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# Anti-HBV and cytotoxic activities of pyranocoumarin derivatives

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ABSTRACT

Four natural pyranocoumarins clausenidin (**1**), nordentatin (**2**), clausarin (**3**), and xanthoxyletin (**4**) were isolated from the medicinal plant *Clausena excavata*. Recently, we found that **1** and **2** suppressed hepatitis B virus surface antigen in HepA2 cells, and in addition, **1**–**3** showed cytotoxic activity against four human cancer cell lines (A549, MCF7, KB, and KB-VIN). To explore the SAR of **1**–**4**, 17 pyranocoumarin analogues (**5**–**21**) were designed and synthesized. Among these analogues, **5** and **10** were the most potent against hepatitis B virus with $EC_{50}$ values of 1.14 and 1.34 μM, respectively. The most interesting result in the cytotoxicity assay was the significant activity of **1**, **5**, and **6** against the multi-drug resistant cell line, KB-VIN, without activity against the KB cell line. These data suggest that these three compounds could be useful hits for developing MDR-inverse drugs.



## 1. Introduction

Coumarins are a widely distributed and important class of natural compounds with broad pharmacological activities. They occur commonly in plants belonging to the families *Rutaceae*, *Simaroubaceae*, *Meliaceae*, and *Burseraceae*. We previously identified pyranocoumarins clausenidin **1**, nordentatin **2**, clausarin **3**, and xanthoxyletin **4** from the roots of *Clausena excavata* (Rutaceae),[1] which are used as a traditional remedy to treat viral hepatitis, cough, asthma, fever, headache, dermatological conditions, and gastrointestinal diseases.[2–4] During our continuing screening for antiviral and cytotoxic activities of traditional drugs and herbal materials, we found that **1** and **2** exhibited strong suppressive activity on hepatitis B virus surface antigen (HBsAg) production in human hepatoma cells. Furthermore, **1**–**3** showed potent cytotoxicity against A549, MCF7, KB, and KB-VIN human cancer cell lines. Therefore, we synthesized additional analogues of the isolated pyranocoumarins to evaluate structure–activity relationships and develop more selective and potent anti-hepatitis B virus (anti-HBV) or antitumor agents.

Hepatitis B virus (HBV) infection frequently results in both acute and chronic hepatitis and remains a major health problem worldwide. The number of chronic HBV-infected people is estimated to be more than 400 million worldwide with an annual death toll of 1.2 million per year.[5] HBV is also associated with a high risk (25–40%) of developing cirrhosis and hepatocellular carcinoma leading to significant mortality.[6–8] Immunization against HBV has helped to prevent new infections,[9] but millions of chronically infected patients will eventually succumb to the infection sequence unless treated with currently available therapies. Currently, the most promising anti-HBV agents are interferon-α (IFN-α) and nucleoside analogues. The efficacy of IFN-α is limited and often associated with severe adverse effects.[10] Nucleoside analogues such as lamivudine (3TC), adefovir dipivoxil (bis-POMP-MEA) and penciclovir (PCV) inhibit reverse transcriptase activity of the viral polymerase and have demonstrated clinical utility.[11] However, some chronic HBV-infected and liver transplant patients often experience a recurrence of HBV after a period of antiviral treatment with 3TC or PCV. This recurrence is due to the emergence of viral resistance.[12,13] Genotypic resistance emerges in 14–32% of patients within the first 12 months of 3TC therapy, increasing to 40% within two years of treatment and 57% by the third year.[14] Adefovir is usually well-tolerated and significantly reduces serum HBV DNA levels.[5] However, the primary limitations of adefovir therapy are dose-related side effects such as nephrotoxicity, lactic acidosis, and severe hepatomegaly with steatosis, and cessation of therapy may result in serious hepatitis. Therefore, novel classes of non-nucleoside anti-HBV agents with different modes of action are urgently needed.

In our studies, certain natural pyranocoumarins not only strongly suppressed HBsAg production in human hepatoma HepA2 cells, but also showed profound cytotoxic activity against four human cancer cell lines. In view of the novel structural template, we

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decided to synthesize additional analogues of the isolated pyranocoumarins to determine structure–activity relationships (SAR) and possibly develop more selective and potent anti-HBV or antitumor agents. Herein, we report our semi-synthesis of several new pyranocoumarin derivatives in excellent yields by using hydrogenation, epoxidation, and methylation techniques. Preliminary SAR studies on anti-HBV and cytotoxic activities are also discussed in this paper (Fig. 1).

## 2. Chemistry

We have seen limited reports of chemical modification on pyranocoumarins **1**–**4**;[15–18] therefore, we probed several structural changes, including hydrogenation, epoxidation, and methylation, at positions C-5, C-6, C-7, C-2′, C-3′, C-2″, and C-3″. Accordingly, semi-synthetic analogues **5**–**21** were prepared from **1–4** as illustrated in Schemes 1 and 2.

Compounds **1**, **2**, and **3** were reacted separately with methyl iodide in basic medium to yield **7**, **11**, and **16**, respectively. Catalytic hydrogenation of **1–4**, **7**, **11**, and **16** in the presence of Pd/C afforded target compounds **5**, **10**, **15**, **20**, **8**, **12**, and **17**, respectively. Compound **1** and its methyl derivative **7** were converted successfully to epoxides **6** and **9** by treatment with *m*-CPBA in dichloromethane. Unfortunately, compounds **2** and **3**, which have a hydroxy group at C-5, failed to produce corresponding epoxides under similar conditions. All efforts to prepare epoxy derivatives of **2** and **3** were unsuccessful, due to the occurrence of concomitant ring opening of the epoxide under the reaction conditions. However, compounds **4**, **11**, and **16**, which have a methoxy rather than hydroxy group at C-5, did undergo successful epoxidation with the volatile solution-phase reagent dimethyl dioxirane (DMDO) in acetone.[15] The products were epoxidized at the C6–C7 (**13**, **18**, and **21**) and also C2′–C3′ (**14** and **19**) double bonds.

## 3. Results and discussion

In our screening program, HepA2 cells were plated into 24-well plates and allowed to attach overnight. The medium was subsequently changed to serum-free DMEM, and various concentrations of semi-synthetic pyranocoumarin analogues were added. The production of HBsAg in the medium was determined after 48 h incubation. The 50% efficacy concentrations ($EC_{50}$, in μM) for these analogues are listed in Table 1.

Among the tested pyranocoumarin analogues, **1**, **5**, **10**, **11**, and **12** had anti-HBV $EC_{50}$ values of 1.88, 1.14, 1.34, 1.64, and 1.63 μM, respectively. Compounds **2**, **8**, **13**, **17**, and **18** exhibited good but lower antiviral potency ($EC_{50}$ < 7 μM). Compound **7** displayed only moderate activity, with an $EC_{50}$ value of 11.25 μM. However, **3**, **4**, **6**, **9**, **14–16**, and **19–21** showed no activity at concentrations up to 15 μM.

For **1**-analogues, the rank order of potency was **5** > **1** > **8** > **7** > **6** = **9**, with **5** being the most potent anti-HBV agent. Analogues of **2** demonstrated significant anti-HBV activities in the decreasing order **10** > **12** = **11** > **13** > **2** > **14**. Interestingly, although **3** exhibited no anti-HBV activity, its analogues **17** and **18** showed good antiviral activities. Finally, all **4**-analogues (**4**, **20**, **21**) were inactive. On the basis of the structural characteristics and the $EC_{50}$ values, the following SAR conclusions were established.

With **1** versus **7**, conversion of the C-5 OH to $OCH_3$ decreased activity significantly, postulated to possibly be due to the loss of intramolecular hydrogen bonding to the oxygen of the carbonyl group at C-6. A more modest decrease was found between **5** (OH) and **8** ($OCH_3$). However, the analogous modification of **2**, which has a C6–C7 double bond, to give **11** resulted in increased activity. Thus, the carbonyl functionality at C-6 had a greater effect on activity when a hydroxy group was present at C-5.

Compounds **4**, **20**, and **21**, which do not contain dimethylallyl or dimethylpropyl groups, showed no activity. However analogues **11**, **12**, **13**, **17**, and **18**, which do contain these groups, were active. Thus, these side chain groups are essential functionalities for the biological activity of these pyranocoumarins. Compounds with these side chains at only the C-10 position (**2**, **10**, **12**, **13**) were generally more or as active than compounds substituted at both the C-3 and C-10 positions (**3**, **15**, **17**, **18**).

When the double bond in a dimethylallyl side chain was reduced to give a dimethylpropyl group, antiviral potency was retained or increased, as seen by the comparison of **1**/**5**, **2**/**10**, **7**/**8**, and **11**/**12**. However, none of the compounds (**6**, **9**, **14**, and **19**) with epoxidation of this same moiety were active. In comparison, compounds **13** and **18**, which contain an epoxide at the C6–C7 position of the pyran ring together with dimethylallyl side chains, were moderately active.

Overall, our preliminary studies showed that the most potent anti-HIV pyranocoumarins contained a dimethylallyl or dimethylpropyl side chain at the C-10 position, together with various functionalization of the pyran ring, including a C-6 carbonyl (**1**, **5**) with a *peri* C-5 hydroxyl, a C6–C7 double bond (**11**, **12**), and a C6–C7 single bond (**10**).

In addition, pyranocoumarin analogues **1–21** were also assayed for cytotoxicity against A549, MCF-7, KB, and KB-VIN human tumor cell lines, and the $EC_{50}$ values obtained are summarized in Table 2. Compound **3** showed significant cytotoxic activity against A549, KB and KB-VIN cell lines with $EC_{50}$ values ranging from 1.59 to 2.98 μg/mL. Interestingly, compounds **1**, **5**, and **6** showed significant activity only against KB-VIN cells with $EC_{50}$ values of 2.25–2.87 μg/mL; they showed marginal or no activity against the other three cell lines. This type of activity is significant and paradoxical, since KB-VIN cells over-express P-glycoprotein and are either cross-resistant to cytotoxic drugs or have similar susceptibility as the KB parent cell line (if the agent is not a P-glycoprotein substrate). In contrast, compounds **1**, **5**, and **6** behave in the opposite way to P-glycoprotein substrates (i.e., MDR-inverse), and such agents are currently of interest as a new way to exploit the phenomenon of multi-drug resistant cancer development.

Compounds **2**, **10**, **15**, and **18** showed similar and marginal activity against the four cell lines with $EC_{50}$ values ranging from

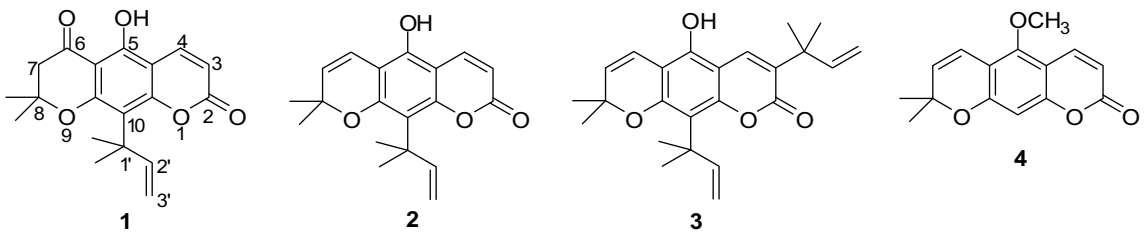
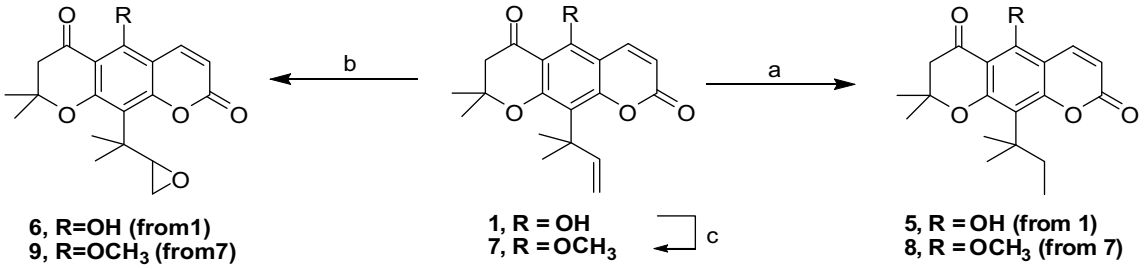
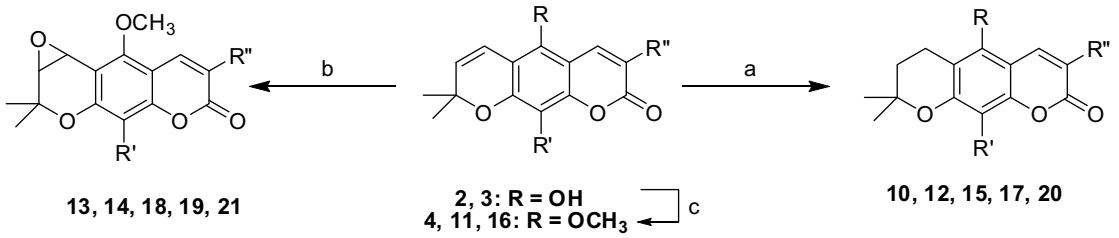



**Figure 1.** Chemical structures of pyranocoumarins isolated from *Clausena excavata*.




**Scheme 1.** Reagents and conditions: (a) $H_2$, Pd/C, EtOAc, rt; (b) *m*-CPBA, $CH_2Cl_2$, rt; (c) $CH_3I$, $K_2CO_3$, acetone, reflux.




See Table below for R groups, and reaction transformations

Reagents and conditions: (a) $H_2$, Pd/C, EtOAc, RT (b) DMDO, acetone, 0 °C (c) $CH_3I$, $K_2CO_3$, acetone, reflux

| Compound | Rxn | R | R' | R'' |
|---|---|---|---|---|
| **2** | — | OH |  | H |
| **10** | a (from **2**) | OH |  | H |
| **11** | c (from **2**) | $OCH_3$ |  | H |
| **12** | a (from **11**) | $OCH_3$ |  | H |
| **13** | b (from **11**)* | $OCH_3$ |  | H |
| **14** | b (from **11**)* | $OCH_3$ |  | H |
| **3** | — | OH |  |  |
| **15** | a (from **3**) | OH |  |  |
| **16** | c (from **3**) | $OCH_3$ |  |  |
| **17** | a (from **16**) | $OCH_3$ |  |  |
| **18** | b (from **16**)* | $OCH_3$ |  |  |
| **19** | b (from **16**)* | $OCH_3$ |  |  |
| **4** | — | $OCH_3$ | H | H |
| **20** | a (from **4**) | $OCH_3$ | H | H |
| **21** | b (from **4**) | $OCH_3$ | H | H |

* Mixture of both compounds obtained in reaction.

**Scheme 2.**

**Table 1**
Suppression of HBV HBsAg by **1**–**22** in HepA2 cells

| Compound | EC50[a] (μM) | Compound | EC50[a] (μM) |
|---|---|---|---|
| **1** | 1.88 | **12** | 1.63 |
| **2** | 6.38 | **13** | 5.50 |
| **3** | NA[b] | **14** | NA |
| **4** | NA | **15** | NA |
| **5** | 1.14 | **16** | NA |
| **6** | NA | **17** | 6.60 |
| **7** | 11.25 | **18** | 4.56 |
| **8** | 5.35 | **19** | NA |
| **9** | NA | **20** | NA |
| **10** | 1.34 | **21** | NA |
| **11** | 1.64 | | |

All compounds at different concentrations were tested in triplicate for their effect on the production of HBsAg by HepA2 cells for 48 h.

[a] $EC_{50}$ is the concentration that results in suppression of HBsAg to 50% of untreated HepA2 cells.

[b] NA: HBsAg secretion in the presence of the compound at concentrations up to 15 μM did not change when compared with the control.

**Table 2**
Effect of pyranocoumarin derivatives **1**–**21** against tumor cell line replication

| Compound | $EC_{50}$ (μg/mL) | | | |
|---|---|---|---|---|
| | A549[a] | MCF7[a] | KB[a] | KB-VIN[a] |
| **1** | 19.36 | NA[b] | 19.83 | 2.25 |
| **2** | 8.70 | 17.32 | 9.63 | 9.96 |
| **3** | 2.98 | 7.96 | 1.61 | 1.59 |
| **5** | 19.73 | NA | 19.49 | 2.87 |
| **6** | NA | NA | NA | 2.71 |
| **8** | NA | NA | NA | 13.05 |
| **10** | 8.84 | 16.34 | 9.25 | 9.11 |
| **11** | NA | NA | 13.26 | 14.71 |
| **12** | NA | NA | 11.54 | 10.16 |
| **15** | 12.21 | 17.73 | 8.21 | 7.73 |
| **18** | 11.78 | 11.42 | 6.50 | 6.12 |

[a] Cell line: A549 = lung; MCF-7 = breast; KB = nasopharynx; KB-VIN = nasopharynx MDR

[b] NA = Not active; $EC_{50}$ > 20 μg/mL. Compounds **4**, **7**, **9**, **13**, **14**, **16**, **17**, **19**–**21** were not active against any tested cell line.

6.12 to 17.7 μg/mL. Compounds **11** and **12** were marginally active against both KB and KB-VIN, and **8** against only KB-VIN cells. Moreover, all three **4**-analogues (**4**, **20**, **21**) as well as **7**, **13**, **14**, **16**, and **17** were inactive against any of the four tested cell lines.

From a SAR viewpoint, the data for **7**, **11**, and **16** indicated that methylation of the C-5 OH decreased or abolished activity. This conclusion was also confirmed from comparison of the data for **11** and **12** with those of **2** and **10**, respectively, which again showed that methylation of the C-5 OH decreased activity, but perhaps enhanced selectivity against different cell lines. Furthermore, comparison of **1** and **5** with **2** and **10** showed that the carbonyl group at C-6 seems to be an important factor for cancer cell line selectivity. All compounds (**1**, **5**, **6**, **8**) that were most selective against KB-VIN were analogues of **1**. Epoxidation or hydrogenation of the double bonds in the pyran ring or dimethylallyl groups had no significant effect on activity. However, the presence of a dimethylallyl group at the C-3 position increased overall cytotoxic activity as seen from the higher potency of **3** compared with **2**.

From the above structure–activity relationship analysis, the C-6 carbonyl, C-5 hydroxy groups, and C-10 dimethylpropyl group play important roles in the anticancer activity and selectivity of these pyranocoumarin compounds.

In conclusion, these studies present that pyranocoumarin derivatives display both anti-HBV and cytotoxic activities. The SAR studies demonstrated that the carbonyl and hydroxy groups at peri positions (C-6 and C-5) and dimethylpropyl groups on the coumarin skeleton will affect the activity and selectivity. Interestingly, in contrasting the two assays, certain compounds displayed good anti-HBV activity but no cytotoxic activity (e.g., **11**, **12**), and vice versa (e.g., **3**).

Synthesized analogues **5** and **10**, which showed the highest anti-HBV activity, were selectively or moderately cytotoxic. Therefore, the activities of these pyranocoumarin derivatives do not seem to correlate directly and the mechanism(s) of action should be further investigated to enhance our understanding on the regulation of these compounds. The strong anti-HBV activity, selective cytotoxic activity, particularly against the multi-drug resistant cell line, and ease of preparation make these pyranocoumarin valuable for further exploration and development as novel non-nucleoside anti-HBV and antitumor agents.

## 4. Experimental

### 4.1. General experimental procedures

Melting points were determined on a Yanaco MP-S3 melting point apparatus and are uncorrected. IR spectra were determined in KBr discs on a Shimadzu FT-IR Prestige 21. $^1H$ and $^{13}C$ NMR spectra were recorded on a Bruker Avance-300 NMR spectrometer, using tetramethylsilane (TMS) as internal standard; all chemical shifts were reported in ppm ($\delta$). All MS and HRMS spectra (EI) were obtained on a VG-70-250S mass spectrometer. Elemental microanalysis were determined by Elementar Vario EL III and gave combustion values for C, H, N within 0.4% of the theoretical values. Preparative flash column chromatography was performed using silica gel (Merck) G60 230–400 mesh and aluminum oxide 90 active neutral gel (Merck) 70–230 mesh.

### 4.2. Extraction and isolation

*C. excavata* was collected from Pigntung Hsien, Taiwan, and verified by Prof. C. S. Kuoh. A voucher specimen of this plant was deposited in the herbarium of National Cheng Kung University, Tainan, Taiwan. The acetone extract of the roots of *C. excavata* (0.9 kg) was subjected to silica gel chromatography and successively eluted with $CHCl_3$–MeOH and $C_6H_6$–$Me_2CO$ to obtain **1** (4.2 g), **2** (7.4 g), **3** (5.2 g), and **4** (5.3 g).

### 4.3. General procedure for compounds 5, 8, 10, 12, 15, 17, and 20

A solution of the corresponding coumarin (100 mg) in EtOAc (15 mL) was hydrogenated at atmospheric pressure in the presence of 10% Pd/C (25 mg). After filtration of the catalyst and evaporation of the solvent, the residue obtained was purified by column chromatography over silica gel for each compound.

#### 4.3.1. 10-(1,1-Dimethylpropyl)-5-hydroxy-8,8-dimethyl-7,8-dihydropyrano[3,2-γ]chromen-2,6-dione (5)

89.4 mg (90.2%), mp 124–125 °C; NMR ($CDCl_3$, 300 MHz) $\delta$: 13.05 (1H, br s, $D_2O$-exchangeable, OH-5), 8.05 (1H, d, $J$ = 9.6 Hz, H-4), 6.16 (1H, d, $J$ = 9.6 Hz, H-3), 2.76 (2H, s, H-7), 1.92 (2H, q, $J$ = 7.4 Hz, H-2′), 1.57 (6H, s, $(CH_3)_2$-1′), 1.50 (6H, s, $(CH_3)_2$-8), 0.73 (3H, t, $J$ = 7.4 Hz, H-3′); $^{13}C$ NMR ($CDCl_3$, 75 MHz) $\delta$: 198.4, 161.2, 160.6, 160.1, 158.9, 138.7, 114.2, 110.6, 104.0, 103.3, 79.7, 47.6, 40.2, 35.0, 30.6 (2×), 26.6 (2×), 10.1; EIMS *m/z* (rel. int.%): 330 ($M^+$, 13), 301 (100); HR-EIMS *m/z* 330.1466 (calcd for $C_{19}H_{22}O_5$: 330.1467); Anal. ($C_{19}H_{22}O_5$) C, H.

#### 4.3.2. 10-(1,1-Dimethylpropyl)-5-methoxy-8,8-dimethyl-7,8-dihydropyrano[3,2-γ]chromen-2,6-dione (8)

84.8 mg (83.8%), mp 96–97 °C; $^1H$ NMR ($CDCl_3$, 300 MHz) $\delta$: 7.99 (1H, d, $J$ = 9.6 Hz, H-4), 6.21 (1H, d, $J$ = 9.6 Hz, H-3), 3.90 (1H,

s, $OCH_3$-5), 2.70 (2H, s, H-7), 1.93 (2H, q, $J$ = 7.5 Hz, H-2′), 1.59 (6H, s, $(CH_3)_2$-1′), 1.48 (6H, s, $(CH_3)_2$-8), 0.72 (3H, t, $J$ = 7.5 Hz, H-3′); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 190.4, 162.6, 159.7, 158.1, 156.9, 139.2, 119.3, 112.4, 111.5, 108.5, 79.5, 63.4, 49.7, 40.6, 35.0, 30.5 (2×), 26.3 (2×), 10.1; EIMS $m/z$ (rel. int.%): 344 ($M^+$, 20), 315 (68), 259 (100); HR-EIMS $m/z$ 344.1624 (calcd for $C_{20}H_{24}O_5$: 344.1624); Anal. ($C_{20}H_{24}O_5$) C, H.

#### 4.3.3. 10-(1,1-Dimethylpropyl)-5-hydroxy-8,8-dimethyl-7,8-dihydro-6*H*-pyrano[3,2-γ]chromen-2-one (10)

87.6 mg (87.8%), mp 195–196 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 8.09 (1H, d, $J$ = 9.6 Hz, H-4), 6.24 (1H, br s, $D_2O$-exchangeable, OH-5), 6.11 (1H, d, $J$ = 9.6 Hz, H-3), 2.69 (2H, t, $J$ = 7.0 Hz, H-6), 1.90 (3H, q, $J$ = 7.4 Hz, H-2′), 1.81 (2H, t, $J$ = 7.0 Hz, H-7), 1.56 (6H, s, $(CH_3)_2$-1′), 1.36 (6H, s, $(CH_3)_2$-8), 0.67 (3H, t, $J$ = 7.5 Hz, H-3′); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 162.4, 157.7, 153.1, 149.2, 139.9, 114.8, 109.2, 103.8, 102.7, 75.1, 40.2, 35.1, 31.2, 30.8 (2×), 26.4 (2×), 17.2, 10.1; EIMS $m/z$ (rel. int.%): 344 ($M^+$, 20), 315 (68), 259 (100); HR-EIMS $m/z$ 316.1575 (calcd for $C_{19}H_{24}O_4$: 316.1675); Anal. ($C_{19}H_{24}O_4$) C, H.

#### 4.3.4. 10-(1,1-Dimethylpropyl)-5-methoxy-8,8-dimethyl-7,8-dihydro-6*H*-pyrano[3,2-γ]chromen-2-one (12)

91.3 mg (90.7%), mp 91–92 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 7.86 (1H, d, $J$ = 9.6 Hz, H-4), 6.15 (1H, d, $J$ = 9.6 Hz, H-3), 3.80 (3H, s, $OCH_3$-5), 2.79 (2H, t, $J$ = 6.9 Hz, H-6), 1.92 (2H, q, $J$ = 7.4 Hz, H-2′), 1.77 (2H, t, $J$ = 6.9 Hz, H-7), 1.58 (6H, s, $(CH_3)_2$-1′), 1.37 (6H, s, $(CH_3)_2$-8), 0.68 (3H, t, $J$ = 7.4 Hz, H-3′); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 161.2, 157.8, 153.2, 153.1, 139.2, 118.5, 111.4, 111.0, 106.5, 75.5, 61.8, 40.4, 35.0, 31.4, 30.6 (2×), 26.7 (2×), 17.4, 10.1; EIMS $m/z$ (rel. int.%): 330 ($M^+$, 48), 301 (100), 245 (99); HR-EIMS $m/z$ 330.1832 (calcd for $C_{20}H_{26}O_4$: 330.1831); Anal. ($C_{20}H_{26}O_4$) C, H.

#### 4.3.5. 3,10-Bis-(1,1-dimethylpropyl)-5-hydroxy-8,8-dimethyl-7,8-dihydro-6*H*-pyrano[3,2-γ]chromen-2-one (15)

82.9 mg (82.6%), mp 237–238 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 7.83 (1H, s, H-4), 5.52 (1H, br s, $D_2O$-exchangeble, OH-5), 2.66 (2H, t, $J$ = 7.0 Hz, H-6), 1.89 (2H, q, $J$ = 7.5 Hz, H-2′), 1.86 (2H, q, $J$ = 7.7 Hz, H-2″), 1.83 (2H, t, $J$ = 7.0 Hz, H-7), 1.63 (6H, s, $(CH_3)_2$-1′), 1.35 (6H, s, $(CH_3)_2$-8), 1.31 (6H, s, $(CH_3)_2$-1″), 0.71 (2H, t, $J$ = 7.7 Hz, H-3″), 0.68 (2H, t, $J$ = 7.5 Hz, H-3′); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 160.6, 156.1, 152.4, 148.4, 133.9, 128.5, 114.2, 102.9, 102.8, 74.6, 40.1, 38.5, 35.1, 32.2, 31.4, 30.7 (2×), 26.8 (2×), 26.4 (2×), 17.3, 10.1, 9.4; EIMS $m/z$ (rel. int.%): 386 ($M^+$, 8), 371 (78), 357 (38); HR-EIMS $m/z$ 386.2460 (calcd for $C_{24}H_{34}O_4$: 386.2457); Anal. ($C_{24}H_{34}O_4$) C, H.

#### 4.3.6. 3,10-Bis-(1,1-dimethylpropyl)-5-methoxy-8,8-dimethyl-7,8-dihydro-6*H*-pyrano[3,2-γ]chromen-2-one (17)

88.4 mg (88.3%), mp 102–103 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 7.66 (1H, s, H-4), 3.82 (3H, s, $OCH_3$-5), 2.80 (2H, t, $J$ = 6.8 Hz, H-6), 1.93 (2H, q, $J$ = 7.4 Hz, H-2′), 1.85 (2H, q, $J$ = 7.4 Hz, H-2″), 1.77 (2H, t, $J$ = 6.8 Hz, H-7), 1.59 (6H, s, $(CH_3)_2$-1′), 1.36 (6H, s, $(CH_3)_2$-8), 1.32 (6H, s, $(CH_3)_2$-1″), 0.71 (2H, t, $J$ = 7.4 Hz, H-3″), 0.69 (2H, t, $J$ = 7.4 Hz, H-3′); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 160.0, 156.5, 152.7, 152.3, 133.6, 129.8, 117.7, 110.6, 106.9, 75.1, 61.5, 40.3, 40.0, 35.1, 32.1, 31.6, 30.6 (2×), 26.7 (4×), 17.4, 10.1, 9.3; EIMS $m/z$ (rel. int.%): 400 ($M^+$, 37), 371 (100), 315(100); HR-EIMS $m/z$ 400.2617 (calcd for $C_{25}H_{36}O_4$: 400.2614); Anal. ($C_{25}H_{36}O_4$) C, H.

#### 4.3.7. 5-Methoxy-8,8-dimethyl-7,8-dihydro-6*H*-pyrano[3,2-γ]chromen-2-one (20)

94.3 mg (94.4%), mp 142–145 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 7.84 (1H, d, $J$ = 9.6 Hz, H-4), 6.54 (1H, s, H-10), 6.17 (1H, d, $J$ = 9.6 Hz, H-3), 3.85 (3H, s, $OCH_3$-5), 2.77 (2H, t, $J$ = 6.7 Hz, H-6), 1.81 (2H, t, $J$ = 6.7 Hz, H-7), 1.36 (6H, s, $(CH_3)_2$-8); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 161.4, 158.5, 155.3, 154.2, 138.6, 112.0, 111.6, 106.6, 100.9, 75.7, 62.0, 31.8, 26.7 (2×), 17.0; EIMS $m/z$ (rel. int.%): 260 ($M^+$, 45), 205 (100); HR-EIMS $m/z$ 260.1046 (calcd for $C_{15}H_{16}O_4$: 260.1049); Anal. ($C_{15}H_{16}O_4$) C, H.

### 4.4. General procedure for compounds 7, 11, and 16

To a solution of corresponding coumarin (1 mmol) in acetone (20 mL) was added $K_2CO_3$ (830 mg, 6 mmol) and $CH_3I$ (283.9 mg, 2 mmol). After 3 h stirring at the refluxing temperature, the reaction mixture was extracted with EtOAc, washed with water, dried over $Na_2SO_4$, evaporated and the obtained residue was purified by column chromatography over silica gel for each compound.

#### 4.4.1. 10-(1,1-Dimethylallyl)-5-methoxy-8,8-dimethyl-7,8-dihydropyrano[3,2-γ]chromen-2,6-dione (7)

247 mg (72.3%), mp 195–196 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 7.97 (1H, d, $J$ = 9.8 Hz, H-4), 6.23 (1H, dd, $J$ = 16.8, 10.1 Hz, H-2′), 6.22 (1H, d, $J$ = 9.8 Hz, H-3), 4.92 (1H, d, $J$ = 16.8 Hz, $H_b$-3′), 4.87 (1H, d, $J$ = 10.1 Hz, $H_a$-3′), 3.91 (3H, s, $OCH_3$-5), 2.62 (2H, s, H-7), 1.66 (6H, s, $(CH_3)_2$-1′), 1.46 (6H, s, $(CH_3)_2$-8); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 190.3, 162.0, 159.7, 157.5, 157.1, 149.6, 139.2, 119.5, 112.5, 111.6, 108.5, 108.2, 79.9, 63.5, 49.9, 41.3, 28.8 (2×), 26.2 (2×); EIMS $m/z$ (rel. int.%): 342 ($M^+$, 50), 327 (22), 271 (100); HR-EIMS $m/z$ 342.1465 (calcd for $C_{20}H_{22}O_5$: 342.1467); Anal. ($C_{20}H_{22}O_5$) C, H.

#### 4.4.2. 10-(1,1-Dimethylallyl)-5-methoxy-8,8-dimethyl-8*H*-pyrano[3,2-γ]chromen-2-one (11)

296.0 mg (90.2%), mp 86–87 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 7.86 (1H, d, $J$ = 9.5 Hz, H-4), 6.56 (1H, d, $J$ = 10.0 Hz, H-6), 6.30 (1H, dd, $J$ = 17.3, 10.6 Hz, H-2′), 6.18 (1H, d, $J$ = 9.5 Hz, H-3), 5.69 (1H, d, $J$ = 10.0 Hz, H-7), 4.93 (1H, d, $J$ = 17.3 Hz, $H_b$-3′), 4.87 (1H, d, $J$ = 10.6 Hz, $H_a$-3′), 3.82 (3H, s, $OCH_3$-5), 1.66 (6H, s, $(CH_3)_2$-1′), 1.44 (6H, s, $(CH_3)_2$-8); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 160.6, 155.9, 153.8, 151.1, 149.7, 138.8, 130.2, 119.0, 116.1, 111.5, 108.0, 107.4, 77.3, 63.3, 41.0, 29.3 (2×), 27.4 (2×); EIMS $m/z$ (rel. int.%): 326 ($M^+$, 100), 165 (54), 151 (89); HR-EIMS $m/z$ 326.1517 (calcd for $C_{20}H_{22}O_4$: 326.1518); Anal. ($C_{20}H_{26}O_4$) C, H.

#### 4.4.3. 3,10-Bis-(1,1-dimethylallyl)-5-methoxy-8,8-dimethyl-8*H*-pyrano[3,2-γ]chromen-2-one (16)

379.9 mg (96.3%), mp 142–143 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 7.73 (1H, s, H-4), 6.57 (1H, d, $J$ = 9.9 Hz, H-6), 6.30 (1H, dd, $J$ = 17.4, 10.4 Hz, H-2′), 6.18 (1H, dd, $J$ = 17.6, 10.5 Hz, H-2″), 5.67 (1H, d, $J$ = 9.9 Hz, H-7), 5.09 (1H, d, $J$ = 17.6 Hz, H-3b″), 5.08 (1H, d, $J$ = 10.5 Hz, H-3a″), 4.95 (1H, d, $J$ = 17.4 Hz, H-3b′), 4.87 (1H, d, $J$ = 10.4 Hz, H-3a′), 3.82 (3H, s, $OCH_3$-5), 1.65 (6H, s, $(CH_3)_2$-1′), 1.47 (6H, s, $(CH_3)_2$-1″), 1.44 (6H, s, $(CH_3)_2$-8); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 159.4, 154.9, 153.1, 151.0, 149.8, 145.5, 132.8, 130.3, 130.1, 118.4, 116.5, 111.9, 111.5, 108.0, 107.7, 77.2, 63.2, 41.0, 40.2, 29.2 (2×), 27.4 (2×), 26.0 (2×); EIMS $m/z$ (rel. int.%): 394 ($M^+$, 96), 380(100); HR-EIMS $m/z$ 394.2141 (calcd for $C_{25}H_{30}O_4$: 394.2144); Anal. ($C_{25}H_{30}O_4$) C, H.

### 4.5. General procedure for compounds 6 and 9

To a solution of corresponding coumarin (0.3 mmol) in $CH_2Cl_2$ (10 mL) was added mCPBA (88.8 mg, 0.36 mmol). The mixture was then stirred for 24 h under an argon atmosphere and the solvent was removed to afford a residue, which was purified by column chromatography on aluminum oxide gel.

#### 4.5.1. 5-Hydroxy-8,8-dimethyl-10-(1-methyl-1-oxiranylethyl)-7,8-dihydropyrano[3,2-γ]chromen-2,6-dione (6)

81.0 mg (78.5%), mp 150–151 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 13.10 (1H, br s, $D_2O$-exchangeable, OH-5), 8.04 (1H, d, $J$ = 9.6 Hz,

H-4), 6.17 (1H, d, $J$ = 9.6 Hz, H-3), 3.36 (1H, t, $J$ = 3.4 Hz, H-2′), 2.96 (2H, d, $J$ = 3.4 Hz, H-3′), 2.78 (2H, s, H-7), 1.67 (3H, s, $CH_3$-1′), 1.52 (6H, s, $(CH_3)_2$-8), 1.27 (3H, s, $CH_3$-1′); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 198.1, 160.7, 159.6, 159.3, 159.3, 138.6, 113.3, 110.9, 104.0, 103.3, 80.1, 59.9, 48.2, 47.5, 38.8, 26.6 (2×), 26.2, 21.1; EIMS $m/z$ (rel. int.%): 344 ($M^+$, 79), 329 (74), 301 (93); HR-EIMS $m/z$ 344.1262 (calcd for $C_{19}H_{20}O_6$: 344.1260); Anal. ($C_{19}H_{20}O_6$) C, H.

#### 4.5.2. 5-Methoxy-8,8-dimethyl-10-(1-methyl-1-oxiranylethyl)-7,8-dihydro-pyrano[3,2-γ]chromen-2,6-dione (9)

81.3 mg (77.7%), mp 120–121 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 8.00 (1H, d, $J$ = 9.9 Hz, H-4), 6.23 (1H, d, $J$ = 9.6 Hz, H-3), 3.92 (3H, s, $OCH_3$-5), 3.40 (1H, t, $J$ = 3.4 Hz, H-2′), 2.98 (2H, d, $J$ = 3.4 Hz, H-3′), 1.67 (3H, s, $CH_3$-1′), 1.51 (6H, s, $(CH_3)_2$-8), 1.30 (3H, s, $CH_3$-1′); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 190.0, 162.1, 159.2, 157.5, 157.5, 139.1, 118.3, 112.7, 111.7, 108.6, 80.0, 63.6, 60.0, 49.7, 48.3, 39.2, 26.4, 26.3, 26.1, 20.7; EIMS $m/z$ (rel. int.%): 358 ($M^+$, 38), 315 (32), 259 (100); HR-EIMS $m/z$ 358.1413 (calcd for $C_{20}H_{22}O_6$: 358.1416); Anal. ($C_{20}H_{22}O_6$) C, H.

### 4.6. General procedure for compounds 13, 14, 18, 19, and 21

Dry dimethyldioxiane (DMDO) was prepared and distilled onto molecular sieves according to the previous procedure.[19] To a solution of corresponding coumarin (0.3 mmol) in acetone (5 mL) was added the DMDO stock solution (10 mL, 1 mmol, 3 equiv) at 0 °C. The resulting mixture was stirred at 0 °C for 1 h and the solvents were removed under reduced pressure. The crude epoxide obtained was purified by column chromatography on aluminum oxide gel.

#### 4.6.1. 4-(1,1-Dimethylallyl)-9-methoxy-2,2-dimethyl-1α,9β-dihydro-2*H*-1,3,5-trioxacyclopropa[α]anthracen-6-one (13)

43.4 mg (42.3%), mp 114–115 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 7.88 (1H, d, $J$ = 9.6 Hz, H-8), 6.24 (1H, d, $J$ = 9.6 Hz, H-7), 6.23 (1H, dd, $J$ = 16.6, 9.0 Hz, H-2′), 4.91 (1H, d, $J$ = 16.6 Hz, H-3b′), 4.87 (1H, d, $J$ = 9.0 Hz, H-3a′), 4.31 (1H, d, $J$ = 4.6 Hz, H-9β), 3.95 (3H, s, $OCH_3$-9), 3.47 (1H, d, $J$ = 4.6 Hz, H-1α), 1.65 (3H, s, $CH_3$-1′), 1.60 (3H, s, $CH_3$-1′), 1.59 (3H, s, $CH_3$-2), 1.28 (3H, s, $CH_3$-2); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 160.3, 156.0, 155.2, 154.7, 149.7, 138.4, 120.8, 112.5, 110.1, 108.3, 108.0, 74.8, 64.5, 60.9, 45.9, 41.3, 29.7, 29.1, 25.2, 22.7; EIMS $m/z$ (rel. int.%): 342 ($M^+$, 18), 327 (24), 311 (100); HR-EIMS $m/z$ 342.1465 (calcd for $C_{20}H_{22}O_5$: 342.1467); Anal. ($C_{20}H_{22}O_5$) C, H.

#### 4.6.2. 9-Methoxy-2,2-dimethyl-4-(1-methyl-1-oxiranylethyl)-1α,9β-dihydro-2*H*-1,3,5-trioxacyclopropa[α]anthracen-6-one (14)

60.7 mg (56.5%), mp 125–126 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 7.89 (1H, d, $J$ = 9.7 Hz, H-8), 6.26 (1H, d, $J$ = 9.7 Hz, H-7), 4.33 (1H, d, $J$ = 4.4 Hz, H-9β), 3.82 (3H, s, $OCH_3$-9), 3.50 (1H, d, $J$ = 4.4 Hz, H-1α), 3.40 (1H, t, $J$ = 3.1 Hz, H-2′), 2.98 (1H, d, $J$ = 3.1 Hz, H-3′), 1.67 (3H, s, $CH_3$-1′), 1.62 (3H, s, $CH_3$-1′), 1.30 (3H, s, $CH_3$-2), 1.24 (3H, s, $CH_3$-2); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 159.9, 156.3, 155.3, 154.7, 138.4, 119.7, 112.7, 110.3, 108.1, 74.9, 64.5, 60.9, 59.9, 48.0, 45.9, 39.1, 26.5, 25.4, 22.5, 20.7; EIMS $m/z$ (rel. int.%): 358 ($M^+$, 50), 342 (72), 327 (100); HR-EIMS $m/z$ 358.1416 (calcd for $C_{20}H_{22}O_6$: 358.1416); Anal. ($C_{20}H_{22}O_6$) C, H.

#### 4.6.3. 4,7-Bis-(1,1-dimethylallyl)-9-methoxy-2,2-dimethyl-1α,9β-dihydro-2*H*-1,3,5-trioxacyclopropa[α]anthracen-6-one (18)

51.8 mg (41.2%), mp 96–97 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 7.73 (1H, s, H-8), 6.22 (1H, dd, $J$ = 17.2, 10.5 Hz, H-2′), 6.19 (1H, dd, $J$ = 17.4, 10.6 Hz, H-2″), 5.11 (1H, d, $J$ = 17.4 Hz, H-3b″), 5.07 (1H, d, $J$ = 10.6 Hz, H-3a″), 4.90 (1H, d, $J$ = 17.2 Hz, H-3b′), 4.87 (1H, d, $J$ = 10.5 Hz, H-3a′), 4.31 (1H, d, $J$ = 4.3 Hz, H-9β), 3.93 (3H, s, $OCH_3$-9), 3.45 (1H, d, $J$ = 4.3 Hz, H-1α), 1.64 (3H, s, $CH_3$-1′), 1.61 (3H, s, $CH_3$-1′), 1.48 (6H, s, $(CH_3)_2$-1″), 1.47 (3H, s, $CH_3$-2), 1.26 (3H, s, $CH_3$-2); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 159.1 155.7, 154.1, 153.7, 149.7, 145.3, 132.3, 131.2, 120.8, 112.2, 109.7, 108.3, 108.1, 74.5, 64.2, 61.0, 46.1, 41.1, 40.3, 29.6, 28.9, 25.6, 25.2, 22.5; EIMS $m/z$ (rel. int.%): 410 ($M^+$, 57), 395 (100); HR-EIMS $m/z$ 410.2094 (calcd for $C_{25}H_{30}O_5$: 410.2093); Anal. ($C_{25}H_{30}O_5$) C, H.

#### 4.6.4. 9-Methoxy-2,2-dimethyl-4,7-bis-(1-methyl-1-oxiranylethyl)-1α,9β-dihydro-2*H*-1,3,5-trioxacyclopropa[α]anthracen-6-one (19)

50.6 mg (38.2%), mp 91–92 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 7.84 (1H, s, H-8), 4.32 (1H, d, $J$ = 4.4 Hz, H-9β), 3.95 (3H, s, $OCH_3$-9), 3.49 (1H, d, $J$ = 4.4 Hz, H-1α), 3.40 (1H, t, $J$ = 3.1 Hz, H-2′), 3.29 (1H, t, $J$ = 2.9 Hz, H-2″), 2.97 (1H, d, $J$ = 3.1 Hz, H-3′), 2.79 (1H, d, $J$ = 2.9 Hz, H-3″), 1.67 (3H, s, $CH_3$-1′), 1.62 (3H, s, $CH_3$-1′), 1.35 (3H, s, $CH_3$-1″), 1.29 (3H, s, $CH_3$-1″), 1.28 (3H, s, $CH_3$-2), 1.22 (3H, s, $CH_3$-2); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 159.1 156.2, 154.5, 153.8, 133.5, 129.5, 118.9, 110.1, 108.3, 74.7, 64.3, 61.0, 59.9, 57.3, 48.4, 46.0, 45.3, 38.9, 26.4, 25.3, 22.7, 22.5, 21.2, 20.6; EIMS $m/z$ (rel. int.%): 442 ($M^+$, 67), 399 (71); HR-EIMS $m/z$ 442.1995 (calcd for $C_{25}H_{30}O_7$: 442.1992); Anal. ($C_{25}H_{30}O_7$) C, H.

#### 4.6.5. 9-Methoxy-2,2-dimethyl-1α,9β-dihydro-2*H*-1,3,5-trioxacyclopropa[α]anthracen-6-one (21)

92.6 mg (87.1%), mp 182–183 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$: 7.86 (1H, d, $J$ = 9.6 Hz, H-8), 6.56 (1H, s, H-4), 6.24 (1H, d, $J$ = 9.6 Hz, H-7), 4.27 (1H, d, $J$ = 4.3 Hz, H-9β), 3.97 (3H, s, $OCH_3$-9), 3.52 (1H, d, $J$ = 4.3 Hz, H-1α), 1.58 (3H, s, $CH_3$-2), 1.30 (3H, s, $CH_3$-2); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$: 160.5, 157.6, 156.6, 155.9, 137.9, 113.0, 109.8, 107.6, 102.2, 74.5, 64.5, 61.0, 45.3, 25.2, 23.1; EIMS $m/z$ (rel. int.%): 274 ($M^+$, 70), 245 (40), 218 (100); HR-EIMS $m/z$ 274.0842 (calcd for $C_{15}H_{14}O_5$: 274.0841); Anal. ($C_{15}H_{14}O_5$) C, H.

### 4.7. Quantification of HBsAg

Cells were seeded in 24-well plates at a density of $8 \times 10^4$ cells/well in DMEM medium containing 10% fetal calf serum. After 24 h of incubation, the cells were washed twice with phosphate-buffered saline (PBS), pH 7.0, and treated with various concentrations of drugs in serum-free DMEM for the time indicated. The HBsAg in the culture medium were measured by enzyme immunoassay (EIA) kit (Bio-Rad, CA, USA). The viability of cells was determined by a WST-1 cell proliferation assay. For this assay,[20] WST-1 (Roche, Germany) was added to each well and incubated for 0.5 h. The amount of formazan dye formed correlates to the number of metabolically active cells and was quantitated using a scanning multiwell spectrophotometer (ELISA reader) at the absorbance 450 nm.

### 4.8. In vitro anticancer assay

All stock cultures were grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 1500–7500 cells per well with compounds added from DMSO-diluted stock solutions. After 3 days in culture, attached cells were fixed with cold 50% trichloroacetic acid and then stained with 0.4% sulforhodamine B (SRB). The absorbance at 562 nm was measured using a microplate reader after solubilizing the bound dye. The mean $EC_{50}$ is the concentration of agent that reduces cell growth by 50% under the experimental conditions and is the average from at least three independent determinations that were reproducible and statistically significant. The following human tumor cell lines were used in the assay: A549 (non-small cell lung cancer), MCF-7 (human breast cancer), KB (nasopharyngeal carcinoma), and KB-VIN (vincristine-resistant KB subline). All cell lines were obtained from the

Lineberger Comprehensive Cancer Center (UNC-CH) or from ATCC (Rockville, MD) and were cultured in RPMI-1640 medium supplemented with 25 μM HEPES, 0.25% sodium bicarbonate, 10% fetal bovine serum, and 100 μg/mL kanamycin.[21]

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