d, J = 8.4 Hz, 1H), 7.14 (d, J = 8.4 Hz, 1H), 7.31 (dd, J = 0.8, 1.6 Hz, 1H), 7.77 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.4, 20.9, 23.0, 48.7, 56.1, 60.8, 106.4, 109.2, 110.7, 113.4, 120.6, 123.5, 123.9, 132.3, 133.1, 141.7, 143.1, 151.5, 151.9, 154.0, 156.0, 165.2, 165.5, 168.8 ppm; GC–MS (EI): m/e 482.2 $[M]^+$.

4.3.2. (*Z*)-Ethyl 2-(4-Methoxybenzylidene)-5-(4-acetoxy-3-methoxyphenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (12)

A reaction mixture of ethyl 4-(4-hydroxy-3-methoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**10**) (0.32 g, 1.0 mmol), 4-methoxybenzaldehyde (0.14 g, 1.0 mmol), chloroacetic acid (0.10 g, 1.03 mmol), and NaOAc (0.08 g, 1.0 mmol) in acetic anhydride (2 mL) and acetic acid (3 mL) was refluxed for 11 h. The residue was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1:3) to afford compound **12** as an orange solid (0.37 g, 70.8%). mp: 194–196 °C; R_f 0.51 (ethyl acetate/*n*-hexane = 1:1); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.20 (t, J = 7.2 Hz, 3H), 2.29 (s, 3H), 2.52 (s, 3H), 3.81 (s, 3H), 3.86 (s, 3H), 4.13 (dq, J = 4.0, 7.2 Hz, 1H), 6.20 (s, 1H), 6.95 (d, J = 1.2 HZ,2H), 6.98 (d, J = 8.8 Hz, 2H), 7.06 (s,1H), 7.44 (d, J = 8.8 Hz, 2H), 7.73 (S,1H); ^{13}C NMR ($CDCl_3$, 100 MHz) 14.3, 20.9, 22.9, 55.1, 55.7, 56.1, 60.8, 108.7, 112.8, 115.0, 117.4, 120.3, 123.1, 126.0, 132.3, 139.0, 140.0, 151.2, 153.1, 156.9, 161.7, 165.6, 165.7, 169.0 ppm. One peak is missed; GC–MS (EI): m/e 522.2 $[M]^+$.

4.3.3. (*Z*)-Ethyl 2-(4-acetoxy-3-bromo-5-methoxybenzylidene)-5-(4-methoxyphenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (13)

A reaction mixture of ethyl 4-(4-methoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**1**) (0.31 g, 1.0 mmol), 3-bromo-4-hydroxy-5-methoxybenzaldehyde (0.23 g, 1.0 mmol), chloroacetic acid (0.10 g, 1.03 mmol), and NaOAc (0.08 g, 1.0 mmol) in acetic anhydride (3 mL) and acetic acid (4 mL) was refluxed for 7 h. The residue was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1:3 → 1:2) to afford compound **13** as an orange solid (0.19 g, 31.6%); mp: 86–88 °C; R_f 0.19 (ethyl acetate/*n*-hexane = 1:3); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.19 (t, J = 7.2 Hz, 3H), 2.37 (s, 3H), 2.52 (s, 3H), 3.78 (s, 3H), 3.86 (s, 3H), 4.11 (dq, J = 2.8, 7.2 Hz, 2H), 6.16 (s, 1H), 6.83 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 2.0 Hz, 1H), 7.28 (d, J = 2.0 Hz, 1H), 7.33 (d, J = 8.8 Hz, 2H), 7.60 (s,1H); ^{13}C NMR ($CDCl_3$, 100 MHz)14.3, 20.6, 22.9, 55.3, 56.5, 60.8, 109.8, 111.9, 114.2, 118.4, 122.3, 126.6, 129.6, 131.2, 132.3, 133.0, 139.5, 152.0, 153.0, 155.4, 160.0, 165.0, 165.7, 167.8 ppm.; GC–MS (EI): m/e 600.2 $[M]^+$.

4.3.4. (*Z*)-Ethyl 2-(4-acetoxy-3-bromo-5-methoxybenzylidene)-5-(4-chlorophenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (14)

A reaction mixture of ethyl 4-(4-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**2**) (0.17 g, 0.56 mmol), 3-bromo-4-hydroxy-5-methoxybenzaldehyde (0.12 g, 0.56 mmol), chloroacetic acid (0.05 g, 0.57 mmol), and NaOAc (0.05 g, 0.56 mmol) in acetic anhydride (2 mL) and acetic acid (3 mL) was refluxed for 16 h. The residue was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1:4) to afford compound **14** as an orange solid (0.20 g, 59.4%). mp: 198–200 °C; R_f 0.28 (ethyl acetate/*n*-hexane = 1:3); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.20 (t, J = 7.2 Hz, 3H), 2.37 (s, 3H), 2.52 (s, 3H), 3.86 (s, 3H), 4.12 (dq, J = 1.6, 7.2 Hz, 2H), 6.17 (s, 1H), 6.96 (d, J = 1.6 Hz, 1H), 7.29 (d, J = 8.4 Hz, 2H), 7.30 (d, J = 1.6 Hz, 1H), 7.35 (d, J = 8.4 Hz, 2H), 7.61 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz)14.3, 20.7, 23.0, 55.2, 56.5, 60.9, 109.1, 111.9, 118.5, 121.9, 126.6, 129.1, 129.7, 131.6, 132.9, 134.9, 138.4, 139.6, 152.7, 153.1, 155.5, 164.9, 165.4, 167.8 ppm.; GC–MS (EI): *m*/*e* 562.1[M-CH_2CO]$^+$.

4.3.5. (*Z*)-Ethyl 2-(4-Acetoxy-2-bromo-5-methoxybenzylidene)-5-(4-chlorophenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (15)

A reaction mixture of ethyl 4-(4-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**2**) (0.62 g, 2.12 mmol), 2-bromo-4-hydroxy-5-methoxybenzaldehyde (0.49 g, 2.12 mmol), chloroacetic acid (0.21 g, 2.18 mmol), and NaOAc (0.17 g, 2.12 mmol) in acetic anhydride (6 mL) and acetic acid (9 mL) was refluxed for 16 h. The residue was triturated with ethyl acetate/*n*-hexane and solid was collected and washed with same solvent. After vacuum drying, compound **15** was obtained as an orange solid (0.69 g, 53.3%). mp: 196–198 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.20 (t, J = 7.2 Hz, 3H), 2.32 (s, 3H), 2.52 (s, 3H), 3.85 (s, 3H), 4.11 (dq, J = 2.4, 7.2 Hz, 2H), 6.17 (s, 1H), 7.10 (s, 1H), 7.29 (d, J = 8 .0 Hz, 2H), 7.35 (s, 1H), 7.36 (d, J = 8.0 Hz, 2H), 7.97(s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz)14.3, 20.7, 23.0, 52.2, 56.4, 60.9, 109.2, 112.0, 116.9, 123.1, 128.2, 129.1, 129.8, 131.7, 132.0, 135.0, 138.3, 141.6, 151.2, 152.6, 155.5, 164.6, 165.4, 168.4 ppm; GC–MS (EI): *m*/*e* 604.1 [M]$^+$.

4.3.6. (*Z*)-Ethyl 2-(4-Acetoxy-3-chloro-5-methoxybenzylidene)-5-(4-chlorophenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (16)

A reaction mixture of ethyl 4-(4-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**2**) (0.10 g, 0.34 mmol), 3-chloro-4-hydroxy-5-methoxybenzaldehyde (0.06 g, 0.34 mmol), chloroacetic acid (0.03 g, 0.35 mmol), and NaOAc (0.03 g, 0.34 mmol) in acetic anhydride (2 mL) and acetic acid (3 mL) was refluxed for 16 h. The residue was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1:3) to afford compound **16** as an orange solid (0.09 g, 44.6%). mp: 184–186 °C; R_f 0.32 (ethyl acetate/*n*-hexane = 1:3); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.21 (t, J = 7.2 Hz, 3H), 2.38 (s, 3H), 2.53 (s, 3H), 3.87 (s, 3H), 4.12 (d, J = 7.2 Hz, 2H), 6.18 (s, 1H), 6.94 (d, J = 1.6 Hz, 1H),7.15 (d, J = 1.6 Hz, 1H), 7.30 (d, J = 8.4 Hz, 2H), 7.35 (d, J = 8.4 Hz, 2H),7.62 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) 14.3, 20.5, 24.7, 55.2, 56.6, 60.9, 109.2, 111.4, 118.3, 122.0, 123.7, 129.1, 129.4, 129.7, 131.8, 132.3, 134.9, 138.4, 152.7, 153.2, 155.5, 164.9, 165.4, 167.8 ppm; GC–MS (EI): *m*/*e* 518.2 [M-CH_2CO]$^+$.

4.3.7. (*Z*)-Ethyl 5-(4-chlorophenyl)-2-(3,4-diacetoxybenzylidene)-7-methyl-3-oxo-3,5-dihydro-2*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (17)

A reaction mixture of ethyl 4-(4-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**2**) (0.5 g, 1.61 mmol), 3,4-dihydroxybenzaldehyde (0.22 g, 1.61 mmol), chloroacetic acid (0.17 g, 1.83 mmol) and NaOAc (0.14 g, 1.75 mmol) in acetic anhydride (10 mL) and acetic acid (15 mL) was refluxed for 16 h. Ethanol was added to the residue and the mixture was heated to around 50 °C. The solid was filtered and washed with EtOH to give compound **17** as a gray-orange solid (0.47 g, 52.6%). mp: 98–100 °C; R_f 0.20 (ethyl acetate/*n*-hexane = 1:3); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.20 (t, J = 6.8 Hz, 3H), 2.31 (s, 6H), 2.51 (s, 3H), 4.11 (q, J = 6.8 Hz, 2H), 6.16 (s, 1H), 7.27–7.33 (m, 7H), 7.66 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) 14.3, 20.9, 23.0, 23.1, 55.2, 60.9, 109.0, 121.5, 124.6, 125.1, 128.3, 129.1, 129.7, 131.8, 131.9, 132.0, 134.9, 138.5, 142.8, 143.8, 152.8, 165.1, 165.5, 168.0, 168.1 ppm; HRMS (ESI) [M+H]$^+$ $C_{27}H_{24}ClN_2O_7S$ calcd 555.0987, found 555.0990.

4.3.8. (*Z*)-Ethyl 5-(4-chlorophenyl)-7-methyl-3-oxo-2-(3,4,5-triacetoxybenzylidene)-3,5-dihydro-2*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (18)

A reaction mixture of ethyl 4-(4-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**2**) (0.1 g, 0.34 mmol), 3,4,5-trihydroxybenzaldehyde (49.6 mg, 0.34 mmol), chloroacetic acid (33.0 mg, 0.35 mmol), and NaOAc (27.9 mg, 0.34 mmol) in acetic anhydride (2 mL) and acetic acid (3 mL) was refluxed for 16 h. Ethanol was added to the residue and the mixture was heated to around 50 °C. The solid was filtered and washed with EtOH to give a gray-orange solid (96.0 mg, 48.7%). mp: 168–170 °C; R_f 0.81 (ethyl acetate/*n*-hexane = 1:1); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.20 (t, J = 7.2 Hz, 3H), 2.31 (s, 9H), 2.51 (s, 3H), 4.11 (q, J = 7.2 Hz, 2H), 6.16 (s, 1H), 7.24 (s, 2H), 7.29 (d, J = 8.4 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 7.62 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) 14.3, 20.4, 20.9, 23.0, 55.2, 60.9, 109.1, 122.1, 122.6, 129.1, 129.7, 131.0, 131.1, 131.6, 134.9, 136.2, 138.4, 144.2, 152.8, 155.5, 164.9, 165.4, 166.8, 167.7 ppm; HRMS (ESI) [M+H]$^+$ $C_{29}H_{26}ClN_2O_9S$ calcd 613.1042, found 613.1044.

4.4. General synthetic method for benzylidenethiazolopyrimidone compounds 19–23

Corresponding *O*-acetylbenzylidenethiazolopyrimidone was dissolved in pyrrolidine and the reaction mixture was kept stirring for 10–20 min, at room temperature. After dilution of mixture with ethyl acetate, the organic layer was sequentially washed with 1 M H_2SO_4, satd NH_4Cl, and brine and dried over Na_2SO_4. Solvent was removed under reduced pressure and the residue was triturated with ethyl acetate/*n*-hexane.

4.4.1. (*Z*)-Ethyl 2-(4-hydroxy-3-methoxybenzylidene)-5-(furan-2-yl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (19)

Ethyl 2-(4-acetoxy-3-methoxybenzylidene)-5-(furan-2-yl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (**11**) (53.3 mg, 0.10 mmol) was dissolved in pyrrolidine (0.5 mL) and the reaction mixture was kept stirring for 10 min. After evaporation of solvent, compound **19** was obtained as an orange solid (50.5 mg, quantitative). Mp: 224–226 °C; R_f 0.53 (ethyl acetate/*n*-hexane = 1:1); HPLC: R_T 12.37 min (purity; 100%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.25 (t, J = 7.2 Hz, 3H), 2.51(s, 3H), 3.93 (s, 3H), 4.10–4.25 (m, 2H), 6.28 (dd, J = 1.6, 3.6 Hz, 1H), 6.34 (d, J = 3.6 Hz, 1H), 6.35 (s, 1H), 6.99 (d, J = 1.6 Hz, 1H), 7.00 (d, J = 8.4 Hz, 1H), 7.07 (dd, J = 1.6, 8.4 Hz, 1H), 7.30 (dd, J = 0.8, 0.8 Hz, 1H), 7.75 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.4, 23.0, 48.6, 56.1, 60.8, 106.0, 109.0, 110.7, 111.9, 115.6, 117.2, 125.7, 125.9, 134.3, 142.9, 147.2, 148.5, 151.8, 154.2, 156.6, 165.5, 165.6 ppm; HRMS (ESI) [M+H]$^+$ $C_{22}H_{21}N_2O_6S$ calcd 441.1115, found 441.1115.

4.4.2. (*Z*)-Ethyl 2-(4-methoxybenzylidene)-5-(4-hydroxy-3-methoxyphenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (20)

Ethyl 2-(4-Methoxybenzylidene)-5-(4-acetoxy-3-methoxyphenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (**12**) (21 mg, 0.04 mmol) was dissolved in pyrrolidine (0.5 mL) and the reaction mixture was kept stirring for 10 min. After evaporation of solvent, compound **20** was obtained as an orange solid (18.0 mg, 93.3%). Mp: 188–190 °C; R_f 0.12 (ethyl acetate/*n*-hexane = 1:3); HPLC: R_T 6.38 min (purity; 100%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.23 (t, J = 7.2 Hz, 3H), 2.53 (s, 3H), 3.87 (s, 3H), 3.88 (s, 3H), 4.13 (dq, J = 2.0, 7.2 Hz, 2H), 5.62 (s, 1H), 6.15 (s, 3H), 6.83 (d, J = 8.4 Hz, 1H), 6.90 (dd, J = 2.0, 8.4 Hz, 1H), 6.95 (d, J = 2.0 Hz, 2H), 6.98 (d, J = 8.8 Hz, 2H), 7.44 (d, J = 8.8 Hz, 2H), 7.72 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.4, 23.0, 55.4, 55.7, 56.2, 60.7, 109.2, 111.1, 114.6, 115.0, 117.5, 121.2, 126.1, 132.3, 132.4, 133.6, 146.1, 146.5, 152.5, 156.6, 161.7, 165.8, 165.9 ppm; HRMS (ESI) $[M+H]^+$ $C_{25}H_{25}N_2O_6S$ calcd 481.1428, found 481.1438.

4.4.3. (*Z*)-Ethyl 2-(4-hydroxy-3-bromo-5-methoxybenzylidene)-5-(4-chlorophenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (21)

Ethyl 2-(4-Acetoxy-3-bromo-5-methoxybenzylidene)-5-(4-chlorophenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (**14**)(36 mg, 0.06 mmol) was dissolved in pyrrolidine (0.5 mL) and the reaction mixture was kept stirring for 10 min. The residue was treated with EtOAc/*n*-hexane to afford compound **21** as an orange solid (28.0 mg, 83.6%). Mp: 232–233 °C; R_f 0.57 (ethyl acetate/*n*-hexane = 1:1); HPLC: R_T 10.48 min (purity; 99%);^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.21 (t, J = 7.2 Hz, 3H), 2.52 (s, 3H), 3.95 (s, 3H), 4.12 (dq, J = 1.2, 7.2 Hz, 2H), 6.17 (s, 1H), 6.91 (d, J = 1.6 Hz, 1H), 7.27 (d, J = 1.6 Hz, 1H), 7.29 (d, J = 8.4 Hz, 2H), 7.35 (d, J = 8.4 Hz, 2H), 7.60 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 23.0, 55.1, 56.6, 60.9, 108.9, 109.3, 110.7, 118.9, 126.6, 128.2, 129.1, 129.7, 132.6, 134.8, 138.6, 145.6, 147.6, 152.9, 156.1, 165.2, 165.5 ppm; HRMS (ESI) $[M+H]^+$ $C_{24}H_{21}BrClN_2O_5S$ calcd 563.0038, found 563.0047.

4.4.4. (*Z*)-Ethyl 2-(4-hydroxy-2-bromo-5-methoxybenzylidene)-5-(4-chlorophenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (22)

Ethyl 2-(4-acetoxy-2-bromo-5-methoxybenzylidene)-5-(4-chlorophenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (**15**) (0.63 g, 1.04 mmol) was dissolved in pyrrolidine (5 mL) and the reaction mixture was kept stirring for 20 min.The residue was treated with CH_2Cl_2/*n*-hexane to afford compound **22** as an orange solid (0.54 g, 92.0%). Mp: 234–235 °C; R_f 0.53 (ethyl acetate/*n*-hexane = 1:1); HPLC: R_T 4.99 min (purity; 99%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.11 (t, J = 7.2 Hz, 3H), 2.38 (s, 3H), 3.85 (s, 3H), 4.03 (dq, J = 3.2, 7.2 Hz, 2H), 6.01 (s, 1H), 7.01 (s, 1H), 7.13 (s, 1H), 7.32 (d, J = 8.8 Hz, 2H), 7.42 (d, J = 8.8 Hz, 2H), 7.77 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) 13.9, 22.5, 54.5, 55.8, 60.3, 108.2, 111.3, 117.9, 119.0, 119.9, 122.5, 128.8, 129.6, 131.2, 133.2, 139.1, 147.8, 150.6, 151.6, 155.5, 164.1, 164.7 ppm; HRMS (ESI) $[M+H]^+$ $C_{24}H_{21}BrClN_2O_5S$ calcd 563.0038, found 563.0040.

4.4.5. (*Z*)-Ethyl 2-(4-hydroxy-3-chloro-5-methoxybenzylidene)-5-(4-chlorophenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (D)

Ethyl 2-(4-Acetoxy-3-chloro-5-methoxybenzylidene)-5-(4-chlorophenyl)-7-methyl-3-oxo-2,3-dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (**16**) (19.7 mg, 0.04 mmol) was dissolved in pyrrolidine (0.5 mL) and the reaction mixture was kept stirring for 10 min. The residue was treated with to CH_2Cl_2/*n*-hexane afford compound **23** as an orange solid (16.5 mg, 90.5%). Mp: 212–214 °C; R_f 0.61 (ethyl acetate/*n*-hexane = 1:1); HPLC: R_T 9.83 min (purity; 99%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.18 (t, J = 7.2 Hz, 3H), 2.50 (s, 3H), 3.93 (s, 3H), 4.11 (q, J = 7.2 Hz, 2H), 6.14 (s, 1H), 6.86 (s, 1H), 7.10 (s, 1H), 7.26 (d, J = 8.0 Hz, 2H), 7.33 (d, J = 8.0 Hz, 2H), 7.57 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) 14.3, 23.1, 55.1, 56.7, 60.9, 108.9, 110.2, 118.9, 120.8, 125.3, 125.9, 129.1, 129.7, 132.8, 134.8, 138.6, 144.5, 147.9, 152.9, 156.0, 165.2, 165.5 ppm; HRMS (ESI) $[M+H]^+$ $C_{24}H_{21}Cl_2N_2O_5S$ calcd 519.0543, found 519.0550.

4.4.6. (*Z*)-Ethyl 5-(4-chlorophenyl)-2-(3,4-dihydroxybenzylidene)-7-methyl-3-oxo-3,5-dihydro-2*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (24)

A reaction mixture of ethyl 4-(4-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**2**) (10.0 g, 32.2 mmol), 3,4-dihydroxybenzaldehyde (4.44 g, 32.1 mmol), chloroacetic acid (3.04 g, 32.2 mmol), and NaOAc (7.92 g, 96.6 mmol) in acetic acid (100 mL) was refluxed (140 °C) for 5 h. The reaction mixture was cooled to room temperature and poured into ice water. Solid was collected and washed with water and *n*-hexane and dissolved in EtOAc. Organic solvent was dried over Na_2SO_4 and solvent was removed under reduced pressure. The residue was treated with CH_2Cl_2 and small amount of *n*-hexane to give compound **24** as a weak green solid (9.2 g, 60.7%). Mp: 272–274 °C; HPLC: R_T 8.66 min (purity; 100%); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.12 (t, J = 7.2 Hz, 3H), 2.34 (s, 3H), 4.08 (q, J = 7.2 Hz, 2H), 6.01 (s, 1H), 6.87 (d, J = 8.0 Hz, 1H), 6.97 (d, J = 8.0 Hz, 1H), 7.00 (d, J = 2.0 Hz, 1H), 7.31 (d, J = 8.4 Hz, 2H), 7.40 (d, J = 8.4 Hz, 2H), 7.60 (s, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz) 13.9, 22.6, 54.3, 60.2, 107.8, 114.7, 116.4, 116.5, 124.1, 124.2, 128.7, 129.5, 133.1, 134.3, 139.5, 146.0, 149.2, 152.0, 156.2, 164.5, 164.8 ppm: HRMS (ESI) $[M+H]^+$ $C_{23}H_{20}ClN_2O_5S$ calcd 471.0776, found 471.0779.

4.4.7. (*Z*)-Ethyl 5-(4-chlorophenyl)-7-methyl-3-oxo-2-(3,4,5-trihydroxybenzylidene)-3,5-dihydro-2*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate (25)

A reaction mixture of ethyl 4-(4-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylate (**2**) (0.2 g, 0.64 mmol), 3,4,5-trihydroxybenzaldehyde (99.0 mg, 0.64 mmol), chloroacetic acid (91.2 mg, 0.97 mmol), and NaOAc (105.5 g, 1.29 mmol) in acetic acid (5 mL) was refluxed (140 °C) for 6 h. The reaction mixture was cooled to room temperature and poured into ice water. Solid was collected and washed with water and *n*-hexane and dissolved in EtOAc. Organic solvent was dried over Na_2SO_4 and solvent was removed under reduced pressure. The residue was treated with CH_2Cl_2 and small amount of *n*-hexane to give compound **25** as a weak green solid (136.0 mg, 30.3%). mp: 256–258 °C; HPLC: R_T 4.30 min (purity; 100%); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.12 (t, J = 6.8 Hz, 3H), 2.38 (s, 3H), 4.04 (brs, 2H), 6.02 (s, 1H), 6.58 (s, 2H), 7.31 (d, J = 8.4 Hz, 2H), 7.41 (d, J = 8.4 Hz, 2H), 7.51 (s, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz) 14.5, 23.2, 54.9, 60.8, 108.4, 110.4, 115.3, 123.6, 129.3, 130.1, 133.7, 135.2, 138.0, 140.1, 147.1, 152.6, 156.9, 165.1, 165.4 ppm; HRMS (ESI) $[M+H]^+$ $C_{23}H_{20}ClN_2O_6S$ calcd 487.0720, found 487.0733.

4.5. In vitro kinase assay

Inhibitory effects of PKCK2 inhibitors were measured using in vitro kinase assay as previously described.[35] Briefly, 3 μg of bacterially expressed GST-CS (CK2 Substrate) protein was incubated with glutathione Sepharose 4B beads for 60 min and washed twice with 1× kinase buffer (4 mM MOPS, pH 7.2, 5 mM β-glycerolphosphate, 1 mM EGTA, 200 μM sodium orthovanadate, and 200 μM DTT). The beads were incubated with 100 μg cell lysate in a final volume of 50 μL kinase reaction buffer [10 μL of 5× kinase buffer,

10 μL magnesium/ATP cocktail solution (90 μL of 75 mM $MgCl_2$/500 mM ATP plus 10 μL (100 μCi) γ-[^{32}P]-ATP] for 20 min at 30 °C. Reactions were stopped by washing twice with 1× kinase buffer. Samples were resuspended with 30 μL of 2× sodium dodecyl sulfate (SDS) sample loading buffer, subjected to 12% SDS–polyacrylamide gel electrophoresis (PAGE) followed by staining with Coomassie brilliant blue, and dried on Whatman paper. Incorporation of ^{32}P was detected by autoradiography.

4.6. Kinase selectivity

To examine kinase selectivity, we used KinaseProfiler service (Millipore). 773, TBB, compounds **24**, and **25** were screened against 31 human kinases. The kinases were tested for inhibition with 10 μM concentrations of each inhibitor. Radioactive phosphorylated product was quantitated with and without inhibitor. Kinase activity is expressed as a percentage of the mean kinase activity in the positive control samples. Positive-control wells contain all components of the reaction except inhibitors. DMSO is included in these wells to control for solvent effects. IC_{50} of TBB, compounds **24**, and **25** for inhibition of PKCK2 were determined using the IC_{50} profiler assay service (Millipore).

4.7. Kinetic determination

For kinetic analysis, increasing TBB and compound **24** concentrations were applied. Initial velocities were determined at each of the substrate concentration tested. Kinetic parameters (K_m and V_{max}) were calculated either in the presence or absence of increasing concentrations of inhibitor using the sigma plot program (enzyme kinetics module). Inhibition constants were subsequently calculated by linear regression analyses of K_m/V_{max} against inhibitor concentration plots. K_i values were determined based on these averages. The rationale underlying this approach was that kinase inhibition is competitive with respect to ATP.

4.8. Cell culture and transfection

The human esophageal cancer cell lines HCE4, TE2-GFP, and TE2-CK2α were grown as previously described.[33] Human stomach cancer cell lines AGS, MKN-1, MKN-28, MKN-45, MKN-74, NCI-N87, SNU484, and SNU668 and human liver cancer cell lines HepG2, Hep3B, SK-HEP1, SNU739, SNU761, and SNU878 were obtained from the Korea Cell Line Bank (KCLB; Seoul, Korea). MKN-1, MKN-28, MKN-45, MKN-74, NCI-N87, SNU484, SNU668, SK-HEP1, SNU739, SNU761, and SNU878 cells were maintained in RPMI1640 medium (Gibco, Gaithersburg, MD). AGS and HepG2 cells were maintained in Modified Eagle's Medium (Gibco). Hep3B and SK-HEP-1 cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco). Each medium was supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Sigma, St. Louis, MO). The cells were cultured at 37 °C in a 95% air/5% CO_2 incubator, and the medium was replaced every three days.

4.9. Cell viability assay

Cell viability was determined using MTT (Sigma) assays. Briefly, cells were seeded at a density of 1×10^4 cells/well on 96-well cell culture plates. After 24 h incubation, the cells were treated with PKCK2 inhibitors for 24 h. Each well was incubated with MTT solution (final concentration 4 mg/mL) for 2 h. The culture medium in each well was removed, and the formazan dye was resuspended with 100 μL DMSO. The absorbance of each well was measured at a wavelength of 570 nm using a microtiter plate reader.

4.10. Apoptosis detection

The apoptotic effect of PKCK2 inhibitors on stomach and liver cancer cell lines were examined by Annexin-V-FLUOS staining kits (Roche Diagnostics, Mannheim, Germany), as previously described.[36] In brief, the cells were treated with TBB or compound **24** as described above, washed with phosphate-buffered saline, and stained with 100 μL Annexin-V-FLUOS/propidium iodide mixture for 15 min. Finally, apoptotic cells were analyzed by fluorescence microscopy (Nikon TE2000U).

4.11. Molecular docking studies

The coordinates for the catalytic subunit of human CK2 (CK2α) were retrieved from the Protein Data Bank (PDB code 1JWH).[37] All the water molecules, ligands, and cofactors were removed except for the conserved water molecule as following the previous work[38] and the hydrogen atoms were added. The structures of compound **24** were constructed by Sybyl 8.0 and minimized energetically using a Tripos force field with Gasteiger-Huckel charges. The receptor and ligand file were prepared according to the original publication protocols.[38,39] Docking was carried out with Autodock 4.0/ADT using the Lamarckian genetic algorithm search parameters.[40]

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Supplementary data

Supplementary data (compounds library screening data for PKCK2) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.07.037.

References and notes

1. Pinna, L. A. *Cell Mol. Biol. Res.* **1994**, *40*, 383.
2. Meggio, F.; Pinna, L. A. *FASEB J.* **2003**, *17*, 349.
3. Pinna, L. A. *J. Cell Sci.* **2002**, *115*, 3873.
4. Litchfield, D. W. *Biochem. J.* **2003**, *369*, 1.
5. Ahmed, K.; Gerber, D. A.; Cochet, C. *Trends Cell Biol.* **2002**, *12*, 226.
6. Ahmad, K. A.; Wang, G.; Slaton, J.; Unger, G.; Ahmed, K. *Anticancer Drugs* **2005**, *16*, 1037.
7. Song, D. H.; Sussman, D. J.; Seldin, D. C. *J. Biol. Chem.* **2000**, *275*, 23790.
8. Shin, S.; Lee, Y.; Kim, W.; Ko, H.; Choi, H.; Kim, K. *EMBO J.* **2005**, *24*, 3532.
9. Pagano, M. A.; Andrzejewska, M.; Ruzzene, M.; Sarno, S.; Cesaro, L.; Bain, J.; Elliott, M.; Meggio, F.; Kazimierczuk, Z.; Pinna, L. A. *J. Med. Chem.* **2004**, *47*, 6239.
10. Oc, P.; Rusch, V.; Talbot, S. G.; Sarkaria, I.; Viale, A.; Socci, N.; Ngai, I.; Rao, P.; Singh, B. *Clin. Cancer Res.* **2004**, *10*, 5792.
11. Faust, M.; Montenarh, M. *Cell Tissue Res.* **2000**, *301*, 329.
12. Laramas, M.; Pasquier, D.; Filhol, O.; Ringeisen, F.; Descotes, J. L.; Cochet, C. *Eur. J. Cancer* **2007**, *43*, 928.
13. Landesman-Bollag, E.; Song, D. H.; Romieu-Mourez, R.; Sussman, D. J.; Cardiff, R. D.; Sonenshein, G. E.; Seldin, D. C. *Mol. Cell Biochem.* **2001**, *227*, 153.
14. Duncan, J. S.; Litchfield, D. W. *Biochim. Biophys. Acta* **2008**, *1784*, 33.
15. Song, D. H.; Dominguez, I.; Mizuno, J.; Kaut, M.; Mohr, S. C.; Seldin, D. C. *J. Biol. Chem.* **2003**, *278*, 24018.
16. Barroga, C. F.; Stevenson, J. K.; Schwarz, E. M.; Verma, I. M. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7637.
17. Wang, G.; Unger, G.; Ahmad, K. A.; Slaton, J. W.; Ahmed, K. *Mol. Cell Biochem.* **2005**, *274*, 77.
18. Unger, G. M.; Davis, A. T.; Slaton, J. W.; Ahmed, K. *Curr. Cancer Drug Targets* **2004**, *4*, 77.
19. Seeber, S.; Issinger, O. G.; Holm, T.; Kristensen, L. P.; Guerra, B. *Apoptosis* **2005**, *10*, 875.

20. Slaton, J. W.; Unger, G. M.; Sloper, D. T.; Davis, A. T.; Ahmed, K. *Mol. Cancer Res.* **2004**, *2*, 712.
21. Siddiqui-Jain, A.; Drygin, D.; Streiner, N.; Chua, P.; Pierre, F.; O'Brien, S. E.; Bliesath, J.; Omori, M.; Huser, N.; Ho, C.; Proffitt, C.; Schwaebe, M. K.; Ryckman, D. M.; Rice, W. G.; Anderes, K. *Cancer Res.* **2010**, *70*, 10288.
22. Golub, A. G.; Yakovenko, O. Y.; Prykhod'ko, A. O.; Lukashov, S. S.; Bdzhola, V. G.; Yarmoluk, S. M. *Biochim. Biophys. Acta* **2008**, *1784*, 143.
23. Sarno, S.; Reddy, H.; Meggio, F.; Ruzzene, M.; Davies, S. P.; Donella-Deana, A.; Shugar, D.; Pinna, L. A. *FEBS Lett.* **2001**, *496*, 44.
24. Zandomeni, R. O. *Biochem. J.* **1989**, *262*, 469.
25. Yim, H.; Lee, Y. H.; Lee, C. H.; Lee, S. K. *Planta Med.* **1999**, *65*, 9.
26. Critchfield, J. W.; Coligan, J. E.; Folks, T. M.; Butera, S. T. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 6110.
27. Sarno, S.; de Moliner, E.; Ruzzene, M.; Pagano, M. A.; Battistutta, R.; Bain, J.; Fabbro, D.; Schoepfer, J.; Elliott, M.; Furet, P.; Meggio, F.; Zanotti, G.; Pinna, L. A. *Biochem. J.* **2003**, *374*, 639.
28. Pagano, M. A.; Poletto, G.; Di Maira, G.; Cozza, G.; Ruzzene, M.; Sarno, S.; Bain, J.; Elliott, M.; Moro, S.; Zagotto, G.; Meggio, F.; Pinna, L. A. *ChemBioChem* **2007**, *8*, 129.
29. Ashok, M.; Holla, B. S.; Poojary, B. *Eur. J. Med. Chem.* **2007**, *42*, 1095.
30. Tozkoparan, B.; Ertan, M.; Kelicen, P.; Demirdamar, R. *Farmaco* **1999**, *54*, 588.
31. Zorkun, I. S.; Sarac, S.; Celebi, S.; Erol, K. *Bioorg. Med. Chem.* **2006**, *14*, 8582.
32. Yu, Y.; Liu, D.; Liu, C.; Luo, G. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3508.
33. Ko, H.; Kim, S.; Jin, C. H.; Lee, E.; Ham, S.; Yook, J. I.; Kim, K. *Mol. Cancer Res.* **2012**, *10*, 1032.
34. Cozza, G.; Bortolato, A.; Moro, S. *Med. Res. Rev.* **2010**, *30*, 419.
35. Yde, C. W.; Ermakova, I.; Issinger, O. G.; Niefind, K. *J. Mol. Biol.* **2005**, *347*, 399.
36. Jin, C. H.; Chae, S. Y.; Kim, T. H.; Yan, H. K.; Lee, E. Y.; Song, Y. W.; Jo, D. J.; Lee, K. C. *J. Pharmacol. Exp. Ther.* **2010**, *332*, 858.
37. Niefind, K.; Guerra, B.; Ermakowa, I.; Issinger, O. G. *EMBO J.* **2001**, *20*, 5320.
38. Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. *J. Comput. Chem.* **1998**, *19*, 1639.
39. Sanner, M. F. *J. Mol. Graph. Model.* **1999**, *17*, 57.
40. Battistutta, R.; Mazzorana, M.; Cendron, L.; Bortolato, A.; Sarno, S.; Kazimierczuk, Z.; Zanotti, G.; Moro, S.; Pinna, L. A. *ChemBioChem* **2007**, *8*, 1804.