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Pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione derivatives: Their cytoprotection effect from rotenone toxicity and preliminary DMPK properties

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ABSTRACT

Pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione derivatives exhibited potent cytoprotective effect from rotenone toxicity. Lead optimization focused on the CC_{50}/EC_{50} ratio and DMPK properties led to the overall improvement of the compound profile of this series with high CC_{50}/EC_{50} ratio (92 for **1f**), good metabolic stability in rat microsomes and medium to high aqueous solubility.

Parkinson's disease (PD) belongs to a group of conditions called motor system disorders, which are the result of the loss of dopamine-producing brain cells. The primary symptoms of PD include tremor or trembling in hands, arms, legs, jaw, and face; rigidity or stiffness of the limbs and trunk; bradykinesia or slowness of movement; and postural instability or impaired balance and coordination. As these symptoms become more pronounced, patients may have difficulty walking, talking or completing other simple tasks. PD is both chronic and progressive.

It is estimated that PD affects at least 500,000 people in the United States, which is expected to increase as the population ages.[1] At present, there is no cure for PD, although a variety of medications provide dramatic relief from the symptoms. In recent years, Parkinson's research has advanced to the point that halting the progression of PD, restoring lost function, and even preventing the disease are all considered realistic goals. However, to cure PD remains a significant challenge. Therefore there is continued significant medical need to discover novel molecules or therapy for treating PD effectively.

Previously we reported that pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione derivatives (**1**, Fig. 1) confer significant cytoprotective effect from rotenone toxicity in a cellular rotenone stress assay, a disease relevant cell model to PD.[2] The biological profile of this series of compounds suggests its potential therapeutic application in neurodegenerative diseases such as PD. We reported both synthesis and the initial structure–activity relationship (SAR) of R^2 and R^3 on their cytoprotective effect from rotenone toxicity.[2] Among them, **1a** (Fig. 1) was discovered as one of the best compounds, which displayed significant cytoprotection from rotenone toxicity (EC_{50} = 0.23 μM).[2]

As our continued effort to optimize this series, here we report the extended SAR of pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione derivatives (**1**) on their cytoprotective potency in the rotenone stress assay, the optimization of the CC_{50}/EC_{50} ratio, as well as their initial DMPK properties.

Rotenone is a broad-spectrum insecticide and pesticide that is known to cause PD-like symptoms in rats.[3] The cellular rotenone

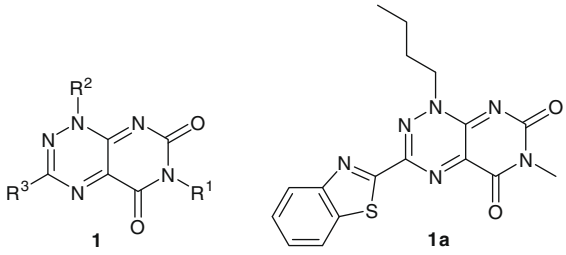

Figure 1. Pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione derivatives.

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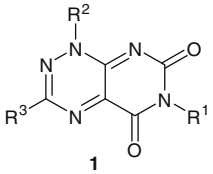
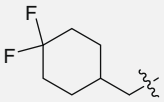
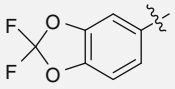
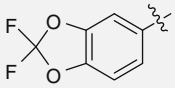
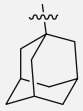
model of PD has been applied by others to study PD-related cytotoxicity.[4–6] Therefore we developed a rotenone toxicity model in SK-N-SH neuroblastoma cells to measure the cytoprotection from rotenone-induced cellular damage.[7] As previously demonstrated, the majority of the compounds tested in this series were very potent in protecting cells from rotenone-induced cytotoxicity with EC_{50} <1 μM.[2] However, most of them had low CC_{50}/EC_{50} ratio (<20) based on their potency in rotenone stress assay and their corresponding cytotoxicity in SK cells without rotenone. In order to have practical application in PD, it is necessity to further improve the CC_{50}/EC_{50} ratio of this series of compounds. For that purpose, new variations and new combinations of R^1, R^2, and R^3 were evaluated. The EC_{50} value, the maximum percent increase of cell viability (compared with the DMSO control with rotenone treatment) and the CC_{50}/EC_{50} ratios are listed in Table 1. For comparison, the previously discovered best compound **1a** was also listed in Table 1.

As shown in Table 1, when R^2 and R^3 were kept constant, the R^1 group with the longer linear carbon chain length led to higher CC_{50}/EC_{50} ratios (compare **1a** or **1c** with **1e** or **1f**). Among them, **1f** with *n*-Bu R^1 moiety had CC_{50}/EC_{50} ratio of 92, close to threefold improvement compared with that of **1a**. Cyclopropylmethylene group is also among the best R^1 substituents (**1i**, **1t**). In addition, increasing the bulkiness of R^2 group also led to an improvement in CC_{50}/EC_{50} ratio (**1c** vs **1d**). In general, most of the compounds listed in Table 1 had improvement in CC_{50}/EC_{50} ratio compared with **1a** via the improvement of either the cytoprotective potency (e.g., **1l** and **1o**, EC_{50} <0.1 μM) or decreased cytotoxicity (e.g., **1d**, **1f**, **1i**, **1k**, **1m–n**, **1p–t**, **1v–w**). The best R^3 groups we discovered include 2-benzothiazole (**1f**) and substituted phenyl (**1m**, **1t**), while the best R^2 are the alkyl groups with the same linear length of carbon chain as *n*-Bu (**1f**, **1t**). It's worth noting that compound **1g** containing the 4-trifluoromethoxyphenyl R^3 moiety not only improved

Table 1
Optimization of CC_{50}/EC_{50} ratio



1

Product[a]	R^1	R^2	R^3	Rotenone EC_{50}[b] (μM)	Max cytoprotection[b] (%)	CC_{50}[c] (μM)	CC_{50}/EC_{50}
1a	Me	*n*-Bu	2-Benzothiazole	0.23	53	8.4	37
1b	Me	*c*-Pr-CH_2CH_2	2-Benzothiazole	0.23	48	10.6	46
1c	Et	*n*-Bu	2-Benzothiazole	0.16	56	4.9	31
1d	Et	Isoamyl[d]	2-Benzothiazole	0.3	46	22.6	77
1e	*n*-Pr	*n*-Bu	2-Benzothiazole	0.12	42	8.6	72
1f	*n*-Bu	*n*-Bu	2-Benzothiazole	0.21	32	19.3	92
1g	Me	*n*-Bu	4-CF_3O-Ph	0.28	85	14.2	51
1h	Me	Et	4-Cl-Ph	0.15	65	5.9	39
1i	*c*-Pr-CH_2	*c*-Pr-CH_2CH_2	4-CF_3-Ph	0.29	62	22.6	78
1j	Me	Me	3,4-Di-Cl-Ph	0.19	30	11	58
1k	*c*-Pr-CH_2	*c*-Pr-CH_2CH_2	3,4-Di-Cl-Ph	0.81	45	43.5	54
1l	Me		3-F-Ph	0.07	46	2.8	40
1m	Me	*c*-C_6H_{11}–CH_2–CH_2	3-F-Ph	0.51	57	42.2	83
1n	Me	Et	3-Cl-Ph	0.4	44	>23	>58
1o	Me	*c*-Pr-CH_2CH_2	3-Cl-Ph	0.09	39	7	77
1p	Me	*c*-C_6H_{11}–CH_2–CH_2	3-CF_3O-Ph	0.35	38	26.5	76
1q	*c*-Pr-CH_2	*c*-Pr-CH_2CH_2	3-CF_3O-Ph	0.59	54	36.6	62
1r	Me	CF_3-$CH_2CH_2CH_2$	3-CF_3O-Ph	0.61	45	31.5	52
1s	Me	CH_3O–CH_2–CH_2–CH_2	3-CF_3O-Ph	0.38	59	26.1	69
1t	*c*-Pr-CH_2	CF_3–$CH_2CH_2CH_2$	3-CF_3-Ph	0.37	43	35.9	83
1u	Me	*c*-Pr-CH_2CH_2		0.2	70	13.1	66
1v	*c*-Pr-CH_2	CF_3-$CH_2CH_2CH_2$		0.57	50	38.6	68
1w	*c*-Pr-CH_2	CF_3-$CH_2CH_2CH_2$		0.78	51	43	55

[a] The expected mass was confirmed for each compound by LC–MS analysis and structure was confirmed by ^1H NMR. Their syntheses are reported in Ref. 2. The data are the mean of multiple testing.
[b] See Ref. 7 for rotenone stress assay condition. EC_{50} is the concentration at which cytoprotection from rotenone is half of the maximum cytoprotection achieved for each compound.
[c] Cell viability was determined by the MTS assay described in Ref. 8 CC_{50} is the concentration at which cytotoxicity is half of maximum.
[d] Isoamyl = 3,3-dimethylpropyl.

the CC_{50}/EC_{50} ratio but was also superior to all others in the maximum cytoprotection (85%) including **1a**. The 85% cytoprotection of **1g** is equivalent to 98% cell survival, close to complete protection from rotenone toxicity, which was a dramatic improvement in cytoprotection over other compounds including the original lead compound **1a**. Therefore, by comparison, a concentration of **1g** below its EC_{50} would result in comparable cytoprotective activity to other compounds, potentially further improving the relative CC_{50}/EC_{50} ratio.

Overall, a few analogs in this series had CC_{50}/EC_{50} ratio of $\geq$70, a 2–3-fold improvement over that of **1a**. The combination of the high CC_{50}/EC_{50} ratio and the high cytoprotective potency in the rotenone stress assay suggests improved therapeutic potential of this series of compounds for PD.

Next, in order to further evaluate the potential applications of this series of pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione derivatives, a rat PK study with both intravenous (IV, 2 mg/kg) and oral (PO, 20 mg/kg) administration of compound **1a** was conducted. We found that this compound was well distributed (V_z = 9.26 L/kg), but the stability in rat measured by the terminal half-life ($t_{1/2}$ = 1.29 h) was relatively short, and the oral bioavailability (F_{po} = 2%) was very poor (Table 2).

To estimate its absorption characteristics, membrane permeability of **1a** was assessed with the Caco-2 assay.[9] Results showed rapid transport in the apical to basal direction (P_{app}AtoB = 34.8 × 10^{-6} cm/s) with efflux ratio (P_{app}BtoA/P_{app}AtoB) of 0.03, suggesting high intestinal permeability and likely lack of P-glycoprotein mediated export.[9] The calculated polar surface area (PSA = 90.1 Å^2) for **1a** also is in the range typically associated with the well-absorbed molecules (PSA <140 Å^2).[10] In addition, this compound (**1a**) has very good aqueous solubility (230 μM) (Table 3) which is expected to have favorable impact on absorption and oral bioavailability. Therefore the poor oral bioavailability of **1a** was most likely not attributed to the absorption.

Since both the poor oral bioavailability and plasma instability could be attributed in part to liver metabolism, compound **1a** was tested for stability in rat liver microsomes (RLM). Although the half-life ($t_{1/2}$) in RLM for this compound was not very short (51 min), the recovery without co-factor NADPH was low (50%, Table 3), suggesting possible non-CyP450 related metabolism.

In an effort to improve the rat liver microsomal stability with high recovery, we first incorporated cyclopropylethyl R^2 motif into our molecule design (**1b**), but the RLM stability did not improve compared with that of **1a** (Table 3). Next we incorporated e-withdrawing substitution at the 3- or 4- position of a phenyl ring R^3, the resulting compounds were very stable in the RLM as expected (compare **1x** or **1n** with **1a**). **1n** is the most stable one with almost no degradation over 45 min with 100% recovery without co-factor NADPH in the RLM assay. When 3-CF_3-Ph, 3-CF_3O-Ph or 2,2-difluorobenzo[*d*][1,3]dioxole group was used as R^3, some of the analogs (**1s** and **1u**) are more stable in RLM than **1a** with $t_{1/2}$ >45 min and recovery $\geqslant$70%, while the others (**1t** and **1v**) are less stable than **1a** in the RLM assay with lower recovery. In these cases, their instability in RLM may be attributed to the R^2 and/or R^1 alkyl groups.

Next, we chose **1n**, the most stable compound in the RLM assay, to do a rat PK study with both IV (2.5 mg/kg) and PO (10 mg/kg) administrations hoping to see some level of improvement. However, although the oral bioavailability of this compound was significantly improved (from 2% to 11%) compared with **1a**, it was still not adequate. As shown in Table 2, this compound (**1n**) also had a very short terminal half-life ($t_{1/2}$ = 0.12 h) following IV administration in rats, which was inconsistent with its in vitro RLM stability results. On the other hand, **1n** was far less well distributed in rats than **1a** (V_z = 1.62 vs 9.26 L/kg) following IV administration. This may explain why the terminal half-life ($t_{1/2}$) of the compound **1n** in rats following IV dose was so short since it is directly affected by the volume distribution (V) and clearance (CL) [$t_{1/2}$ = (0.693 × V)/CL]. Considering the fact that **1n** had comparable in vivo clearance as **1a**, the much lower volume distribution of **1n** (compared with **1a**) was likely the main reason to cause the very short terminal half-life. The low volume distribution of **1n** may indicate possible plasma protein binding of this compound. Furthermore, the low oral bioavailability of **1n**, despite dramatically increased RLM stability of this compound, could have been offset by its low aqueous solubility (23 μM, Table 3) resulting in improved but still poor oral bioavailability. Therefore we also focused our efforts on improving the aqueous solubility of this series of compounds, in addition to the other properties.

When we incorporated a CF_3-substituted phenyl ring as R^3, a significant improvement of the aqueous solubility was observed (compare **1i** or **1t** with **1n** or **1o**) (Table 3). Likewise all the compounds with a CF_3O-substituted phenyl ring as R^3 had good aqueous solubility ranging from 117 to 491 μM (**1q–t**), with **1s** maintaining good RLM stability and high CC_{50}/EC_{50} ratio. The significant improvement of the aqueous solubility is likely due to the formation of hydrogen bonds between F-atoms and water molecules. Interestingly, 2,2-difluorobenzo[*d*][1,3]-dioxole R^3 group led to poor aqueous solubility (**1u–y**). However, when a CF_3 moiety was incorporated into a R^2 alkyl group, the aqueous solubility of the resulting compound **1v** was improved 10-fold as compared with that of **1y,** likely due to the R^2 group containing F-atoms which enable the formation of H-bonds with water molecules. Finally, we incorporated the morpholine motif into our target molecule design, as expected the aqueous solubility of **1z** was very high (482 μM).

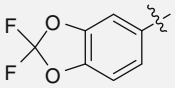
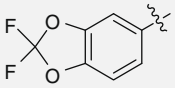
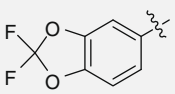
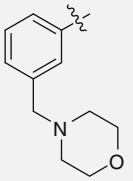
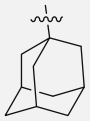
Our next step of the research is to select analogs we have discovered that had excellent cytoprotective potency or overall good balance between potency, CC_{50}/EC_{50} ratio, RLM stability, and aqueous solubility such as **1l–o** and **1s** for continued DMPK evaluation. Results from such studies may provide additional insights into how to simultaneously improve PK characteristics for the compounds in this series, and ultimately for animal efficacy studies. As there are often multiple factors that simultaneously influence the animal PK profile even with different analogs in the same chemical series as demonstrated by the above cases of **1a** and **1n**, it will be worth-

Table 2
Pharmacokinetic parameters and calculated physical properties for selected compounds

Compd	Dose (mg/kg) (PO/IV)	AUC_{inf}[a] (μg/h/L) (PO/IV)	$t_{1/2}$ (h) (PO/IV)	V_z[b] (L/kg) (IV)	CL[c] (L/h/kg) (IV)	F_{po} (%)	*t*PSA[d]	Clog *P*[d]
1a	20/2	79/392	2.36/1.29	9.26	5.13	2	90.1	1.93
1n	10/2.5	130/288	1.85/0.12	1.62	8.88	11	77.7	1.39

[a] Male rats: formulation (for both IV PO and IV administration) for **1a**: 5% DMSO, 20% PEG400, 9.5% Cremophor EL in 50 mM sodium phosphate buffer, pH 7.4; for **1n**: 7% DMA, 20% PEG400, 73% (30% HP-β-CD water), pH 6.
[b] Ref.: The total body water in the rats is 0.67 L/kg.
[c] Ref.: The rat hepatic blood flow is 3.31 L/h/kg.
[d] tPSA means the calculated polar surface area using ChemDraw Ultra 11.0; *C*log P values were also calculated using ChemDraw Ultra 11.0.

Table 3
Optimization of ADME properties

Product[a]	R^1	R^2	R^3	Rotenone EC_{50} (μM)	CC_{50}/EC_{50}	RLM $t_{1/2}$[b] (min)	RLM recovery[c] (%, -NADPH)	Solubility[d] (μM)
1a	Me	*n*-Bu	2-Benzothiazole	0.23	37	51	50	230
1b	Me	*c*-Pr-CH_2CH_2	2-Benzothiazole	0.23	46	40	44	34
1d	Et	Isoamyl	2-Benzothiazole	0.3	77	24	25	ND[e]
1x	Me	Me	4-CF_3-Ph	0.12	32	140	105	ND
1i	*c*-Pr-CH_2	*c*-Pr-CH_2CH_2	4-CF_3-Ph	0.29	78	ND	ND	75.3
1m	Me	*c*-C_6H_{11}–CH_2–CH_2	3-F-Ph	0.51	83	ND	ND	49
1n	Me	Et	3-Cl-Ph	0.4	>58	2890	101	28.5
1o	Me	*c*-Pr-CH_2CH_2	3-Cl-Ph	0.09	77	ND	ND	21.4
1q	*c*-PrCH_2	*c*-Pr-CH_2CH_2	3-OCF_3-Ph	0.59	62	ND	ND	117
1r	Me	CF_3–$CH_2CH_2CH_2$	3-OCF_3-Ph	0.61	52	ND	ND	171
1s	Me	CH_3O–CH_2–CH_2–CH_2	3-OCF_3-Ph	0.38	69	101	71	491
1t	*c*-PrCH_2	CF_3–$CH_2CH_2CH_2$	3-CF_3-Ph	0.37	83	23	26	200
1u	Me	*c*-Pr-CH_2CH_2		0.2	66	72	73	20.7
1y	*c*-PrCH_2	*c*-Pr-CH_2CH_2		0.47	>35	79	68	16.1
1v	*c*-PrCH_2	CF_3–$CH_2CH_2CH_2$		0.57	68	31	41	168
1z	Me	*c*-C_6H_{11}–CH_2–CH_2		0.82	42	ND	ND	482
1aa	Me	*c*-Pr-CH_2CH_2		0.99	41	19	90	47.3

[a] The desired mass was found for each compound in LC–MS analysis and structure was confirmed by ^1H NMR. Their syntheses can be referred to the procedure described in Ref. 2. The data are the average of multiple testing.
[b] See Ref. 11 for RLM assay condition. The $t_{1/2}$ (min) value is the extrapolated value from a 45 min run of the assay.
[c] The compound recovery (%) without co-factor NADPH in RLM assay.
[d] See Ref. 12 for the solubility test condition.
[e] ND—not done.

while with these and future compounds to also measure plasma protein binding as it could affect distribution as well as oral bioavailability.

In summary, we synthesized a number of new pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione analogs using the synthetic procedures described previously for lead optimization, which focused on the improvement of CC_{50}/EC_{50} ratio and DMPK properties from the previously discovered lead compound **1a**. Our continued lead optimization effort in this series (as described here) led to highly potent compounds (EC_{50} <0.1 μM) in the rotenone cytoprotection assay and high CC_{50}/EC_{50} ratio of up to 92, close to threefold improvement from that of **1a**. This lead optimization effort also resulted in a number of stable compounds in rat liver microsomes as well as compounds with medium-high aqueous solubility. Most importantly, we were able to discover compounds that had good overall balance between potency, CC_{50}/EC_{50} ratio, metabolic stability in RLM and aqueous solubility such as **1l–o** and **1s**. As the rotenone cell model is directly relevant to PD, the results presented here provided us much encouragement and hope for this series of compounds to be potentially used in treating PD in the future.

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7. *Rotenone stress assay condition:* SK-N-SH neuroblastoma cells (purchased from ATCC, Manassas, VA, within passage 6) were seeded in a 96 well plate with 8000 cells /well in DMEM containing 0.1% FBS for 18 h before Rotenone treatment. Using Sciclone liquid handling workstation (Caliper Life Science, Hopkinton, MA), fresh media containing rotenone with or without testing compounds were added to the assay plates with a final concentration of rotenone at 200 nM and compound concentration ranging from 0.019 μM to 10 μM. Cells were incubated for 48 h followed by MTS viability assay. % Cytoprotection (percent increase in viable cells compared to DMSO control) was determined using the following formula: $[(MTS_{\text{compound with rotenone}} - MTS_{\text{rotenone alone}})/MTS_{\text{rotenone alone}}] \times 100\%$. The mean % cell survival of the

DMSO control with rotenone alone averaged 53%, and therefore 89% cytoprotection corresponds to 100% cell survival and complete protection from rotenone toxicity. EC_{50} is the concentration at which cytoprotection from rotenone is half of maximum protection achieved for each compound. EC_{50} values and curve fitting were calculated using Prism 4.0 (GraphPad Software, San Diego, CA) with nonlinear regression analysis. Maximum percent cytoprotection <20% was considered inactive.

8. *MTS viability assay*: SK-N-SH (14,000 cells/well) cells were seeded in DMEM containing 10% FBS in 96-well plates (Catalog # Costar 3598, Cornings, MA) for 18 h before experiment. Compounds or DMSO control were added to the culture at 1–200 dilutions with a final concentration ranging from 0.3 μM and 80 μM (final DMSO concentration is 0.5% v/v). Cells were incubated with compounds for 72 h followed by MTS/PMS addition into the plates and incubated for four additional hours. SDS was added to a final concentration of 1.4% (w/v %). Plates were then measured for absorbance at 492 nm using Envision Excite (Perkin Elmer, Wellesley, MA). The absorbance at 492 nm is directly proportional to the living cells in the culture. Percent inhibition was determined using the following formula: $[(MTS_{DMSO} - MTS_{compound})/MTS_{DMSO}] \times 100\%$. IC_{50} values and curve fitting were calculated using Prism 4.0 (GraphPad Software, San Diego, CA) with nonlinear regression analysis.
9. *Caco-2 permeability (bi-directional; pH 7.4/pH 7.4) assay condition:* the Caco-2 cells are seeded on Transwell™ plates and form a confluent monolayer over 20 days prior to the experiment. On day 20, the test compound (10 μM) is added to the apical side of the membrane and the transport of the compound across the monolayer is monitored over a 2 h time period. The measurement from the basolateral compartment to the apical compartment was done similarly. Permeability is measured by monitoring the appearance of the test compound on the opposite side of the membrane using LC–MS/MS. The permeability coefficient (P_{app}) is calculated from the following equation: $P_{app} = [(dQ/dt)/(C_0 \times A)]$, where dQ/dt is the rate of permeation of the drug cross the cells, C_0 is the donor compartment concentration at time zero and A is the area of the cell monolayer. Percentage of the recovery is calculated as following: $\% R = (\text{Total compound in donor and receiver at end of the experiment/Initial compound present}) \times 100$. For compound **1a**, the recovery in the apical to basolateral direction in the Caco-2 assay was very low, which may indicate problems with binding of this compound to the plate or accumulation on the cell monolayer considering the fact that it has good aqueous solubility. The control compounds used in this assay are atenolol and propranolol. Their P_{app} values in A-to-B direction (apical-to-basolateral) are listed below. P_{app} atenolol (low) = 0.41×10^{-6} (cm/s); P_{app} propranolol (high) = 51.3×10^{-6} (cm/s).
10. (a) Veber, D. F.; Johnson, S. R.; Cheng, H.-Y.; Smith, B. R.; Ward, K. W.; Kopple, K. D. *J. Med. Chem.* **2002**, *45*, 2615; (b) Palm, K.; Stenberg, P.; Luthman, K.; Artursson, P. *Pharm. Res.* **1997**, *14*, 568; (c) Sergeeva, M. V.; Zhou, Y., et al *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3421.
11. *Rat liver microsomal stability test condition*: test compound (3 μM) is incubated with pooled liver microsomes. Test compound is incubated at five time points over the course of a 45 min experiment and the test compound is analyzed by LC–MS/MS. The half-life ($t_{1/2}$) value was calculated according to the following formula. Half-life ($t_{1/2}$) (min) = $0.693/k$, where elimination rate constant (k) = (−gradient).
12. *Aqueous solubility test condition*: the aqueous solubility of the test compounds were determined based on UV absorbance in an aqueous (pH 7.4) buffer solution containing 5% DMSO. The method was referred to the 'solubility multiscreen filter plate protocol' with reference No. PC2445EN00 from Millipore.