

Cite this: *RSC Adv.*, 2017, 7, 48031

Synthesis, cytotoxic activity and drug combination study of tertiary amine derivatives of 2',4'-dihydroxyl-6'-methoxyl-3',5'-dimethylchalcone†

Chen Wang,^{ac} Ping Wu,^a Xiao-Ling Shen,^d Xiao-Yi Wei^{*a} and Zi-Hua Jiang^{id} ^{*b}

In an effort to develop more water soluble anticancer drugs based on 2',4'-dihydroxyl-6'-methoxyl-3',5'-dimethylchalcone (DMC), a group of DMC derivatives bearing polar and ionizable tertiary amine functionalities were synthesized. Cell based MTT assay resulted in the discovery of one compound (2b) which shows a broad spectrum of cytotoxic activity ($IC_{50} < 5 \mu M$ against all tested sensitive cancer cells) and moderate selectivity between normal and tumor cells. Further drug combination study uncovered that all of the semi-synthetic DMC derivatives (2a–2f) acted synergistically with anticancer drug Taxol® to achieve improved anti-proliferative efficacy against drug resistant HeLa/Tax cells. The combinations of Taxol® with each of the two most effective compounds (2b and 2d) displayed weighted average Combination Index (CI) values of 0.14 and 0.15, respectively, and significantly reduced the dosage of Taxol® while maintaining the same level of efficacy. Our findings suggested that the introduction of a polar and ionizable tertiary amine functionality into the 4'-OH of DMC is a feasible way to improve its aqueous solubility, anticancer activity, and cancer selectivity.

Received 4th August 2017
Accepted 5th October 2017

DOI: 10.1039/c7ra08639c

rsc.li/rsc-advances

1. Introduction

Chalcones constitute an important class of plant-derived natural products with a wide range of biological and pharmacological properties. Several review articles comprehensively summarized their anticancer, anti-infection, anti-inflammatory, antioxidant, and anti-hyperglycemic activities as well as their identified molecular targets.^{1–9} A number of chalcones have been approved for clinical use, including the choleretic drug metochalcone and the gastrointestinal medication sofalcone (Fig. 1).⁸ Structurally, chalcones carry an α,β -unsaturated carbonyl system, which is further conjugated with two aromatic rings at both end (Fig. 1). As a member of this huge family, 2',4'-dihydroxyl-6'-methoxyl-3',5'-dimethylchalcone (DMC, Fig. 1), featuring a 3',5'-dimethyl substituted ring A, was originally isolated from the leaves of *Myrica gale* in 1977 (ref. 10) and later found in several other

species including *Syzygium samarangense*,¹¹ *Psoralea polydenius*,¹² and *Cleistocalyx operculatus*.¹³ It is abundantly accumulated in the buds of *Cleistocalyx operculatus* which has been used for preparing tonic drinks in southern China for centuries.

The pharmacological profile of this C-methylated chalcone has been extensively studied.^{14–22} Ye *et al.* disclosed that DMC not only effectively inhibited the growth of six human cancer cell lines at micromolar concentration in cell based assay,¹⁴ but also significantly suppressed the tumorous development of solid human carcinoma in xenograft model.¹⁵ More interestingly, Qian *et al.* discovered that DMC was able to potentiate the cytotoxicity of doxorubicin against drug resistant KB-A1 cells both *in vitro* and *in vivo* by increasing the intracellular accumulation of doxorubicin and modulating the expression of *MDR1* gene which encodes the transporter P-glycoprotein.¹⁶ Similar multidrug resistance (MDR) reversal effect of DMC was also observed by Huang *et al.* in a human hepatocellular carcinoma BEL-7402/5-FU cell line.^{17,18} This intriguing pharmacological profile of DMC makes it a promising anticancer drug lead in a world crying for alternative drugs to treat patients who are suffering from multidrug resistant cancers.

However, DMC is barely soluble in aqueous solution due to its lipophilic nature. This physicochemical shortcoming severely hampers its development for clinical use. Earlier studies have shown that the incorporation of polar and ionizable functional group(s) into the structure of a lead compound is a feasible approach to solve this troublesome issue.²³ In a pool of suitable ionizable groups, tertiary amine functionality appears to be a superior choice by virtue of its excellent ability to

^aKey Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, Chinese Academy of Sciences, Xingke Road 723, Tianhe District, Guangzhou 510650, People's Republic of China. E-mail: wxy@scbg.ac.cn; Tel: +86-20-37252538

^bDepartment of Chemistry, Lakehead University, 955 Oliver Road, Thunder Bay, Ontario P7B 5E1, Canada. E-mail: zjiang@lakeheadu.ca; Tel: +1-807-766-7171

^cUniversity of Chinese Academy of Sciences, Yuquanlu 19A, Beijing 100049, People's Republic of China

^dLaboratory of Herbal Drug Discovery, Tropical Medicine Institute, Guangzhou University of Chinese Medicine, Guangzhou 510405, People's Republic of China

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7ra08639c

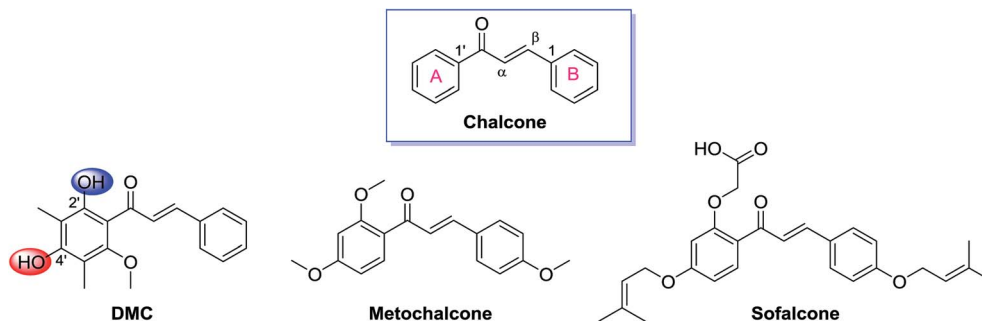


Fig. 1 Structures of chalcone, DMC, and two clinically approved drugs, metochalcone and sofalcone.

ionize, the easiness of introducing an aminoalkyl group onto the phenolic hydroxyl of DMC, and the fact that installation of heterologous atom containing side chain usually benefits biological activities of the lead compound. By following these designing logic, we synthesized a group of DMC derivatives bearing various polar and ionizable tertiary amine groups using DMC as the starting material. The cytotoxicity of these semi-synthetics was investigated against five cell lines (A549, HepG2, MCF-7, HeLa, and Vero), and a drug combination study was conducted to evaluate the synergistic effect of these compounds with Taxol® against a multi-drug resistant cancer cell line (HeLa/Tax). To the best of our knowledge, this is the first chemical modification study of DMC aiming to improve its aqueous solubility and anticancer property.

2. Results and discussion

2.1. Synthesis and structural characterization

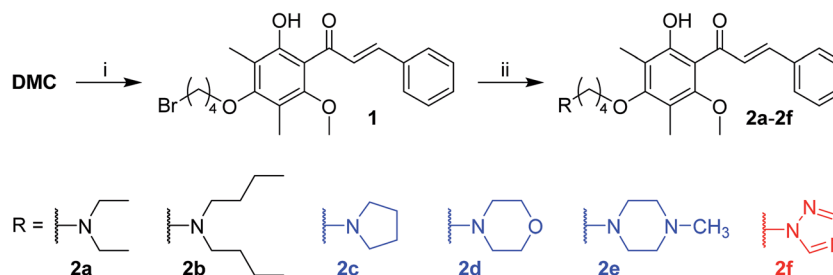
DMC possesses two free aromatic hydroxyl groups in the ring A (Fig. 1). The one attached to C-2' is unfavorable for chemical modification because it is involved in an intra-molecular hydrogen bond with the adjacent carbonyl group and its reactivity is reduced. The reactivity of 4'-OH is much higher and could act as an effective nucleophile in S_N2 reactions in the presence of a relatively weak base. Herein, we linked the nitrogen atom of different secondary amines, which represent non-cyclic, cyclic, and aromatic subgroups, with the 4'-OH of DMC via a four carbon bridge (Scheme 1). The starting material DMC was isolated from *Cleistocalyx operculatus* as previously described (see the Experimental section, 4.1 General, for more

details) and treated with 1,4-dibromobutane in the presence of K_2CO_3 in acetone under reflux to give bromobutyl-substituted **1** in 59% yield. The following coupling of **1** with different secondary amines was conducted in chloroform using K_2CO_3 as base, providing corresponding tertiary amine derivatives **2a–2f** in good yield. All the synthesized compounds were purified by silica gel CC and/or preparative HPLC and their structures were confirmed by comprehensive analysis of their HR-ESIMS data, 1H -NMR and ^{13}C -NMR spectral data.

2.2. Biological evaluation

2.2.1. Cytotoxicity. The cytotoxicity of these synthetic compounds were evaluated against four sensitive human cancer cell lines, including human lung carcinoma (A549), human hepatocellular carcinoma (HepG2), human breast carcinoma (MCF-7), and human cervical carcinoma (HeLa), one Taxol®-resistant HeLa cell subline HeLa/Tax, and one normal cell line Vero by standard MTT assay.²⁴ These cancer cell lines represent some of the most common types of cancer with high rate of occurrence. DMC as well as the anticancer drug doxorubicin and Taxol® were used as references, and activities were expressed as the concentration of drug inhibiting 50% cell growth (IC_{50}). The IC_{50} values of all semisynthetic DMC derivatives (**1** and **2a–2f**) were listed in Table 1.

In general, all tertiary amine derivatives (**2a–2f**) exhibited good activity against all tested cancer cell lines with IC_{50} values ranging from 1.3 to 24.7 μM . Compounds **2a**, **2e**, and **2f** displayed higher activity against HeLa cell line ($IC_{50} < 8 \mu M$) while **2b–2d** were more potent against MCF-7 cells. Among these DMC



Scheme 1 Reagents and conditions: (i) 1,4-dibromobutane, acetone, K_2CO_3 , reflux, 10 h, 59%; (ii) secondary amines, K_2CO_3 , $CHCl_3$, reflux, 40–88%.



Table 1 Cytotoxicity (IC₅₀, μM)^a of **1** and **2a–2f**

Compound	Cell lines					
	A549	HepG2	MCF-7	HeLa	HeLa/Tax	Vero
1	7.6 ± 0.76	8.5 ± 0.32	5.2 ± 0.12	10.9 ± 0.10	n.t. ^b	4.1 ± 0.81
2a	10.3 ± 0.21	14.3 ± 0.07	9.6 ± 0.68	7.4 ± 0.27	13.5 ± 0.004	11.7 ± 1.19
2b	3.8 ± 0.55	4.3 ± 0.57	1.3 ± 0.16	2.3 ± 0.08	7.9 ± 0.63	14.9 ± 0.20
2c	14.3 ± 0.64	17.9 ± 0.21	6.5 ± 0.96	17.6 ± 0.88	19.0 ± 0.57	25.6 ± 4.15
2d	24.7 ± 3.94	19.1 ± 0.44	15.4 ± 1.99	20.2 ± 1.45	22.0 ± 1.18	11.2 ± 0.75
2e	13.0 ± 0.89	10.8 ± 1.09	11.3 ± 1.39	4.9 ± 0.23	12.8 ± 0.71	5.6 ± 0.88
2f	9.4 ± 1.34	11.8 ± 0.43	15.1 ± 0.14	6.1 ± 0.61	7.8 ± 0.16	12.3 ± 0.12
DMC	2.3 ± 0.44	8.3 ± 0.22	15.1 ± 0.72	11.8 ± 0.42	15.3 ± 0.19	20.3 ± 4.08
Doxorubicin	0.7 ± 0.06	1.2 ± 0.02	1.0 ± 0.17	0.5 ± 0.05	n.t. ^b	0.4 ± 0.01
Taxol®	n.t. ^b	n.t. ^b	n.t. ^b	0.2 ± 0.01 ^c	0.5 ± 0.002	n.t. ^b

^a Values represent means ± SD based on three individual experiments. ^b n.t. not tested. ^c Values expressed in nM.

derivatives, **2b** is the most active compound, having IC₅₀ values all below 5 μM against tested sensitive cancer cell lines and specifically an IC₅₀ of 1.3 μM against MCF-7 cells. **2b** is 11.6 times and 5.1 times more potent than DMC against MCF-7 cells and HeLa cells, respectively, and its efficacy on MCF-7 cells is comparable to the clinically approved anticancer drug doxorubicin. **2a**, **2c**, **2e**, and **2f** also showed potency enhancement against certain cancer cell line when compared to DMC (**2c**: 6.5 μM vs. DMC: 15.1 μM against MCF-7 cells; **2a**: 7.4 μM, **2e**: 4.9 μM and **2f**: 6.1 μM vs. DMC: 11.8 μM against HeLa cells). Additionally, the bromobutyl-substituted synthetic intermediate (**1**) exhibited a broad range of cytotoxic activity as well (Table 1).

Several studies have shown that DMC is able to reverse cancer cell multidrug resistance by suppressing the expression of *MDR1* gene.^{16–18} In this study we included a Taxol®-resistant HeLa cell subline, HeLa/Tax, to show whether our DMC derivatives are active against multidrug-resistant cancer cells. The HeLa/Tax cells were established by continuous exposing HeLa cells to Taxol® at a gradient concentration, which became resistant to Taxol® and other chemodrugs (see Experimental for more details). Most of the synthesized compounds that are cytotoxic to HeLa cells also display cytotoxicity to the Taxol®-resistant HeLa/Tax cells, albeit at somewhat reduced potency (Table 1). Compounds **2b** and **2f** have IC₅₀ below 10 μM, indicating that they are quite potent against Taxol®-resistant HeLa/Tax cells. Whether these DMC derivatives are able to reverse multidrug resistance requires further studies (see also the Drug combination study in section 2.2.2 below).

In order to find out whether these DMC derivatives are toxic to normal cells, their anti-proliferative activity against monkey kidney epithelial cells (Vero) were evaluated, and the results were shown in Table 1. Although the tertiary amine derivatives showed considerable growth inhibitory activity to Vero cells, some of them, such as **2b** and **2c**, were found to be less toxic to normal cells than to certain type of cancer cells (**2b**: 1.3 μM to MCF-7 cells vs. 14.9 μM to Vero cells; **2c**: 6.5 μM to MCF-7 cells vs. 25.6 μM to Vero cells), and hence these compounds have moderate selectivity towards cancer cells over normal cells.

A preliminary structure–activity relationship (SAR) analysis of these DMC derivatives indicates that, for DMC derivatives

carrying non-cyclic tertiary amine groups, longer aliphatic chains on the nitrogen atom is superior to shorter ones in improving cytotoxicity (**2b** is 7 times more potent than **2a** against MCF-7 cells and 3 times more potent than **2a** against A549, HepG2, and HeLa cells). In the case of cyclic tertiary amine substituents, compound **2d** with morpholine moiety is less active than those with *N*-methyl piperazine and pyrrolidine (**2e** and **2c**), which might be related to the reduced basicity of the nitrogen atom in morpholine. Compound **2f** with a 1,2,4-triazole moiety displays higher potency than **2c** (with a pyrrolidine moiety) against most cell lines except MCF-7. In fact, **2f** is the most potent compound against the Taxol®-resistant HeLa/Tax cells. These observations indicate that multiple nitrogen atoms introduced in DMC are tolerated and may benefit the anticancer effect of such derivatives.

DMC is known to promote apoptosis in human breast adenocarcinoma MCF-7 cells by activating poly(adenosine diphosphate-ribose) polymerase (PARP),²⁵ which is one of the best biomarker of apoptosis. Ye *et al.* also reported that DMC was able to induce apoptosis in human hepatocarcinoma SMMC-7721 cells, as indicated by the fragmentation and condensation of chromatin in the cells and the increase on the percentage of hypodiploid cells showed by flowcytometric analysis.¹⁴ Furthermore, Zhu *et al.* demonstrated that DMC inhibited tumor growth of human hepatocarcinoma Bel7402 and lung cancer GLC-82 xenografts in nude mice, and their study showed that DMC inhibited vascular endothelial growth factor receptor (kinase-inserting domain-containing receptor, KDR) tyrosine kinase and shut down KDR-mediated signal transduction.²² The detailed anticancer mechanism of our DMC derivatives needs further investigation.

2.2.2. Drug combination study. Drug combinations have long been used for the treatment of human diseases ever since the ancient times, as exemplified by the Traditional Chinese Medicine, in which multiple herbal medicines are used simultaneously. Over the past century, extensive research has been focused on this subject in order to find new ways of treating the most dreadful diseases like cancer^{26–28} and AIDS.^{29–31} The ultimate goal of applying drug combination is to achieve synergism, which will result in beneficial therapeutic effects such as



(1) enhancing drug efficacy, (2) decreasing dosage to reduce toxicity while increasing or maintaining the efficacy, and (3) minimizing or circumventing drug resistance.³² The accurate definition and determination method for drug synergism have been discussed and argued for quite a long time.^{32–34}

The term Combination Index (CI) was introduced by Chou and Talaly in 1980s to quantitatively describe drug combination effect as synergism ($CI < 1$), additive effect ($CI = 1$), or antagonism ($CI > 1$).^{35,36} A corresponding computer software for dose-effect analysis, CI calculation, and Fa–CI plot simulation was developed by Joseph Chou in 1989, and its second (CalcuSyn, 1997)³⁷ and third (CompuSyn, 2005)³⁸ generations were written by Mike Hayball and Nick Martin, respectively. In this study, a drug combination assay using Chou–Talalay method was carried out to investigate whether the synthesized DMC derivatives could work synergistically with Taxol®. For specific drug combination assay, the IC_{50} values of Taxol® and DMC derivatives against HeLa/Tax cells (Table 1) were used as a guide to choose individual drug concentration.

Each drug combo was prepared at a constant ratio of $(IC_{50})_1/(IC_{50})_2$ so that each drug contributes equally to the cytotoxic effect.^{32,36} This was followed by serial duplicate dilutions to afford six gradient concentrations. The Taxol®-resistant HeLa/Tax cells were treated with Taxol® alone, each DMC derivative alone, and the corresponding combination for 72 h. CI was calculated by the CompuSyn software using the general equation: $CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2}$, in which the denominator $(Dx)_1$ and $(Dx)_2$ are the concentration of Drug 1 and Drug 2 alone that inhibit the growth of cells by $x\%$. Similarly, the numerator $(D)_1$ and $(D)_2$ represent the respective dosage of Drug 1 and Drug 2 at which their drug combo inhibits the cell growth by the same $x\%$. The CI values of each drug combo at 50%, 75% and 90% growth inhibition (GI_{50} , GI_{75} , and GI_{90}) were listed in Table 2.

The concept of dose-reduction index (DRI) was originally introduced by Chou JH and Chou TC in 1988 (ref. 39) to describe how many-folds the dose of each drug was reduced at certain effect levels in the drug combo compared with each drug alone.³² The equation for calculating DRI is $(DRI)_1 = \frac{(Dx)_1}{(D)_1}$ and $(DRI)_2 = \frac{(Dx)_2}{(D)_2}$, which is a simple inversion of CI equation, and

DRI could be generated by CompuSyn software. The DRI values of Taxol® in each drug combo at 50%, 75%, and 90% cell growth inhibition (GI_{50} , GI_{75} and GI_{90}) were calculated and listed in Table 2.

All tested DMC derivatives display synergistic effects with Taxol® against HeLa/Tax cells, having the weighted average CI (CI_{avg}) values all below 0.5. The combinations of Taxol®/2b and Taxol®/2d exhibit the strongest synergy at different levels of growth inhibition (GI_{50} , GI_{75} , and GI_{90} , Table 2), which result in CI_{avg} of 0.14 and 0.15, respectively. Compounds 2b and 2d reduced the dosage of Taxol® by more than 15-folds at high effect level (with DRI value >15 at GI_{90} , Table 2). Compounds 2a, and 2c are also able to work synergistically with Taxol® ($CI_{avg} = 0.22$, and 0.29, respectively) and markedly enhance the potency of Taxol® with DRI values around 10 at GI_{50} , GI_{75} and GI_{90} effect levels. The other two derivatives 2e and 2f are not as effective, but their drug combos with Taxol® still show stronger synergistic effect than the Taxol®/DMC combination.

The combination index (CI) for each drug combo usually has a different value at a different effect level. A plot of CI values at different effect levels (Fa, fraction affected, i.e., % growth inhibition in this case) can be determined by computer simulation. Such a combination index plot is also called the Fa–CI plot.³⁶ As examples, the Fa–CI plots for drug combos of Taxol® with DMC, 2b or 2d were simulated by CompuSyn and presented in Fig. 2. The plots clearly show the trend that the CI value decreases as the effect level (Fa) increases, indicating that a higher level of synergism is observed at a higher effect level for a drug combo.

Structurally speaking, compound 2b with a di-butylamino substituent displays the highest synergistic effect with Taxol® among all the DMC derivatives tested. Compound 2b alone also shows the highest cytotoxicity against all cancer cells tested (Table 1). In contrast, compound 2d with a morpholine residue as the substituent exhibits a similar level of synergism with Taxol® as 2b does but the lowest cytotoxicity against all cancer cells tested including HeLa/Tax cells. Furthermore, compound 2f (with a triazole-moiety) is most cytotoxic against HeLa/Tax cells among all DMC derivatives but shows the lowest synergistic effect in combination with Taxol®. These observations indicate that the level of synergism for each drug combo does not correlate with the cytotoxic potency of individual DMC

Table 2 Combination index (CI)^a and dose-reduction index (DRI)^b of 2a–2f

Comp.	Drug ratio Comp./Taxol® ^c	CI at				DRI of Taxol® at			
		GI_{50}	GI_{75}	GI_{90}	CI_{avg} ^a	GI_{50}	GI_{75}	GI_{90}	
2a	30 : 1	0.33 ± 0.01	0.24 ± 0.01	0.18 ± 0.001	0.22	9.36 ± 2.4	11.61 ± 0.21	14.84 ± 3.30	
2b	14 : 1	0.26 ± 0.03	0.15 ± 0.01	0.10 ± 0.005	0.14	8.60 ± 1.97	11.31 ± 0.66	15.09 ± 1.72	
2c	40 : 1	0.36 ± 0.04	0.30 ± 0.009	0.25 ± 0.02	0.29	10.49 ± 1.48	10.74 ± 0.52	11.20 ± 2.65	
2d	40 : 1	0.28 ± 0.02	0.17 ± 0.007	0.10 ± 0.02	0.15	8.26 ± 0.95	11.95 ± 1.09	17.64 ± 5.20	
2e	30 : 1	0.45 ± 0.06	0.39 ± 0.05	0.34 ± 0.03	0.38	7.41 ± 0.98	8.03 ± 0.41	8.86 ± 2.07	
2f	14 : 1	0.60 ± 0.02	0.47 ± 0.007	0.41 ± 0.007	0.46	3.39 ± 0.08	6.29 ± 0.32	11.68 ± 1.44	
DMC	30 : 1	0.67 ± 0.05	0.55 ± 0.03	0.45 ± 0.02	0.52	4.40 ± 0.49	5.29 ± 0.31	6.38 ± 0.03	

^a CI values of each compounds are presented at 50%, 75%, and 90% of growth inhibition (GI_{50} , GI_{75} , and GI_{90}). $CI_{avg} = (CI_{50} + 2CI_{75} + 3CI_{90})/6$, is the weighted average CI, and $CI < 1$, $CI = 1$, and $CI > 1$ represents synergism, additive effect, and antagonism, respectively. ^b DRI value of Taxol® in each combination assay is presented at GI_{50} , GI_{75} , and GI_{90} effect levels. ^c Each drug combination was prepared at equipotency ratio [$(IC_{50})_1/(IC_{50})_2$ ratio].



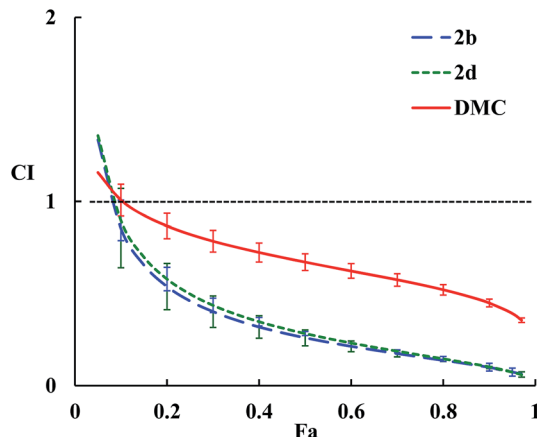


Fig. 2 Fa–CI plots for drug combos of Taxol® with **2b**, **2d**, or DMC. The dashed horizontal line expresses CI = 1, and error bars show standard deviations.

derivative. These data confirm the earlier findings that drug synergism is a complicated issue.³² The potential of these DMC derivatives to reverse multidrug resistance in HeLa/Tax cells might be a contributing factor for the strong synergism observed between Taxol® and individual DMC derivative.

2.3. Solubility

The solubility of DMC and selected synthetic derivatives in pure water with 0.1% formic acid (pH = 2.9) were determined by quantitative ultra-performance liquid chromatography (UPLC) analysis. The tested DMC derivatives bearing tertiary amine functional groups (**2a** and **2e**) showed good solubility in acidic aqueous solution (2.27 and 1.04 $\mu\text{mol mL}^{-1}$, respectively) while DMC is barely soluble (0.004 $\mu\text{mol mL}^{-1}$). On the other hand, compound **2f** (with a triazole moiety) shows a much lower solubility in the same medium (0.04 $\mu\text{mol mL}^{-1}$), which correlates with the reduced basicity of the nitrogen atoms in the aromatic triazole ring.

3. Conclusion

In summary, a group of DMC derivatives bearing different tertiary amine functionalities were readily synthesized. Their cytotoxicity and drug–drug interaction effects with Taxol® were investigated using cell based assays. All semi-synthetic DMC derivatives exhibited moderate to strong cytotoxicity against tested cell lines, with **2b** being the most active compound which improved the potency of DMC by about 2-folds against HepG2 cells, 11-folds against MCF-7 cells, and 5-folds against HeLa cells. Compound **2b** also showed considerable selectivity toward cancer cells over normal cells.

The drug combination study uncovered that all the cytotoxic derivatives displayed moderate to strong drug synergism with Taxol®. The most cytotoxic derivative **2b** also possesses the strongest synergistic effect with Taxol® with a combination index (CI) of 0.10 and a dose-reduction index (DRI) of 15.09 for Taxol® at 90% growth inhibition (GI₉₀). Solubility study

suggested that the tertiary amine derivatives were more soluble than DMC in pure water containing 0.1% formic acid (pH = 2.9). Overall, the study shows that the introduction of a tertiary amine functional group into the 4'-OH of DMC can improve the aqueous solubility, anticancer activity, and cancer selectivity of DMC. These findings may shed lights on the rational design of other chalcone or flavonoid derivatives aiming to improve their biological activities and physicochemical properties.

4. Experimental

4.1. General

All reagents and solvents were purchased from commercial sources, and further purification and drying by standard methods were employed when necessary. ¹H and ¹³C NMR spectra were collected on a Bruker AVIII 500M instrument at 500 (¹H) and 125 (¹³C) MHz or a Bruker Avance-600 instrument at 600 (¹H) and 150 (¹³C) MHz. Chemical shifts are reported as ppm (δ units) using the residual solvent peak as reference and the coupling constants as *J* in hertz. The splitting pattern abbreviations are as follows: s = singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet, brs = broad singlet. ESIMS data were obtained on an MDS SCIEX API 2000 LC/MS instrument. HRESIMS data were obtained on a Bruker maXis Q-TOF mass spectrometer. Preparative HPLC was run on a Waters 600 pump and a Waters 2487 dual λ absorbance detector. All separations were carried out with an SHIMADZU Shim-Pack Pro-ODS C₁₈ column (20 mm \times 25 cm) at a flow rate of 5 mL min⁻¹. Reactions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10% H₂SO₄ in EtOH.

Our previous phytochemical study on the buds of *Cleistocalyx operculatus* demonstrated the isolation and structural characterization of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (DMC), 5,7-dihydroxy-6,8-dimethylflavanone, 7-hydroxy-5-methoxy-6,8-dimethylflavanone, ethyl gallate, gallic acid, ursolic acid, β -sitosterol, and cinnamic acid.⁴⁰ Similar process was applied herein to afford adequate amount of DMC for chemical modification. Briefly, 5 kg fresh buds of *C. operculatus* were soaked in 95% aqueous ethanol for 24 h. The ethanol solution was collected after filtration and then concentrated under vacuum to afford the crude extract. The crude extract was re-suspended in water and consecutively extracted with diethyl ether, ethyl acetate, and *n*-pentane (5 times each). The ethyl acetate extract was concentrated to provide a paste 13 g which was subjected to silica gel column chromatography eluted with gradient solution of chloroform–methanol (100 : 0, 99 : 1, 92 : 2, and 97 : 3, v/v). Orange precipitates were formed and collected from the 100 : 0 chloroform–methanol eluted fraction and then washed by methanol to afford DMC (0.69 g) in good purity.

4.2. Preparation of (*E*)-1-(4-(2-bromoethoxy)-2-hydroxy-6-methoxy-3,5-dimethylphenyl)-3-phenylprop-2-en-1-one (**1**)

1,4-Dibromobutane (1.45 g, 6.72 mmol) was added to a mixture of DMC (500 mg, 1.68 mmol) and anhydrous K₂CO₃ (927 mg,



6.72 mmol) in dry acetone (150 mL) and stirred under reflux for 6 h. After removing the solvent under reduced pressure, the residue was dissolved in EtOAc and sequentially washed with 1% aqueous HCl solution, distilled water and dried over anhydrous Na₂SO₄. The crude product was purified by silica gel column chromatography eluting with petroleum ether/acetone (v/v, from 99 : 1 to 85 : 15) to afford compound **1** as an orange waxy solid; 59% yield; ¹H NMR (500 MHz, CDCl₃) δ: 13.11 (s, 1H, 2'-OH), 7.97 (d, *J* = 15.7 Hz, 1H, H-β), 7.86 (d, *J* = 15.7 Hz, 1H, H-α), 7.64 (m, 2H, H-2, H-6), 7.41 (m, 3H, H-3, H-4, H-5), 3.84 (t, *J* = 6.2 Hz, 2H, H-1''), 3.66 (s, 3H, 6'-OCH₃), 3.52 (t, *J* = 6.6 Hz, 2H, H-4''), 2.16 and 2.15 (s, each 3H, 3'-CH₃ and 5'-CH₃), 2.13 (m, 2H, H-2''), 1.98 (m, 2H, H-3''); ¹³C NMR (125 MHz, CDCl₃) δ: 194.10 (CO), 162.65 (C-6'), 161.74 (C-4'), 158.79 (C-2'), 143.51 (C-β), 135.29 (C-1), 130.48 (C-4), 129.06 (C-3, C-5), 128.60 (C-2, C-6), 126.63 (C-α), 115.78 and 115.74 (C-3' and C-5'), 111.91 (C-1'), 71.87 (C-1''), 62.41 (6'-OCH₃), 33.57 (C-4''), 29.61 and 29.07 (C-2'' and C-3''), 9.19 and 9.06 (3'-CH₃ and 5'-CH₃); HRESIMS *m/z*: 455.0844 [M + Na]⁺ (calcd for C₂₂H₂₅BrNaO₄, 455.0828).

4.3. General procedure for the synthesis of compounds 2a–2f

Different secondary amine (0.48 mmol) was added into a mixture of compound **1** (50 mg, 0.12 mmol) and anhydrous K₂CO₃ (0.48 mmol) in CHCl₃ (20 mL) and stirred under reflux for 10 h. The reaction mixture was washed with distilled water three times and dried over anhydrous Na₂SO₄. The crude products were subjected to preparative HPLC using CH₃OH/H₂O (v/v, from 50 : 50 to 100 : 0, linear gradient, 60 min) as eluents to afford compounds **2a–2f**.

4.3.1. (E)-1-(4-((Dimethylamino)butoxy)-2-hydroxy-6-methoxy-3,5-dimethylphenyl)-3-phenylprop-2-en-1-one (2a). Orange solid; *t_R* = 28 min; 65% yield; ¹H NMR (500 MHz, CDCl₃) δ: 7.95 (d, *J* = 15.7 Hz, 1H, H-β), 7.85 (d, *J* = 15.7 Hz, 1H, H-α), 7.63 (m, 2H, H-2, H-6), 7.40 (m, 3H, H-3, H-4, H-5), 3.82 (t, *J* = 5.9 Hz, 2H, H-1''), 3.65 (s, 3H, 6'-OCH₃), 3.18 (q, 4H, H-5'' and H-7''), 3.13 (m, 2H, H-4''), 2.14 and 2.12 (s, each 3H, 3'-CH₃ and 5'-CH₃), 1.99 (m, 2H, H-2''), 1.88 (m, 2H, H-3''), 1.33 (t, *J* = 7.2 Hz, 6H, H-6'' and H-8''); ¹³C NMR (125 MHz, CDCl₃) δ: 194.17 (CO), 162.29 (C-6'), 161.68 (C-4'), 158.86 (C-2'), 143.68 (C-β), 135.24 (C-1), 130.56 (C-4), 129.09 (C-3, C-5), 128.63 (C-2, C-6), 126.55 (C-α), 115.69 and 115.60 (C-3' and C-5'), 112.06 (C-1'), 71.74 (C-1''), 62.46 (6'-OCH₃), 51.09 (C-4''), 46.13 (C-5'' and C-7''), 27.65 (C-2''), 20.70 (C-3''), 9.24 and 9.10 (3'-CH₃ and 5'-CH₃), 8.53 (C-6'' and C-8''); HRESIMS *m/z*: 426.2645 [M + H]⁺ (calcd for C₂₆H₃₆NO₄, 426.2639).

4.3.2. (E)-1-(4-((Dibutylamino)butoxy)-2-hydroxy-6-methoxy-3,5-dimethylphenyl)-3-phenylprop-2-en-1-one (2b). Orange solid; *t_R* = 35 min; 43% yield; ¹H NMR (500 MHz, CDCl₃) δ: 13.11 (s, 1H, 2'-OH), 7.97 (d, *J* = 15.7 Hz, 1H, H-β), 7.86 (d, *J* = 15.7 Hz, 1H, H-α), 7.64 (m, 2H, H-2, H-6), 7.41 (m, 3H, H-3, H-4, H-5), 3.81 (t, *J* = 6.5 Hz, 2H, H-1''), 3.66 (s, 3H, 6'-OCH₃), 2.48 (m, 2H, H-4''), 2.42 (m, 4H, H-5'' and H-9''), 2.17 and 2.15 (s, each 3H, 3'-CH₃ and 5'-CH₃), 1.82 (m, 2H, H-2''), 1.66 (m, 2H, H-3''), 1.43 (m, 4H, H-6'' and H-10''), 1.31 (m, 4H, H-7'' and H-11''), 0.92 (t, *J* = 7.3 Hz, 6H, H-8'' and H-12''); ¹³C NMR (125 MHz, CDCl₃) δ: 194.16 (CO),

163.02 (C-6'), 161.77 (C-4'), 158.78 (C-2'), 143.45 (C-β), 135.36 (C-1), 130.48 (C-4), 129.08 (C-3, C-5), 128.63 (C-2, C-6), 126.73 (C-α), 115.97 and 115.87 (C-3' and C-5'), 111.84 (C-1'), 73.08 (C-1''), 62.42 (6'-OCH₃), 54.06 (C-4''), C-5'' and C-9''), 29.37 (C-6'' and C-10''), 28.48 (C-2''), 23.87 (C-3''), 20.91 (C-7'' and C-11''), 14.25 (C-8'' and C-12''), 9.23 and 9.11 (3'-CH₃ and 5'-CH₃); HRESIMS *m/z*: 482.3274 [M + H]⁺ (calcd for C₃₀H₄₄NO₄, 482.3265).

4.3.3. (E)-1-(2-Hydroxy-6-methoxy-3,5-dimethyl-4-(4-(pyrrolidin-1-yl)butoxy)phenyl)-3-phenylprop-2-en-1-one (2c). Orange solid; *t_R* = 31 min; 79% yield; ¹H NMR (500 MHz, CDCl₃) δ: 13.07 (s, 1H, 2'-OH), 7.96 (d, *J* = 15.7 Hz, 1H, H-β), 7.86 (d, *J* = 15.7 Hz, 1H, H-α), 7.64 (m, 2H, H-2, H-6), 7.41 (m, 3H, H-3, H-4, H-5), 3.82 (t, *J* = 6.0 Hz, 2H, H-1''), 3.65 (s, 3H, 6'-OCH₃), 3.23 (br s, 4H, H-5'' and H-8''), 3.07 (m, 2H, H-4''), 2.14 and 2.13 (s, each 3H, 3'-CH₃ and 5'-CH₃), 2.17 (m, 6H, H-2'', H-6'', and H-7''), 1.88 (m, 2H, H-3''); ¹³C NMR (125 MHz, CDCl₃) δ: 194.20 (CO), 162.41 (C-6'), 161.73 (C-4'), 158.87 (C-2'), 143.65 (C-β), 135.31 (C-1), 130.56 (C-4), 129.11 (C-3, C-5), 128.67 (C-2, C-6), 126.63 (C-α), 115.76 and 115.70 (C-3' and C-5'), 112.06 (C-1'), 71.96 (C-1''), 62.49 (6'-OCH₃), 55.18 (C-4''), 53.19 (C-5'' and C-8''), 27.82 (C-2''), 23.44 (C-2'' and C-3''), 23.10 (C-3''), 9.28 and 9.14 (3'-CH₃ and 5'-CH₃); HRESIMS *m/z*: 424.2485 [M + H]⁺ (calcd for C₂₆H₃₄NO₄, 424.2482).

4.3.4. (E)-1-(2-hydroxy-6-methoxy-3,5-dimethyl-4-(4-morpholinobutoxy)phenyl)-3-phenylprop-2-en-1-one (2d). Orange solid; *t_R* = 25 min; 72% yield; ¹H NMR (500 MHz, CDCl₃) δ: 13.10 (s, 1H, 2'-OH), 7.97 (d, *J* = 15.7 Hz, 1H, H-β), 7.86 (d, *J* = 15.7 Hz, 1H, H-α), 7.64 (m, 2H, H-2, H-6), 7.41 (m, 3H, H-3, H-4, H-5), 3.82 (t, *J* = 6.3 Hz, 2H, H-1''), 3.75 (m, 4H, H-6'' and H-7''), 3.66 (s, 3H, 6'-OCH₃), 2.49 (m, 6H, H-4'', H-5'', and H-8''), 2.16 and 2.15 (s, each 3H, 3'-CH₃ and 5'-CH₃), 1.86 (m, 2H, H-2''), 1.75 (m, 2H, H-3''); ¹³C NMR (125 MHz, CDCl₃) δ: 194.16 (CO), 162.89 (C-6'), 161.78 (C-4'), 158.81 (C-2'), 143.53 (C-β), 135.36 (C-1), 130.52 (C-4), 129.10 (C-3, C-5), 128.65 (C-2, C-6), 126.70 (C-α), 115.90 and 115.84 (C-3' and C-5'), 111.90 (C-1'), 72.79 (C-1''), 67.05 (C-6'' and C-7''), 62.46 (6'-OCH₃), 58.90 (C-4''), 53.86 (C-5'' and C-8''), 28.36 (C-2''), 23.26 (C-3''), 9.24 and 9.12 (3'-CH₃ and 5'-CH₃); HRESIMS *m/z*: 440.2449 [M + H]⁺ (calcd for C₂₆H₃₄NO₅, 440.2431).

4.3.5. (E)-1-(2-Hydroxy-6-methoxy-3,5-dimethyl-4-(4-(4-methylpiperazin-1-yl)butoxy)phenyl)-3-phenylprop-2-en-1-one (2e). Orange solid; *t_R* = 28 min; 88% yield; ¹H NMR (500 MHz, CDCl₃) δ: 7.95 (d, *J* = 15.7 Hz, 1H, H-β), 7.84 (d, *J* = 15.7 Hz, 1H, H-α), 7.63 (m, 2H, H-2, H-6), 7.39 (m, 3H, H-3, H-4, H-5), 3.79 (t, *J* = 5.9 Hz, 2H, H-1''), 3.64 (s, 3H, 6'-OCH₃), 2.90 and 2.81 (br s, each 4H, H-5'', H-6'', H-7'' and H-8''), 2.60 (m, 2H, H-4''), 2.52 (s, 3H, -NCH₃), 2.14 and 2.12 (s, each 3H, 3'-CH₃ and 5'-CH₃), 1.81 (m, 4H, H-2'' and H-3''); ¹³C NMR (125 MHz, CDCl₃) δ: 194.13 (CO), 162.65 (C-6'), 161.70 (C-4'), 158.79 (C-2'), 143.56 (C-β), 135.27 (C-1), 130.51 (C-4), 129.07 (C-3, C-5), 128.61 (C-2, C-6), 126.60 (C-α), 115.80 and 115.71 (C-3' and C-5'), 111.91 (C-1'), 72.40 (C-1''), 62.42 (6'-OCH₃), 57.42 (C-4''), 53.18 and 50.38 (C-5'', C-6'', C-7'', and C-8''), 44.21 (-NCH₃), 28.07 (C-2''), 22.80 (C-3''), 9.21 and 9.09 (3'-CH₃ and 5'-CH₃); HRESIMS *m/z*: 453.2756 [M + H]⁺ (calcd for C₂₇H₃₇N₂O₄, 453.2748).

4.3.6. (E)-1-(4-(4-(1H-1,2,4-Triazol-1-yl)butoxy)-2-hydroxy-6-methoxy-3,5-dimethylphenyl)-3-phenylprop-2-en-1-one (2f). Orange solid; *t_R* = 38 min; 40% yield; ¹H NMR (600 MHz, CDCl₃) δ: 13.08 (s, 1H, 2'-OH), 8.11 and 7.97 (br s, each 1H, H-5'' and H-



6''), 7.96 (d, $J = 15.7$ Hz, 1H, H- β), 7.86 (d, $J = 15.7$ Hz, 1H, H- α), 7.64 (m, 2H, H-2, H-6), 7.41 (m, 3H, H-3, H-4, H-5), 4.31 (t, $J = 7.0$ Hz, 2H, H-4''), 3.81 (t, $J = 6.1$ Hz, 2H, H-1''), 3.65 (s, 3H, 6'-OCH₃), 2.19 (m, 2H, H-2''), 2.14 and 2.13 (s, each 3H, 3'-CH₃ and 5'-CH₃), 1.82 (m, 2H, H-3''); ¹³C NMR (150 MHz, CDCl₃) δ : 194.15 (CO), 162.44 (C-6'), 161.73 (C-4'), 158.83 (C-2'), 152.23 (C-5''), 143.63 (C- β and C-6''), 135.29 (C-1), 130.54 (C-4), 129.09 (C-3, C-5), 128.64 (C-2, C-6), 126.60 (C- α), 115.73 (C-3' and C-5'), 112.04 (C-1'), 71.98 (C-1''), 62.47 (6'-OCH₃), 49.62 (C-4''), 27.33 (C-2''), 27.05 (C-3''), 9.20 and 9.08 (3'-CH₃ and 5'-CH₃); HRESIMS m/z : 422.2073 [M + H]⁺ (calcd for C₂₄H₂₈N₃O₄, 422.2074).

4.4. Cytotoxic activity

4.4.1. Cell culture. A549, HepG2, HeLa, MCF-7, and Vero cell lines were purchased from Kunming cell bank, Chinese Academy of Sciences (Kunming, the People's Republic of China). MCF-7 and Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco) as well as 1% (v/v) penicillin and streptomycin. A549, HepG2, HeLa cells were cultured in RPMI1640 (Gibco) cell culture medium supplemented with 10% FBS and 1% penicillin and streptomycin. HeLa/Tax cells were cultured in RPMI1640 cell culture medium supplemented with 10% FBS, 1% penicillin and streptomycin, and 100 ng mL⁻¹ Taxol®. All cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C.

4.4.2. Establishment of Taxol®-resistant HeLa/Tax cells. Taxol®-resistant HeLa/Tax cells were established by continuous exposure to Taxol® according to published procedures^{41,42} with modification. Briefly, HeLa cells were cultured with a gradient dose of Taxol® beginning at 2.5 ng mL⁻¹. After the cells were stable in proliferation without significant death, the next dose was given (5 ng mL⁻¹, then 10 ng mL⁻¹, etc.). Eventually the cells were cultured with 100 ng mL⁻¹ of Taxol® and this concentration was maintained. The whole induction process took about 4–6 months to establish the HeLa/Tax cell subline. Western blot analysis revealed that the cells overexpressed glycoprotein-1 (Pgp, aka. MDR1) and became resistant to Taxol® and other chemotherapeutic agents. The full characterization of this drug-resistant HeLa/Tax cell subline is to be communicated elsewhere.

4.4.3. Cell viability assay. A549, HepG2, HeLa, MCF-7, HeLa/Tax, and Vero cells (5×10^4 mL⁻¹) were seeded into flat-bottomed 96 well microplates, and allowed to attach overnight. Indicated concentration of test compounds was added into wells, and doxorubicin as well as DMC were used as references. Cell viability was assessed using MTT assay as previously reported.²⁴

4.4.4. Drug combination assay. HeLa/Tax cells were first subcultured into a new culture flask containing Taxol® free culture media and incubated at 37 °C. After growing to 70–80% confluence, the cells were passaged again and then incubated at 37 °C for 72 h before seeding into 96 well microplates. Cells were allowed to attach overnight, and were treated with gradient concentrations of Taxol® (2, 1, 0.5, 0.25, 0.125 μ M) and test

compounds alone, as well as their equipotency ratio combination on the same plate. Cell viability was measured using aforementioned method after 72 h incubation. The drug–drug interaction effects were expressed as CI and DRI (calculated by the CompuSyn software, CompuSyn, Inc., U.S.).

4.5. Solubility

The solubility of selected compounds (2a, 2e, and 2f) were evaluated using UPLC quantitative analysis with DMC as reference. The UPLC runs were carried out on a Waters ACQUITY UPLC H-Class system (Waters, Milford, MA, USA) equipping a Waters ACQUITY UPLC BEH C18 column (1.7 μ m, 2.1 \times 50 mm). Samples were eluted with gradient acetonitrile/water (A/W) solvent system (initial A/W = 30/70, 10 min linear gradient to A/W = 80/20) at a flow rate of 0.5 mL min⁻¹. Six standard samples of each test compounds with gradient concentrations were prepared by dilution of a methanol stock solution of which the exact concentration was known. Prepared samples were injected into the UPLC system and the standard curves were automatically generated by the software Empower3 (Waters) with r^2 no less than 0.995. Excess amount of the tertiary amine derivatives 2a, 2e, and 2f were suspended in pure water containing 0.1% formic acid (pH = 2.9). The mixtures were sonicated for 5 min at room temperature and followed by centrifugation at 12 000 rpm for 2 min using a Thermo Fisher microcentrifuge (Thermo Fisher Scientific, Boston, MA, USA). An aliquot of the clear supernates was injected into the UPLC system, and the peak area values of each sample was substituted into the equation, which was generated by the corresponding standard curve, to calculate the solubility.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

We thank Mr Yunfei Yuan, South China Botanical Garden, Chinese Academy of Sciences, for NMR spectroscopic measurements, and Ms Aijun Sun, South China Sea Institute of Oceanology, Chinese Academy of Sciences, for HRESIMS measurements. This work was supported by National Science Foundation of China (NSFC) (Grant no. 31470423 and 31528002).

References

- 1 A. J. Leon-Gonzalez, N. Acero, D. Munoz-Mingarro, I. Navarro and C. Martin-Cordero, *Curr. Med. Chem.*, 2015, **22**, 3407–3425.
- 2 D. K. Mahapatra, S. K. Bharti and V. Asati, *Eur. J. Med. Chem.*, 2015, **98**, 69–114.
- 3 D. K. Mahapatra, S. K. Bharti and V. Asati, *Eur. J. Med. Chem.*, 2015, **101**, 496–524.
- 4 Z. Nowakowska, *Eur. J. Med. Chem.*, 2007, **42**, 125–137.



- 5 C. Kontogiorgis, M. Mantzanidou and D. Hadjipavlou-Litina, *Mini-Rev. Med. Chem.*, 2008, **8**, 1224–1242.
- 6 J. R. Dimmock, D. W. Elias, M. A. Beazely and N. M. Kandepu, *Curr. Med. Chem.*, 1999, **6**, 1125–1149.
- 7 P. Singh, A. Anand and V. Kumar, *Eur. J. Med. Chem.*, 2014, **85**, 758–777.
- 8 N. K. Sahu, S. S. Balbhadra, J. Choudhary and D. V. Kohli, *Curr. Med. Chem.*, 2012, **19**, 209–225.
- 9 B. Zhou and C. Xing, *Med. Chem.*, 2015, **5**, 388.
- 10 K. E. Malterud, T. Anthonsen and G. B. Lorentzen, *Phytochemistry*, 1977, **16**, 1805–1809.
- 11 R. Srivastava, A. K. Shaw and D. K. Kulshreshtha, *Phytochemistry*, 1995, **38**, 687–689.
- 12 M. M. Salem and K. A. Werbovetz, *J. Nat. Prod.*, 2005, **68**, 108–111.
- 13 C.-L. Ye, Y.-H. Lu and D.-Z. Wei, *Phytochemistry*, 2004, **65**, 445–447.
- 14 C.-L. Ye, J.-W. Liu, D.-Z. Wei, Y.-H. Lu and F. Qian, *Pharmacol. Res.*, 2004, **50**, 505–510.
- 15 C.-L. Ye, J.-W. Liu, D.-Z. Wei, Y.-H. Lu and F. Qian, *Cancer Chemother. Pharmacol.*, 2005, **56**, 70–74.
- 16 F. Qian, C.-L. Ye, D.-Z. Wei, Y.-H. Lu and S.-L. Yang, *J. Chemother.*, 2005, **17**, 309–314.
- 17 H.-Y. Huang, J.-L. Niu and Y.-H. Lu, *J. Sci. Food Agric.*, 2012, **92**, 135–140.
- 18 H.-Y. Huang, J.-L. Niu, L.-M. Zhao and Y.-H. Lu, *Phytomedicine*, 2011, **18**, 1086–1092.
- 19 Y. Sone, J. K. Moon, T. T. Mai, N. Thu, E. Asano, K. Yamaguchi, Y. Otsuka and T. Shibamoto, *J. Sci. Food Agric.*, 2011, **91**, 2259–2264.
- 20 W.-G. Yu, J. Qian and Y.-H. Lu, *J. Agric. Food Chem.*, 2011, **59**, 12821–12829.
- 21 Y.-C. Hu, D.-M. Hao, L.-X. Zhou, Z. Zhang, N. Huang, M. Hoptroff and Y.-H. Lu, *J. Agric. Food Chem.*, 2014, **62**, 1602–1608.
- 22 X.-F. Zhu, B.-F. Xie, J.-M. Zhou, G.-K. Feng, Z.-C. Liu, X.-Y. Wei, F.-X. Zhang, M.-F. Liu and Y.-X. Zeng, *Mol. Pharmacol.*, 2005, **67**, 1444–1450.
- 23 V. J. Stella and K. W. Nti-Addae, *Adv. Drug Delivery Rev.*, 2007, **59**, 677–694.
- 24 J.-F. Shi, P. Wu, Z.-H. Jiang and X.-Y. Wei, *Eur. J. Med. Chem.*, 2014, **71**, 219–228.
- 25 A. Subarnas, A. Diantini, R. Abdulah, A. Zuhrotun, Y. E. Hadisaputri, I. M. Puspitasari, C. Yamazaki, H. Kuwano and H. Koyama, *Oncol. Lett.*, 2015, **9**, 2303–2306.
- 26 M. Figul, A. Söling, H. Dong, T.-C. Chou and N. Rainov, *Cancer Chemother. Pharmacol.*, 2003, **52**, 41–46.
- 27 M. Balzarotti, E. Ciusani, C. Calatuzzolo, D. Croci, A. Boiardi and A. Salmaggi, *Oncol. Res.*, 2004, **14**, 325–330.
- 28 A. Martins, N. Tóth, A. Ványolós, Z. Béni, I. Zupkó, J. Molnár, M. Báthori and A. Hunyadi, *J. Med. Chem.*, 2012, **55**, 5034–5043.
- 29 X. Kong, Q. Zhu, R. M. Ruprecht, K. A. Watanabe, J. M. Zeidler, J. Gold, B. Polsky, D. Armstrong and T.-C. Chou, *Antimicrob. Agents Chemother.*, 1991, **35**, 2003–2011.
- 30 K. Y. Hostetler, J. L. Hammond, G. D. Kini, S. E. Hostetler, J. R. Beadle, K. A. Aldern, T.-C. Chou, D. D. Richman and J. W. Mellors, *Antiviral Chem. Chemother.*, 2000, **11**, 213–219.
- 31 K. L. Hartshorn, M. W. Vogt, T.-C. Chou, R. S. Blumberg, R. Byington, R. Schooley and M. Hirsch, *Antimicrob. Agents Chemother.*, 1987, **31**, 168–172.
- 32 T.-C. Chou, *Pharmacol. Rev.*, 2006, **58**, 621–681.
- 33 A. Goldin and N. Mantel, *Cancer Res.*, 1957, **17**, 635–654.
- 34 W. R. Greco, G. Bravo and J. C. Parsons, *Pharmacol. Rev.*, 1995, **47**, 331–385.
- 35 T.-C. Chou and P. Talalay, *Trends Pharmacol. Sci.*, 1983, **4**, 450–454.
- 36 T.-C. Chou and P. Talalay, *Adv. Enzyme Regul.*, 1984, **22**, 27–55.
- 37 T.-C. Chou and M. Hayball, *CalcuSyn for windows: multiple drug dose-effect analyzer and manual*, Biosoft, Cambridge, UK, 1997.
- 38 T.-C. Chou and N. Martin, *CompuSyn for drug combinations: PC software and user's guide: a computer program for quantitation of synergism and antagonism in drug combinations, and the determination of IC₅₀ and ED₅₀ and LD₅₀ values*, ComboSyn, Paramus, NJ, 2005.
- 39 J. Chou and T.-C. Chou, Computerized simulation of dose reduction index (DRI) in synergistic drug combinations, *Pharmacologist*, 1988, **30**, A231.
- 40 F.-X. Zhang, M.-F. Liu and R.-R. Lu, *Acta Bot. Sin.*, 1990, **32**, 469–472.
- 41 X. Peng, F. Gong, Y. Chen, Y. Jiang, J. Liu, M. Yu, S. Zhang, M. Wang, G. Xiao and H. Liao, *Cell Death Dis.*, 2014, **5**, e1367.
- 42 K. Takara, Y. Obata, E. Yoshikawa, N. Kitada, T. Sakaeda, N. Ohnishi and T. Yokoyama, *Cancer Chemother. Pharmacol.*, 2006, **58**, 785–793.

