Bioorganic & Medicinal Chemistry 21 (2013) 824–831

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Exploration of 1-(3-chloro-4-(4-oxo-4*H*-chromen-2-yl)phenyl)-3-phenylurea derivatives as selective dual inhibitors of Raf1 and JNK1 kinases for anti-tumor treatment

Feng Jin [a,b], Dan Gao [b], Cunlong Zhang [b], Feng Liu [b], Bizhu Chu [a,b], Yan Chen [b], Yu Zong Chen [c], Chunyan Tan [b,*], Yuyang Jiang [b,d,*]

[a] *Department of Chemistry, Tsinghua University, Beijing 100084, China*
[b] *The Ministry-Province Jointly Constructed Base for State Key Lab-Shenzhen Key Laboratory of Chemical Biology, the Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055, China*
[c] *Bioinformatics and Drug Design Group, Department of Pharmacy, Centre for Computational Science and Engineering, National University of Singapore, Singapore 117543, Singapore*
[d] *Department of Pharmacology and Pharmaceutical Sciences, School of Medicine, Tsinghua University, Beijing, 100084, China*

ARTICLE INFO

Article history:
Received 1 February 2012
Revised 31 March 2012
Accepted 4 April 2012
Available online 11 April 2012

Keywords:
Flavones
Aromatic urea
Raf
JNK
Kinases inhibitors
Hepatocellular carcinoma

ABSTRACT

Based on the roles of Raf1 and JNK1 in hepatocarcinoma development, scaffold-based drug design was employed to produce a series of compounds, which subsequently were synthesized and explored as potential dual inhibitors Raf1 and JNK1 kinases for anti-tumor treatment. The compound 1-(3-chloro-4-(6-ethyl-4-oxo-4*H*-chromen-2-yl)phenyl)-3-(4-chloro-phenyl)urea (**3d**) showed 66%, 67% and 13% inhibition rate at 50 μM against Raf1, JNK1 and p38-alpha, respectively, but no effect on ERK1 and ERK2, and inhibited the expression of pERK1/2 markedly and HepG2 cells proliferation with IC_{50} at 8.3 μM. Furthermore, **3d** showed lower toxicity against normal liver cell-lines QSG7701 and HL7702. Molecular docking study further showed that **3d** can fit into binding domain of JNK1 and Raf1. Our data suggested the activities of **3d** were associated with dual inhibition of JNK1 and Raf1 kinases.

1. Introduction

The Raf protein is a key component of the Ras–Raf–ERK mitogen-activated protein kinases (MAPKs) pathway, which regulates cellular processes, for example cell proliferation, migration and apoptosis.[1] c-Jun-N-terminal Kinase (JNK), another MAPK member, is activated in some cancers by Ras to collectively promote cancer together with the Ras–Raf–ERK signaling.[2] Dysregulation of both Raf-MAPKs and JNK pathways had been well elucidated in various human cancers, for example, the mutant of Ras proteins often result in carcinogenesis by continuously activating downstream effectors, and it is well known that Raf-MAPKs are not the only downstream targets of mutant Ras, another an important target is the JNK pathway.[3] Some compounds targeting either Raf or JNK had been approved or in preclinical trails for anti-cancer treatment.[4,5] However, because of the heterogenetic nature of cancers, multi-target drugs or drug combinations directed at multiple components of cancer proliferation and survival pathways produce more effective anti-cancer effects,[6–8] in particular, dual-targeting of Ras–Raf–ERK and JNK pathways by drug combinations has shown promising potential in inducing anti-cancer effect.[9] Therefore, development of multi-target inhibitors of Raf and JNK may be a promising strategy for specific cancer therapy.

Hepatocellular carcinoma (HCC) accounts for up to 70% of cancer deaths in Eastern Asia and Central Africa, in HCC as well as other cancers, pathways of mitogen-activated protein kinases, including extracellular signal-regulated kinase (ERK), JNK and p38 kinase, had been implicated,[10,11] and ERK regulators such as Raf have been explored as target against HCC.[4] High JNK activation has been found in various cancer cell lines and is linked to cancer development,[12,13] JNK1 is over-expressed in 55% Chinese HCC patients,[12,14] and JNK inhibitor has been found to facilitate the inhibition of HCC growth.[15] In addition to their individual roles in HCC, Raf1 and JNK1 synergistically regulate MAPK signaling via a positive feedback loop established by functional interaction between Raf1 and JNK1, which enables both JNK1 activation of Raf1 (and thus subsequent activation of ERK)[16] and Raf1 activation of JNK1. On the other hand, p38-alpha antagonized JNK pathway in liver cancer development.[17] Thus, MAPKs map had been identified as promising target against HCC,[18,19] multi-target agents that

* Corresponding authors. Tel./fax: +86 755 26036017 (Y.J.).
E-mail addresses: tancy@sz.tsinghua.edu.cn (C. Tan), jiangyy@sz.tsinghua.edu.cn (Y. Jiang).

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2012.04.006

simultaneously inhibit Raf1 and JNK1 with no or minimal inhibition of p38-alpha may produce enhanced anti-HCC effect than agents targeting either Raf1 or JNK1 alone.

A key step towards the design of multi-target Raf1 and JNK1 inhibitors is to select relevant appropriate scaffolds from chemical libraries. There are accumulating evidences suggesting the potential of flavones scaffold in driving protein kinases inhibitors due to their suitable molecular skeleton.[20,21] Many flavonoids exert extensive biological benefits including inhibition of MAPKs.[22–24] For example, quercetin shows potent anti-tumor activities partly due to inhibition of Raf1 and ERK1.[20,25] Some naturally occurring flavonoids with various substituted groups exhibit moderate inhibitory activity against p38-alpha and JNK3, 6-substituted flavones displayed selectivity for JNK3 over p38-alpha.[26] On the other hand, it has been reported that aromatic urea moiety has been identified as a promising fragment to produce novel inhibitors of Raf kinase. One urea derivative sorafenib had been approved as Raf1 inhibitors for treatments of advanced HCC,[27] two others urea derivatives, CP-547623 and KRN951, were in clinical trials. Aromatic urea derivatives of benzothiazole was also explored as Raf1 inhibitors.[28] Also, certain compounds containing aminopyrimidines and urea moiety have been designed as JNK kinase inhibitors for anti-inflammation therapeutics.[29]

To the best of our knowledge, flavones scaffold has not been specifically explored for developing multi-target Raf and JNK inhibitors. Thus, novel multi-target Raf1 and JNK1 inhibitors may be potentially derived from this scaffold. It was well noted that 4′-amino or 2′-chloro substituent of flavones was interesting for kinases inhibition activity.[30,31] In this study, a 6-ethyl-2′-chloro-4′ -aminoflavones compound (**1d**) was found to have moderate kinases inhibition activity against Raf1 and p38-alpha, further structural optimization was conducted by scaffold-based drug designed and thereby produced a series of 1-(3-chloro-4-(4-oxo-4*H*-chromen-2-yl)phenyl)-3-phenylurea derivatives, then evaluated as multi-target Raf1 and JNK1 inhibitors, which were tested by in vitro Raf1, JNK1 and p38-alpha kinase inhibition assays, antiproliferative activities against hepatocellular carcinoma cell-line HepG2, lung adenocarcinoma epithelial cell-line A549, and colon cancer cell-line HCT116, and toxicity activities against normal human liver cells QSG7701 and HL7702.

2. Results and discussions

2.1. Scaffold-based drug design and synthesis of compounds

A general strategy for designing multi-target agents is to conjugate or combine pharmacophoric elements for each target by connecting them with a metabolically stable linker,[32] which is highly effective if certain degrees of commonalities exist in the pharmacophoric elements of each target. Scaffold-based design strategy was especially suitable for kinase inhibitors discovery because of the conserved construction of kinase domain.[33] Firstly, a 6-ethyl-2′-chloro-4′-aminoflavone compound (**1d**) was tested as low-affinity scaffold, which inhibits 14% p38-alpha activity at 100 μM and 27% raf1 activity at 50 μM, based on this scaffold novel compound **3d** was generated by merging 6-ethyl-2′-chloro-4′-aminoflavones and diphenylurea (Scheme 1). In order to clarify structure–activity relationship, some analogs also were designed and synthesized. As shown in Scheme 2, the designed compounds were synthesized by a one-step routine. 2′-chloro-4′-aminoflavones derivatives (**1a**–**1e**) and phenylisocyanate derivatives (**2a**–**2c**) were refluxed in dry tetrahydrofuran (THF), 4-dimethylamiopryidine (DMAP) as catalyst, until raw materials disappeared in solution (monitored by thin-layer chromatography, TLC). After evaporation of the solvent, the residue was washed sequentially with deionizer water and ether, dried in vacuo to obtain **3a**–**3g**. A solution of **1d**, phenylacetic acid derivatives (**2d**–**2e**), *N*,*N*-diisopropylcarbodiimide (DIC), *N*,*N*-diisopropylethylamine (DIEA) and *N*-hydroxy-benzotriazole (HOBt) in dry THF was stirred at room temperature for about 18 h. Evaporation of the solvent and column chromatography on silica (CH_2Cl_2/CH_3OH = 40:1, v/v) gel afforded **3h** and **3i**.

2.2. In vitro kinase inhibition assay

In vitro kinases inhibition activities of **1b**, **1d** and **3d** at the specific concentration against Raf1, JNK1, ERK1, ERK2 and p38-alpha were tested. As shown in Table 1, **3d** inhibited 66% and 67% of Raf 1 and JNK1 activity at 50 μM, respectively. Notably, only 13% of p38-alpha activity was inhibited, ERK1 and ERK2 activities were not influenced at the same concentration, which indicated the selectivity of **3d** towards the desired targets Raf1 and JNK1. While individual target agents such as SP600125 (a widely used selective JNK inhibitor)[34] and FR180204 (a widely used selective ERK inhibitor)[35] are highly potent (with low nM IC_{50}) against their respective target, multi-target drug may produce good therapeutic effect via balanced activity across targets.[32] For example, some (*S*)-3-aminopyrrolidine derivatives with similar activity against multiple targets were reported to be potent anti-proliferation agents against tumor cell-lines.[8] In a similar manner, our compound **3d** may be balanced dual inhibitor with similar activity between Raf1 and JNK1, and essential selectivity against p38-alpha. Besides, its precursor compound **1d** inhibited 27% Raf1 activity at the same concentration. Our newly designed compound **3d** showed improved activity against Raf1 over its precursor because of structural optimization. Based on comparative analysis of compounds **1b** and **1d**, structural arrangement and physicochemical properties of the substituent at A-ring of flavones seem to significantly influence their inhibitory activity towards their kinase targets. 6-substituted flavone derivatives may be better inhibitor against Raf1 than 7-substituted analogs, and ethyl-substituted at A-ring was more beneficial for inhibition of Raf1 than hydroxyl-substituted analogs.

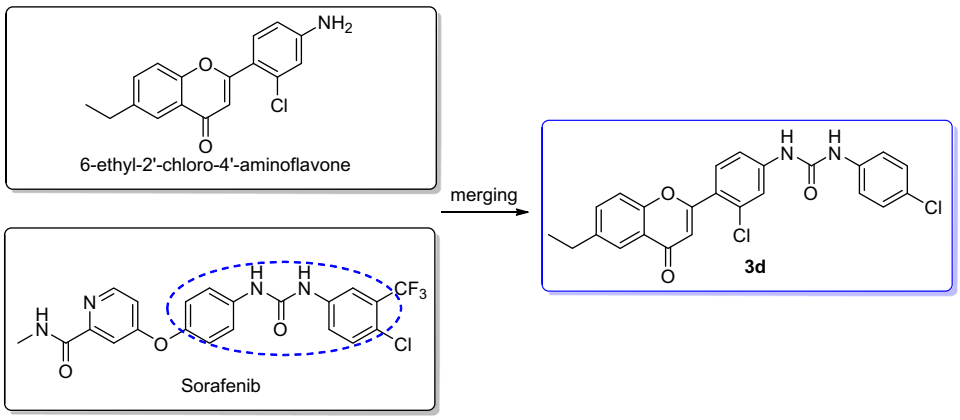
2.3. Western blot analysis

ERK1/2 plays a pivotal role in regulating cell proliferation,[36] and it has specific roles in live tumorigenesis.[37] As the key downstream effector of Raf1, high phosphorylation levels of ERK1/2 have been observed in most human HCC samples.[38] Inhibition of Raf often led to dephosphorylation of ERK1/2.[39,40] Thus, we investigated the effect of 10 μM **3d** on the phosphorylation of ERK1/2 by western blot analysis. As shown in Figure 1(A), **3d** markedly inhibited the phosphorylation of ERK1/2 in HepG2 cells. After a 4 h drug exposure, high activation of pERK1/2 was restrained, but not disappeared, which may contributed to relative low toxicity of **3d** because activation of ERK was critical for a large number of normal cellular response.[41]

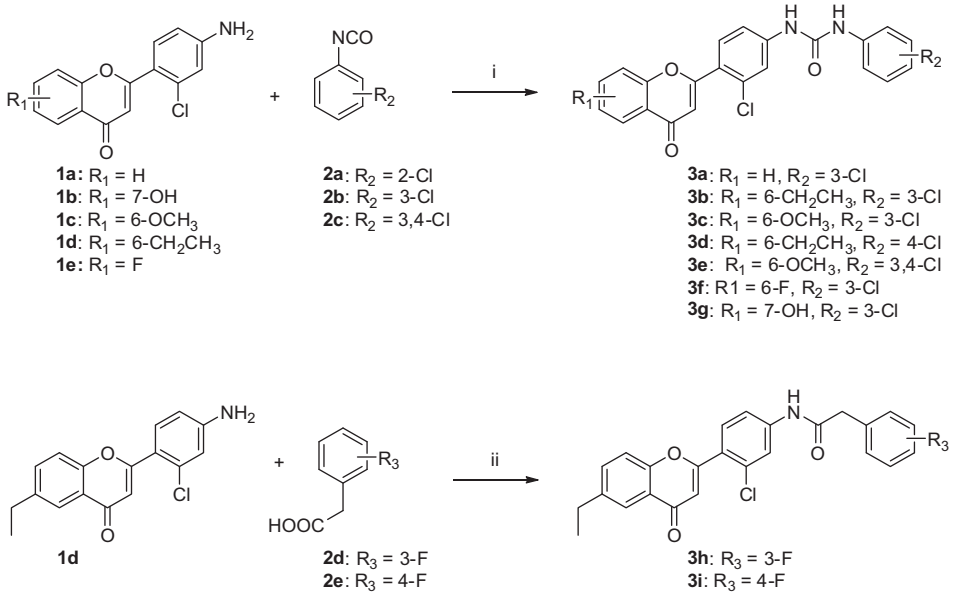
Furthermore, the influences of JNK inhibition upon the phosphorylation levels of c-Jun were studied. Treatment with 10 μM **3d** had no obvious effect on the phosphorylation of c-Jun, a downstream protein of JNK, which might due to low baseline phosphorylation.[42,43] When cells were stimulated by 1 mM MMS (methyl methane sulfonate), the phosphorylation level of c-Jun was strongly enhanced, a detectable inhibition effect was observed after co-treatment with 100 μM **3d** and 1 mM MMS for 2 h, shown in Figure 1(B), which further supported the biological activities of **3d** derived from its dual inhibition against Raf1 and JNK1.

2.4. In vitro cell cytotoxicity assay

In vitro cell cytotoxicity assay of compounds **3a**–**3i** was evaluated against HepG2 cells by MTT methods. As shown in Table 2,



Scheme 1. Scaffold-based drug design to produce 1-(3-chloro-4-(6-ethyl-4-oxo-4*H*-chromen- 2-yl)phenyl)-3-(4-chlorophenyl)urea (**3d**).



Scheme 2. Synthesis of compounds **3a**–**3i**. Reagents and conditions: (i) dry THF, DMAP, reflux; (ii) dry THF, DIC, DIPEA, HoBt, rt for 48 h.

Table 1
Inhibitory activity of **1b**, **1d** and **3d** at 50 μM against different kinases (inhibitory rate, %)

Kinases	1b	1d	3d	Staurosporine	Sorafenib	SB202190
Raf1	na[a]	27.04 ± 3.74	65.57 ± 4.65	nt[b]	101.36 ± 2.09	nt
JNK1	nt	nt	67.40 ± 2.36	95.99 ± 1.22	nt	nt
p38-alpha	na	14.47 ± 0.2[c]	13.51 ± 4.55	nt	nt	105.85 ± 3.21
ERK2	nt	nt	na	100.30 ± 9.53	nt	nt
ERK1	nt	nt	na	99.37 ± 9.35	nt	nt

[a] na: not activity.
[b] nt: not tested.
[c] Inhibitory rate at 100 μM.

compounds **3a**, **3d**, and **3e** exhibited good anti-proliferative activity with <10 μM IC_{50} values. **3d** had an IC_{50} of 8.3 μM, which was comparative with approved inhibitor sorafenib (5.0 μM, a reported IC_{50} was 6.0 μM[44]). By comparing the data, we obtained some preliminary structure–activity relationships (SAR) as follows: (1) when R_2 = 3-Cl, compounds **3b**, **3c** and **3f** with 6-substituted at A-ring of flavones had similar anti-proliferation ability, all three compounds had an IC_{50} at about 30 μM; (2) when R_2 = 3-Cl, 7-hydroxyl substituted **3g** had better activity than 6-substituted analogs **3b**, **3c** and **3f**; (3) when R_2 = 3-Cl, both electron-donating and electron-withdrawing substituents at A-ring of flavones decreased the activity, for example **3a**, an unsubstituted compound at A-ring, had stronger activity than substituted analogs **3b**, **3c**, **3f** and **3g**; (4) compounds with the substitution of R_2 = 4-Cl had better anti-tumor activity than compounds with the substitution of R_2 = 3-Cl, as demonstrated by the results of **3b** and **3d**; Also, R_2 = 3,4-Cl substituted compounds showed stronger inhibitory activity than R_2 = 3-Cl substituted compounds. For example, **3c** with IC_{50} at 26.3 μM, whereas **3e** containing chlorination at 3,4-positions displayed a better effect with IC_{50} at 9.9 μM; (5)

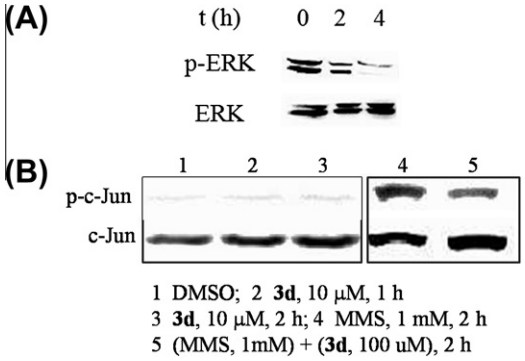


Figure 1. The effect of **3d** on the levels of phosphorylated ERK1/2 and c-Jun proteins. HepG2 cells were treated with indicated compound for specific times, then cell extracts were subjected to western blot analysis using specific antibodies.

Table 2
Anti-proliferative ability of synthetic compounds against HepG2, A549 and HCT116 cells

Compd	IC_{50}* (μM)			Compd	IC_{50} (μM)		
	HepG2	A549	HCT116		HepG2	A549	HCT116
3a	9.7	14.0	25.4	**3e**	9.9	15.6	8.6
3b	30.7	>50	27.9	**3f**	31.7	>50	24.2
3c	26.3	11.0	9.6	**3g**	12.2	16.8	25.9
3d	8.3	>50	28.7	**3h**	19.8	>50	>50
Sorafenib	5.0	4.5	2.3	**3i**	>50	>50	>50

* IC_{50} values were determined from MTT assays after incubation with test compounds for 48 h.

substitution of urea bond with amide bond had a negative effect on cytotoxicity, for example, IC_{50} of **3i** was found to be greater than 50 μM; (6) after substitution of urea bond with amide bond, 3-F substituted compound **3h** had better anti-tumor activity than 4-F substituted compound **3i**.

Furthermore, different activation status of JNK1 and Raf1 in different cell lines might result in different responses. Based on specific role of Raf and JNK in lung cancer and colon cancer,[45] we evaluated anti-proliferative ability of our synthesized compounds against A549 cells and HCT116 cells (Table 2). Excessive activity of Raf1 and JNK1 in HepG2 cells had been well studied, in which **3d** demonstrated the most effective inhibition with the IC_{50} value at 8.3 μM. Moreover, both ERK and JNK signal pathways were activated in about 50% human colon cancer,[46] which may be a major reason why most synthetic compounds had a similar IC_{50} between HCT116 cells and HepG2 cells; On the other hand, it had been reported that activation of JNK pathway was important for cell apoptosis and inhibition of cancer cell growth induced by synthetic compounds or some drugs in clinical trials in A549 cells.[47,48] Our synthetic compound **3d** or some other compounds had little effect on A549 cells (IC_{50} >50 μM), which may derived from its inhibition against JNK.

Drugs targeting cancer-specific pathways need to have minimal adverse effects against normal cells so as to achieve the desired efficacy and safety.[49] Herein, our synthesized compounds were tested against two normal human liver cell lines, QSG7701 and HL7702 cells, at two concentrations of 100 μM and 50 μM, respectively (Table 3). There were no over-expression of Raf1 and JNK1 in both normal cell lines, therefore, most of the compounds have a lower inhibition rate of <45% at 100 μM. In particular, compound **3d** had very weak effect on the two normal liver cell lines, with 32% and 28% inhibition rate for QSG7701 and HL7702 cells, respectively. In contrast, its precursor **1d** had relatively stronger cytotoxicity against both normal liver cell lines (>50% inhibition rate at 50 μM), which indicates that structural optimization of **1d** into **3d** not only substantially enhanced its Raf1 inhibitory activities but also significantly reduced its toxicity towards novel liver cells.

Table 3
Toxicity evaluation of synthetic compounds against QSG7701 cells and HL7702 cells (inhibitory rate%)

Compd	Inhibition rate* (50 μM)		Inhibition rate* (100 μM)	
	QSG7701	HL7702	QSG7701	HL7702
3a	48.9	26.8	62.4	47.1
3b	12.6	9.4	18.0	37.2
3c	15.9	35.7	30.1	40.6
3d	23.7	18.7	31.8	28.3
3e	13.2	66.4	42.5	77.9
3f	26.8	20.1	57.7	54.2
3g	34.6	80.2	43.2	83.8
3h	22.4	20.4	27.4	44.6
3i	16.2	29.1	36.9	45.2
Sorafenib	4.2	5.3	23.2	20.2

* Inhibition rate were determined from MTT assays after incubation with test compounds for 48 h.

A number of studies have suggested that multi-target drugs are often low-affinity binders against each individual targets, but low-affinity against individual targets not necessarily translate into low efficacy against disease models like cell-lines, and in some cases significantly higher efficacy than that of individual targets can be achieve by multi-target drugs due to synergistic actions against multiple components of relevant pathways.[6] Our compound **3d** also showed this behavior, its 8.3 μM IC_{50} value against HepG2 is significantly better than its 65.57% and 67.40% inhibition rate at 50 μM against Raf1 and JNK1. Moreover, compounds of low potency against individual targets might lead to lower toxicity than compounds of higher potency, which may partly explain the relatively lower toxicity of our synthesized compounds.

2.5. Impact of 3d on apoptosis and cell cycle in HepG2 cells

Some widely used JNK1 or Raf1 kinase inhibitor, for example, SP600125 had minimal effect on apoptosis alone,[42] and sorafenib was a poor apoptotic inducer in HCC cells.[50] Some other kinases inhibitors, for example, only highly potent Abl inhibitors were capable of inducing apoptosis, and selective PI3K inhibitors induced apoptosis only at high concentration and prolonged exposure time.[8] In order to further explore similar biological function of **3d** with classical inhibitors of JNK1 or Raf1 kinases, flow cytometry analysis were conducted to identify the effects of **3d** on apoptosis and cell cycle in HepG2 cells. As shown in Figure S1, a minimal apoptotic effect was detected after 10 μM **3d** treatment for varied time (12 h, 24 h, 36 h and 48 h, respectively) in HepG2 cells, which was consistent with classical Raf1 and JNK1 kinases inhibitors. Cell cycle arrest was observed at S phase with the proportion of about 6%, shown in Figure S2.

2.6. Molecular docking

To better understand the interaction of compound **3d** with its target kinases, molecular docking studies were conducted using the Discovery Studio 3.0/CDOCKER protocol. As shown in Figure 2A, specific interactions of **3d** in the Raf1 (PDB ID: 3OMV) model included: (1) two hydrogen bonds, one formed between C=O at B-ring of flavones and NH_2 of Lys431 (C=O···H–NH), which was considered to be an important site of action,[51] with the distance of 2.34 Å; the other formed between the same C=O and OH of Ser427 (C=O···H–O), with the distance of 2.11 Å; (2) three π–π interactions forming between the three rings at flavones backbone (A-ring, B-ring and C-ring) and Trp423; (3) a cation–π interaction existing between 4-chlorination benzene ring and an ammonium

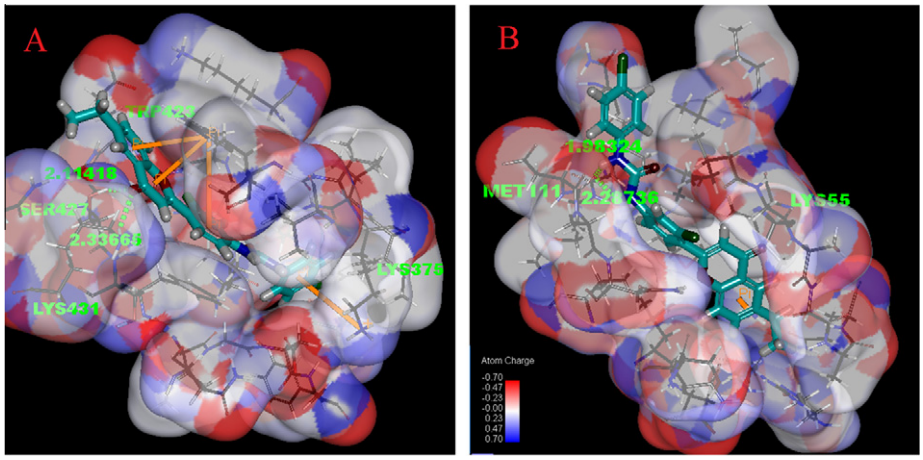


Figure 2. Molecular modeling of **3d** binding to target proteins. Molecules are colored by atom type and hydrogen bonds are represented by green dotted lines. White: hydrogen atom; red: oxygen atom; blue: nitrogen atom; green: chlorine atom; cyan: the backbone and carbon atom of **3d**. (A: Raf1, PDB ID: 3OMV; B: JNK1, PDB ID: 3ELJ).

cation of Lys375. Figure 2B shows two hydrogen bonds (NH···O) between **3d** and Met111 of JNK1 (PDB ID: 3ELJ) with the distance of 1.98 and 2.29 Å, respectively, which contains one of key action model.[52,53] One π–sigma interaction was also observed between A-ring of flavones and Lys55. Molecular docking analysis identified that **3d** could fit into the binding domain of Raf1 and JNK1, thus further supported that anti-tumor activity of **3d** derived from its dual inhibition of Raf1 and JNK1.

A strategy for enhancing the selectivity of kinase inhibitors against selected kinases is to explore distinguished features at binding sites adjacent to the ATP-binding site (non-ATP-competitive sites) where more diverse structural features can be explored, for example an approved inhibitor imatinib largely explores these sites for achieving selectivity against Abl.[54] In our study, we particularly explored some primary difference between JNK1 and p38-alpha MAPKs for preferential selectivity against JNK while maintaining sufficient interactions in Raf1. From the structural analysis of the binding of a highly selective JNK1 inhibitor SP600125 to JNK1, one finds that its selectivity is partly derived from the variations of the crucial hydrophobic residue by analysis of co-crystal structure of JNK1 and SP600125.[55] Our compound **3d** has a common hydrogen bond with SP600125 in MET111. In addition, **3d** had three π–π interactions with Raf1, similar three π–π interactions also could be observed in co-crystal of Raf1 and its inhibitor.[51]

3. Conclusion

Because of the promising potential of the scaffold of flavones to develop inhibitors of MAPKs and essential role of Raf1 and JNK1 in HCC development, a 6-ethyl-2′-chloro-4′-aminoflavone compound (**1d**) was tested to be low affinity inhibitors of Raf1 and p38-alfa. Based on this scaffold, a series of compounds merging 2′-chloro-4′-aminoflavones with diphenylurea were designed, synthesized and evaluated as Raf1, JNK1 and p38-alfa kinases inhibitors for anti-hepatocellular carcinoma treatment. One compound **3d** was found to be a dual inhibitor of Raf1 and JNK1 kinases with substantial selectivity against p38-alpha kinase, good anti-proliferative activity against HepG2 cell-line and inhibition rate against pERK1/2, and relatively lower toxicity against normal liver cell-lines QSG7701 and HL7702. Molecular docking studies showed its binding modes at target binding sites in Raf1 and JNK1, and structure–activity relationship further revealed the structural features in the substructures of flavones and phenylurea on anti-proliferative activity against HepG2 cell-line. Our study suggested that the 2′-chloro-4′-aminoflavones scaffold may be potentially explored for deriving multi-target Raf1 and JNK1 inhibitors with substantial selectivity against p38-alpha for the treatment of HCC at lower toxicity against normal liver cells.

4. Experimental

4.1. Chemistry

All starting materials **1a**–**1e** were from laboratory-made and stored in dryer. Tetrahydrofuran (THF) was dried according to the standard process. All other regents and analytical grade solvents commercial available were directly used without further purification. Thin Layer Chromatography (TLC) was used to monitor the progress of reactions. Melting points were measured with a SGW X-4 electrothermal melting apparatus, and data were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker spectrometer at 400 MHz for ^{1}H NMR and at 101 MHz for ^{13}C NMR, using TMS as an internal standard in $CDCl_3$ or in DMSO-d_6. Abbreviations: singlet (s), doublet (d), triplet (t), broad (br), quadruplet (q), multiplet (m). High-resolution mass spectra were recorded with Waters Q-Tof Premier mass spectrometer.

4.1.1. General procedure for synthesis of compounds 3a–3g

0.10 g 2′-chloro-4′-aminoflavones derivatives (**1a**–**1e**), 1.2 equiv 4-dimethylamio-pyridine (DMAP) and 1.1 equiv (**2a**–**2c**) was added to a 100 mL round-bottomed flask, the resulting mixture was refluxed in 25 mL dry THF. The reaction was monitored with TLC until raw materials were completely consumed. After that, THF was removed by rotating distillation. The crude solids were washed with deionized water and subsequently with ether to obtain pure products.

4.1.1.1. 1-(3-Chloro-4-(4-oxo-4*H*-chromen-2-yl)phenyl)-3-(3-chlorophenyl)urea (3a). Yield 70%, yellow solid, mp: 290–292 °C, HRMS (ESI) *m*/*z* calculated for $[M+H]^+$ 425.0460, found 425.0457. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.33 (s, 1H), 9.13 (s, 1H), 8.08 (dd, J = 7.9, 1.5 Hz, 1H), 7.91 (d, J = 2.0 Hz, 1H), 7.84 (ddd, J = 8.7, 7.2, 1.6 Hz, 1H), 7.76 (d, J = 8.6 Hz, 1H), 7.71 (dd, J = 6.8, 4.9 Hz, 2H), 7.57–7.46 (m, 2H), 7.38–7.26 (m, 2H), 7.11–7.01 (m, 1H), 6.64 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.23, 162.74, 156.49, 152.61, 143.60, 141.25, 134.99, 133.73, 132.58, 132.22, 130.95, 126.19, 125.39, 124.62, 123.68, 122.54, 119.51, 118.99, 118.54, 117.60, 117.42, 112.28.

4.1.1.2. 1-(3-Chloro-4-(6-ethyl-4-oxo-4*H*-chromen-2-yl)phenyl)-3-(3-chlorophenyl)urea (3b). Yield 67%, yellow solid, mp: 229–230 °C, HRMS (ESI) *m*/*z* calculated for $[M+H]^+$ 453.0773, found 453.0772. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.36 (s, 1H), 9.16 (s, 1H), 7.91 (d, *J* = 10.4 Hz, 2H), 7.74 (dt, *J* = 17.9, 8.8 Hz, 3H), 7.62 (d, *J* = 8.6 Hz, 1H), 7.52 (t, *J* = 12.1 Hz, 1H), 7.40–7.24 (m, 2H), 7.06 (d, *J* = 3.4 Hz, 1H), 6.63 (d, *J* = 12.9 Hz, 1H), 2.76 (q, *J* = 7.4 Hz, 2H), 1.24 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.24, 162.58, 154.93, 152.62, 143.56, 141.96, 141.27, 134.96, 133.73, 132.55, 132.16, 130.94, 129.39, 124.69, 123.39, 122.51, 119.49, 118.90, 118.52, 117.58, 117.40, 112.10, 28.07, 15.98.

4.1.1.3. 1-(3-Chloro-4-(6-methoxy-4-oxo-4*H*-chromen-2-yl)phenyl)-3-(3-chlorophenyl)urea (3c). Yield 67%, yellow solid, mp: 240–243 °C, HRMS (ESI) *m*/*z* calculated for $[M+H]^+$ 455.0565, found 455.0562. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.33 (s, 1H), 9.13 (s, 1H), 7.91 (d, *J* = 1.8 Hz, 1H), 7.81–7.69 (m, 2H), 7.67 (d, *J* = 8.8 Hz, 1H), 7.56–7.40 (m, 3H), 7.40–7.26 (m, 2H), 7.12–7.00 (m, 1H), 6.62 (s, 1H), 3.88 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.34, 161.76, 156.61, 151.97, 150.58, 142.90, 140.64, 133.13, 131.93, 131.49, 130.29, 124.01, 123.76, 123.29, 121.88, 119.93, 118.88, 117.89, 116.93, 116.75, 110.79, 104.69, 55.66.

4.1.1.4. 1-(3-Chloro-4-(6-ethyl-4-oxo-4*H*-chromen-2-yl)phenyl)-3-(4-chlorophenyl)urea (3d). Yield 75%, yellow solid, mp: 258–259 °C, HRMS (ESI) *m*/*z* calculated for $[M+H]^+$ 453.0773, found 453.0767. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.29 (s, 1H), 9.06 (s, 1H), 7.90 (dd, *J* = 6.2, 1.8 Hz, 2H), 7.78–7.68 (m, 2H), 7.62 (d, *J* = 8.6 Hz, 1H), 7.50 (dd, *J* = 12.4, 5.4 Hz, 3H), 7.36 (d, *J* = 8.8 Hz, 2H), 6.61 (s, 1H), 2.76 (q, *J* = 7.5 Hz, 2H), 1.24 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.25, 162.60, 154.93, 152.63, 143.65, 141.97, 138.68, 134.97, 132.54, 132.17, 129.18, 126.48, 124.59, 123.46, 123.39, 120.69, 119.40, 118.91, 117.32, 112.09, 28.06, 15.98.

4.1.1.5. 1-(3-Chloro-4-(6-methoxy-4-oxo-4*H*-chromen-2-yl)phenyl)-3-(3,4-dichlorophenyl)urea (3e). Yield 54%, yellow solid, mp: 256–258 °C, HRMS (ESI) *m*/*z* calculated for $[M+H]^+$ 489.0176, found 489.0190. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.37 (s, 1H), 9.23 (s, 1H), 7.89 (s, 2H), 7.75 (d, *J* = 8.8 Hz, 1H), 7.66 (d, *J* = 8.9 Hz, 1H), 7.53 (dd, *J* = 17.5, 8.9 Hz, 2H), 7.44 (d, *J* = 9.5 Hz, 2H), 7.38 (d, *J* = 8.9 Hz, 1H), 6.62 (s, 1H), 3.88 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.96, 162.41, 157.26, 152.55, 151.21, 143.39, 139.95, 132.53, 132.17, 131.59, 131.13, 124.82, 124.39, 124.26, 123.96, 120.59, 120.28, 119.61, 119.29, 117.50, 111.45, 105.33, 56.31.

4.1.1.6. 1-(3-Chloro-4-(6-fluoro-4-oxo-4*H*-chromen-2-yl)phenyl)-3-(3-chlorophenyl)urea (3f). Yield 40%, yellow solid, mp >300 °C, HRMS (ESI) *m*/*z* calculated for $[M-H]^-$ 441.0208, found 441.0217. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.34 (s, 1H), 9.14 (s, 1H), 7.92 (d, *J* = 2.0 Hz, 1H), 7.86–7.81 (m, 1H), 7.79–7.70 (m, 4H), 7.51 (dd, *J* = 8.6, 2.0 Hz, 1H), 7.37–7.30 (m, 2H), 7.07 (dt, *J* = 4.9, 2.2 Hz, 1H), 6.69 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.00, 162.40, 152.32, 151.99, 143.10, 140.62, 133.13, 132.00, 131.68, 130.36, 124.25 (*J* = 7.1 Hz), 123.78, 122.60, 122.35, 121.96, 121.25 (*J* = 9.1 Hz), 118.90, 117.93, 117.00, 116.80, 110.96, 109.43 (*J* = 24.2 Hz).

4.1.1.7. 1-(3-Chloro-4-(7-hydroxy-4-oxo-4*H*-chromen-2-yl)phenyl)-3-(3-chlorophenyl)urea (3g). Yields 45%, yellow solid, mp >300 °C, HRMS (ESI) *m*/*z* calculated for $[M+H]^+$ 441.0409, found 441.0406. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.87 (s, 1H), 9.64 (s, 1H), 7.92 (dd, *J* = 17.9, 9.1 Hz, 2H), 7.73 (d, *J* = 8.6 Hz, 2H), 7.48 (dd, *J* = 8.6, 2.0 Hz, 2H), 7.32 (d, *J* = 4.9 Hz, 2H), 7.09–7.01 (m, 1H), 6.96 (dd, *J* = 8.7, 2.2 Hz, 1H), 6.91 (d, *J* = 2.1 Hz, 1H), 6.49 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 175.90, 162.83, 161.40, 157.67, 152.14, 142.90, 140.77, 133.13, 131.90, 131.54, 130.36, 126.51, 124.04, 121.77, 118.61, 117.62, 116.69, 116.55, 115.84, 115.15, 111.32, 102.33.

4.1.3. Synthesis of compounds 3h and 3i

0.33 mmol (0.1 g) 6-ethyl-2′-chloro-4′-aminoflavone **1d**, equimolar (0.051 g) 3-fluoro-phenylacetic acid **2d**, 63 μL *N*,*N*′-diisopropylcarbodiimide (DIC), 146 μL di-isopropylethylamine (DIEA), 0.40 mmol (0.054 g) 1-hydroxybenzotriazole (HoBt) were added to a 50 mL round-bottomed flask. The resulting mixture was stirred in 15 mL dry THF at room temperature for 48 h. The solvents were removed in vacuo, flash chromatography of the residue on silica gel (CH_2Cl_2/CH_3OH = 40:1, v/v) gave *N*-(3-chloro-4-(6-ethyl-4-oxo-4*H*-chromen-2-yl)phenyl)-2-(3-fluorophenyl)acetamide (**3h**) was obtained as a yellow solid, yield 45%, mp: 181–182 °C, HRMS (ESI) *m*/*z* calculated for $[M+H]^+$ 436.1116, found 436.1125. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (s, 1H), 8.04 (d, *J* = 2.0 Hz, 1H), 7.81 (d, *J* = 1.2 Hz, 1H), 7.62–7.51 (m, 3H), 7.44 (d, *J* = 8.6 Hz, 1H), 7.33 (td, *J* = 7.9, 6.1 Hz, 1H), 7.13 (d, *J* = 7.7 Hz, 1H), 7.08 (dd, *J* = 9.5, 2.0 Hz, 1H), 7.01 (td, *J* = 8.4, 2.1 Hz, 1H), 6.65 (d, *J* = 6.2 Hz, 1H), 3.78 (s, 2H), 2.77 (q, *J* = 7.6 Hz, 2H), 1.29 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.60, 168.96, 163.09 (*J* = 248.5 Hz), 162.34, 155.08, 141.84, 140.94, 136.40 (*J* = 7.07 Hz), 134.31, 133.50, 131.10, 130.66 (*J* = 9. Hz), 127.24, 125.06 (*J* = 2.0 Hz), 123.77, 123.49, 121.42, 118.14, 117.95, 116.46 (*J* = 22.2 Hz), 114.72 (*J* = 21.2 Hz), 112.42, 44.30, 28.41, 15.47.

N-(3-Chloro-4-(6-ethyl-4-oxo-4*H*-chromen-2-yl)phenyl)-2-(4-fluorophenyl)acetamide **3i**, as a light yellow solid from the same synthetic process with **3h**, yield 65%, mp: 237–238 °C, HRMS (ESI) *m*/*z* calculated for $[M+H]^+$ 436.1116, found 436.1116. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.64 (s, 1H), 8.01 (d, *J* = 1.9 Hz, 1H), 7.88 (d, *J* = 1.8 Hz, 1H), 7.77 (d, *J* = 8.5 Hz, 1H), 7.73–7.55 (m, 3H), 7.37 (dd, *J* = 8.5, 5.7 Hz, 2H), 7.16 (t, *J* = 8.9 Hz, 2H), 6.60 (s, 1H), 3.71 (s, 2H), 2.75 (q, *J* = 7.5 Hz, 2H), 1.23 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.21, 170.38, 162.37, 161.72 (*J* = 243.4 Hz), 154.91, 142.88, 141.99, 134.99, 132.42, 132.21, 132.00 (*J* = 3.0 Hz), 131.61 (*J* = 8.1 Hz), 126.02, 123.44, 123.38, 120.43, 118.90, 118.14, 115.55 (*J* = 21.2 Hz), 112.25, 42.77, 28.05, 15.96.

4.2. Molecular docking

The molecular modeling of **3d** was performed with Discovery Studio.3.0/CDOCK protocol (*Accelrys Software Inc.*) according to the reported process.[56,57] Three dimensional structures of Raf-1 (PDB ID: 3OMV) and JNK1 (PDB ID: 3ELJ) were downloaded from Protein Data Bank (PDB). The following process was used to carry out molecular docking: (1) deleting the water crystallization involved in protein kinase structure; (2) optimizing protein structure and ligands; (3) defining receptor and ligand, finding the candidate binding site; (4) deleting small molecular docking in candidate binding site; (5) docking designed compounds into the candidate binding site on the target protein kinase; (6) molecular modeling based on the above docking data.

4.3. Biological assays

4.3.1. Cell culture

All human cell lines, including HepG2, HCT116, A549, QSG7701 and HL7702 cells were obtained from Cell Resources Center of Shanghai Institutes for Biological Science, Chinese Academy of Science. They were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 μg ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

4.3.2. Cell growth inhibition assay

The synthesized compounds were dissolved in DMSO, and then diluted with culture medium to the final concentrations ranging from 0.5 to 50 μM (the final DMSO concentration was less than 1%) for tumor cells assay, 50 and 100 μM for normal liver cells assay. 100 μL of cell solution with the concentration of 5×10^5 cells mL^{-1} was seeded to each well of a 96-well plate and incubated for 24 h at 37 °C in a 5% CO_2 incubator. The medium was then removed from the 96-well plate. The test compound solution was added to each well for the treatment of maintained cells in triplicate per concentration, and incubated at 37 °C in a 5% CO_2 incubator for 48 h. After this treatment, 10 μL MTT solution (5 mg mL^{-1}) was then added to each well and incubated for 4 h at 37 °C. The formazan precipitate was dissolved in 100 μL DMSO and the absorbance at 495 nm was determined using Multimode Detector DTX880 (Beckman Coulter).

4.3.3. In vitro kinase assays

In vitro kinase assays were carried out by HD Biosciences Co., Ltd in Shanghai, China. Kinases inhibitory activities of synthetic compounds against JNK1, p38-alpha, ERK1, ERK2 and Raf1 were evaluated using kinase glo plus. The general procedure for glo plus assay was as follows: mix enzyme, substrate, ATP and compounds in a buffer solution (pH 7.4) of 25 mM HEPES, 10 mM $MgCl_2$, 0.01% Triton X-100, 100 μg/mL BSA, 2.5 mM DTT in a 384-well assay plate, total volume is 10 μL. The assay plate was incubated at 30 °C for 1 h, then the reaction was stopped by the addition of equal volume of kinase glo plus reagent. The luminescence was read at envision. The signal was correlated with the amount of ATP remaining in the reaction and was inversely correlated with the kinase activity.

4.4. Western blot analysis

After treatment with indicated compound for specific time, HepG2 cells were centrifuged and treated with lysis buffer(10 mM HEPES, pH 7.9; 10 mM KCl; 1 mM EDTA; 0.1% NP-40; 0.5 mM Na_3VO_4; 1 mM NaF; and protease inhibitor cocktail (Thermo scientific)) on ice for 50 min, then centrifuged at 15,000 rpm for 15 min. Protein concentration was determined using coomassie brilliant blue according to manufacturer's manual. Cell lysate proteins were subjected to 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), and electrophoretically transferred to PVDF membrane (amc Biobind NT-200). After blotting, the membrane was blocked in 5% skim milk (TBS, 0.5 mM Na_3VO_4; 1 mM NaF) for 2 h, and incubated with the specific primary antibody (ERK1/2, Beyotime, the product No. AM076; p-ERK1/2, Cell Signaling Technology, the product No. #9910; c-Jun, Bioworld, the product No. BS1060; p-c-Jun, Bioworld, the product No. BS4045; ATF-2, Bioworld, the product No. BS1024; p-ATF-2, the procudt No. BS4018) for 2 h at room temperature. Protein bands were detected using the Super Signal West Pico Chemiluminescent Substrate (Thermo scientific) after hybridization with the HRP-conjugated secondary antibody.

Acknowledgements

The authors would like to thank the financial support provided by the Ministry of Science and Technology of China (2012ZX09506001-010, 2012CB722605, 2012AA020305 and 2011DFA30620) and the Science Industry Trade and Information Technology Commission of Shenzhen Municipality (JC201005280602A).

Supplementary data

Supplementary data (cell apoptosis and cell cycle arrest in HepG2 cells induced by 10 μM **3d**, anti-proliferative curve of **3d** against HepG2 and HCT116 cells, and the effect of **3d** on the phosphorylation level of ATF-2. ^1H NMR and ^{13}C NMR spectra and high resolution mass spectrometry) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.04.006.

References and notes

1. Ramurthy, S.; Costales, A.; Jansen, J. M.; Levine, B.; Renhowe, P. A.; Shafer, C. M.; Subramanian, S. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 1678.
2. Castro, A. F.; Campos, T.; Babcock, J. T.; Armijo, M. E.; Martinez-Conde, A.; Pincheira, R.; Quilliam, L. A. *J. Cell Biochem.* **2011**, *113*, 1253.
3. Molina, J. R.; Adjei, A. A. *J. Thorac. Oncol.* **2006**, *1*, 7.
4. Golemis, E. A.; Khazak, V.; Astsaturov, I.; Serebriiskii, I. G. *Expert Opin. Ther. Targets* **2007**, *11*, 1587.
5. Park, W. H. *Mol. Med. Rep.* **2011**, *4*, 193.
6. Korcsmaros, T.; Szalay, M. S.; Csaba, B.; Kovacs, I. A.; Csermely, P. *Expert Opin. Drug Discov.* **2007**, *2*, 799.
7. Petrelli, A.; Valabrega, G. *Expert Opin. Pharmacother.* **2009**, *10*, 589.
8. Zhang, C. L.; Tan, C. Y.; Zu, X. Y.; Zhai, X.; Liu, F.; Chu, B. Z.; Ma, X. H.; Chen, Y. Z.; Gong, P.; Jiang, Y. Y. *Eur. J. Med. Chem.* **2011**, *46*, 1404.
9. Wei, G.; Wang, M.; Carr, B. I. *J. Cell Physiol.* **2010**, *224*, 112.
10. Whittaker, S.; Marais, R.; Zhu, A. X. *Oncogene* **2010**, *29*, 4989.
11. He, S. N.; Krens, S. F. G.; Zhan, H. Q.; Gong, Z. Y.; Hogendoorn, P. C. W.; Spaink, H. P.; Snaar-Jagalska, B. E. *J. Pathol.* **2011**, *225*, 19.
12. Chang, Q. S.; Zhang, Y. D.; Beezhold, K. J.; Bhatia, D.; Zhao, H. W.; Chen, J. G.; Castranova, V.; Shi, X. L.; Chen, F. *J. Hepatol.* **2009**, *50*, 323.
13. Chen, F.; Castranova, V. *Cell Cycle* **2009**, *8*, 1145.
14. Hui, L. J.; Zatloukal, K.; Scheuch, H.; Stepniak, E.; Wagner, E. F. *J. Clin. Invest.* **2008**, *118*, 3943.
15. Aderca, I.; Moser, C. D.; Veerasamy, M.; Bani-Hani, A. H.; Bonilla-Guerrero, R.; Ahmed, K.; Shire, A.; Cazanave, S. C.; Montoya, D. P.; Mettler, T. A.; Burgart, L. J.; Nagorney, D. M.; Thibodeau, S. N.; Cunningham, J. M.; Lai, J. P.; Roberts, L. R. *J. Hepatol.* **2008**, *49*, 373.
16. Adler, V.; Qu, Y. X.; Smith, S. J.; Izotova, L.; Pestka, S.; Kung, H. F.; Lin, M.; Friedman, F. K.; Chie, L.; Chung, D.; Boutjdir, M.; Pincus, M. R. *Biochemistry* **2005**, *44*, 10784.
17. Hui, L. J.; Bakiri, L.; Mairhorfer, A.; Schweifer, N.; Haslinger, C.; Kenner, L.; Komnenovic, V.; Scheuch, H.; Beug, H.; Wagner, E. F. *Nat. Genet.* **2007**, *39*, 741.
18. Chen, F.; Beezhold, K.; Castranova, V. *BBA-Rev. Cancer* **2009**, *1796*, 242.
19. Min, L. H.; He, B. K.; Hui, L. J. *Semin. Cancer Biol.* **2011**, *21*, 10.
20. Hou, D. X.; Kumamoto, T. *Antioxid. Redox. Signal.* **2010**, *13*, 691.
21. Teillet, F.; Boumendjel, A.; Boutonnat, J.; Ronot, X. *Med. Res. Rev.* **2008**, *28*, 715.
22. Verma, A. K.; Pratap, R. *Nat. Prod. Rep.* **2010**, *27*, 1571.
23. Kandaswami, C.; Lee, L. T.; Lee, P. P.; Hwang, J. J.; Ke, F. C.; Huang, Y. T.; Lee, M. T. *In Vivo* **2005**, *19*, 895.
24. Kang, S. R.; Il Park, K.; Park, H. S.; Lee, D. H.; Kim, J. A.; Nagappan, A.; Kim, E. H.; Lee, W. S.; Shin, S. C.; Park, M. K.; Han, D. Y.; Kim, G. S. *Food Chem.* **2011**, *129*, 1721.
25. Lee, K. W.; Kang, N. J.; Heo, Y. S.; Rogozin, E. A.; Pugliese, A.; Hwang, M. K.; Bowden, G. T.; Bode, A. M.; Lee, H. J.; Dong, Z. *Cancer Res.* **2008**, *68*, 946.
26. Goettert, M.; Schattel, V.; Koch, P.; Merfort, I.; Laufer, S. *Chembiochem* **2010**, *11*, 2579.
27. Llovet, J. M.; Ricci, S.; Mazzaferro, V.; Hilgard, P.; Gane, E.; Blanc, J. F.; de Oliveira, A. C.; Santoro, A.; Raoul, J. L.; Forner, A.; Schwartz, M.; Porta, C.; Zeuzem, S.; Bolondi, L.; Greten, T. F.; Galle, P. R.; Seitz, J. F.; Borbath, I.; Haussinger, D.; Giannaris, T.; Shan, M.; Moscovici, M.; Voliotis, D.; Bruix, J. *N. Engl. J. Med.* **2008**, *359*, 378.
28. Song, E. Y.; Kaur, N.; Park, M. Y.; Jin, Y.; Lee, K.; Kim, G.; Lee, K. Y.; Yang, J. S.; Shin, J. H.; Nam, K. Y.; No, K. T.; Han, G. *Eur. J. Med. Chem.* **2008**, *43*, 1519.
29. Alam, M.; Beevers, R. E.; Ceska, T.; Davenport, R. J.; Dickson, K. M.; Fortunato, M.; Gowers, L.; Haughan, A. F.; James, L. A.; Jones, M. W.; Kinsella, N.; Lowe, C.; Meissner, J. W. G.; Nicolas, A. L.; Perry, B. G.; Phillips, D. J.; Pitt, W. R.; Platt, A.; Ratcliffe, A. J.; Sharpe, A.; Tait, L. J. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3463.
30. Cushman, M.; Zhu, H.; Geahlen, R. L.; Kraker, A. J. *J. Med. Chem.* **1994**, *37*, 3353.
31. Cushman, M.; Nagarathnam, D.; Burg, D. L.; Geahlen, R. L. *J. Med. Chem.* **1991**, *34*, 798.
32. Morphy, R.; Rankovic, Z. *J. Med. Chem.* **2005**, *48*, 6523.
33. Zhang, C.; Bollag, G. *Curr. Opin. Genet. Dev.* **2010**, *20*, 79.
34. Bennett, B. L.; Sasaki, D. T.; Murray, B. W.; O'Leary, E. C.; Sakata, S. T.; Xu, W. M.; Leisten, J. C.; Motiwala, A.; Pierce, S.; Satoh, Y.; Bhagwat, S. S.; Manning, A. M.; Anderson, D. W. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 13681.
35. Ohori, M.; Kinoshita, T.; Okubo, M.; Sato, K.; Yamazaki, A.; Arakawa, H.; Nishimura, S.; Inamura, N.; Nakajima, H.; Neya, M.; Miyake, H.; Fujii, T. *Biochem. Biophys. Res. Commun.* **2005**, *336*, 357.
36. Xia, W. L.; Mullin, R. J.; Keith, B. R.; Liu, L. H.; Ma, H.; Rusnak, D. W.; Owens, G.; Alligood, K. J.; Spector, N. L. *Oncogene* **2002**, *21*, 6255.
37. Gailhouste, L.; Ezan, F.; Bessard, A.; Fremin, C.; Rageul, J.; Langouet, S.; Baffet, G. *Int. J. Cancer* **2010**, *126*, 1367.
38. Lee, H. C.; Tian, B.; Sedivy, J. M.; Wands, J. R.; Kim, M. *Gastroenterology* **2006**, *131*, 1208.
39. Li, Q. B.; Wu, J. D.; Zheng, H.; Liu, K.; Guo, T. L.; Liu, Y. Y.; Eblen, S. T.; Grant, S.; Zhang, S. J. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4526.

40. Ramurthy, S.; Aikawa, M.; Amiri, P.; Costales, A.; Hashash, A.; Jansen, J. M.; Lin, S.; Ma, S.; Renhowe, P. A.; Shafer, C. M.; Subramanian, S.; Sung, L.; Verhagen, J. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3286.
41. Lipton, S. A. *Nature* **2004**, *428*, 473.
42. Kuntzen, C.; Sonuc, N.; De Toni, E. N.; Opelz, C.; Mucha, S. R.; Gerbes, A. L.; Eichhorst, S. T. *Cancer Res.* **2005**, *65*, 6780.
43. Bae, M. A.; Song, B. J. *Mol. Pharmacol.* **2003**, *63*, 401.
44. Ou, D.-L.; Shen, Y.-C.; Yu, S.-L.; Chen, K.-F.; Yeh, P.-Y.; Fan, H.-H.; Feng, W.-C.; Wang, C.-T.; Lin, L.-i.; Hsu, C.; Cheng, A.-L. *Cancer Res.* **2010**, *70*, 9309.
45. Roberts, P. J.; Der, C. J. *Oncogene* **2007**, *26*, 3291.
46. Licato, L. L.; Brenner, D. A. *Dig. Dis. Sci.* **1998**, *43*, 1454.
47. Lee, C.-H.; Yao, C.-F.; Huang, S.-M.; Ko, S.; Tan, Y.-H.; Lee-Chen, G.-J.; Wang, Y.-C. *Cancer* **2008**, *113*, 815.
48. Yang, Y.; Ikezoe, T.; Saito, T.; Kobayashi, M.; Koeffler, H. P.; Taguchi, H. *Cancer Sci.* **2004**, *95*, 176.
49. Wilhelm, S.; Carter, C.; Lynch, M.; Lowinger, T.; Dumas, J.; Smith, R. A.; Schwartz, B.; Simantov, R.; Kelley, S. *Nat. Rev. Drug Disc.* **2006**, *5*, 835.
50. Fernando, J.; Sancho, P.; Fernandez-Rodriguez, C. M.; Lledo, J. L.; Caja, L.; Campbell, J. S.; Fausto, N.; Fabregat, I. *J. Cell. Physiol.* **2012**, *227*, 1319.
51. Hatzivassiliou, G.; Song, K.; Yen, I.; Brandhuber, B. J.; Anderson, D. J.; Alvarado, R.; Ludlam, M. J. C.; Stokoe, D.; Gloor, S. L.; Vigers, G.; Morales, T.; Aliagas, I.; Liu, B.; Sideris, S.; Hoeflich, K. P.; Jaiswal, B. S.; Seshagiri, S.; Koeppen, H.; Belvin, M.; Friedman, L. S.; Malek, S. *Nature* **2010**, *464*, 431.
52. Chamberlain, S. D.; Redman, A. M.; Wilson, J. W.; Deanda, F.; Shotwell, J. B.; Gerding, R.; Lei, H. S.; Yang, B.; Stevens, K. L.; Hassell, A. M.; Shewchuk, L. M.; Leesnitzer, M. A.; Smith, J. L.; Sabbatini, P.; Atkins, C.; Groy, A.; Rowand, J. L.; Kumar, R.; Mook, R. A.; Moorthy, G.; Patnaik, S. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 360.
53. Lee, J. Y.; Jeong, K. W.; Heo, Y. S.; Kim, Y. B. *Korean Chem. Soc.* **2010**, *31*, 2147.
54. Kirkland, L. O.; McInnes, C. *Biochem. Pharmacol.* **2009**, *77*, 1561.
55. Heo, Y.-S.; Kim, S.-K.; Seo, C. I.; Kim, Y. K.; Sung, B.-J.; Lee, H. S.; Lee, J. I.; Park, S.-Y.; Kim, J. H.; Hwang, K. Y.; Hyun, Y.-L.; Jeon, Y. H.; Ro, S.; Cho, J. M.; Lee, T. G.; Yang, C.-H. *EMBO J.* **2004**, *23*, 2185.
56. Jiang, Y. Y.; Luan, X. D.; Gao, C. M.; Zhang, N. N.; Chen, Y. Z.; Sun, Q. S.; Tan, C. Y.; Liu, H. X.; Jin, Y. B. *Bioorg. Med. Chem.* **2011**, *19*, 3312.
57. Li, Y. Q.; Tan, C. Y.; Gao, C. M.; Zhang, C. L.; Luan, X. D.; Chen, X. W.; Liu, H. X.; Chen, Y. Z.; Jiang, Y. Y. *Bioorg. Med. Chem.* **2011**, *19*, 4529.