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Original article

Therapeutic effects of cantharidin analogues without bridging ether oxygen on human hepatocellular carcinoma cells

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ABSTRACT

Previous research indicates that cantharidin, norcantharidin and their analogues exhibit anticancer activity due to their inhibition of cancer cell lines such as HL60, HT29 and L1210. The anticancer activities of cantharidin, norcantharidin and their analogues involve the suppression of serine/threonine protein phosphatases (PPs) activity. However, cantharidin is not suitable for cancer therapy because of its high cytotoxicity *in vitro* (IC_{50} = 21 μM in primary cultured rat hepatocytes). In this study, synthetic cantharidin analogues with a structure of aminothiazole compounds **3**–**9** and a structure of anhydride compounds **10**–**12** were screened for anticancer activities and cytotoxic effects on human hepatocellular carcinoma cell (HCC) lines HepG2, Sk-Hep1, and primary cultured rat hepatocytes. Experimental results indicated that compounds **3**–**9** did not perform as expected with regard to anticancer activity and exhibited lower cytotoxicity. Compound **10** promoted apoptosis in HepG2 (IC_{50} = 62 μM) and SK-Hep1 (IC_{50} = 151 μM) cell lines. Compounds **11** and **12** had anticancer potential similar to that of compound **10**. After treatment with compounds **3**–**12**, primary cultured rat hepatocytes exhibited no cytotoxicity (IC_{50} > 200 μM). By investigating the structure–activity relationship (SAR) of these analogues as a whole, this study suggests that the anhydride ether oxygen such as in cantharidin, norcantharidin and compounds **10**–**12** may be correlated with HCC survival suppression. The results further suggest that the elimination of bridging ether oxygen on the ring, such as in compounds **10**–**12**, can decrease cytotoxicity.

1. Introduction

Cantharidin (compound **1**) is a terpenoid isolated from Chinese blister beetles that exhibits significant toxicity against cancer cells [1]. Its pharmacological action includes the inhibition of xanthine oxides [2]. However, cantharidin has limited anticancer therapeutic potential because of its high mammalian toxicity [3]. Previous research shows that cantharidin (compound **1**) and norcantharidin (compound **2**) are potent serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A) inhibitors [4–6]. Both of these terpenoids have a slight inhibitory effect on the activity of PP2B, which is involved in intracellular signal transductions [7] and plays a major role in cell cycle progression. PP2A suppresses tumor growth [8] and is often used as a target molecule for tumor suppression purposes. The detailed mechanism underlying tumor suppression of PP2A remains unknown due to its involvement in diverse signaling pathways [9]. Regarding the binding mode, the structure activity relationship (SAR) of cantharidin analogues exhibits selective inhibitory activity on serine/threonine protein phosphatases [7]. The SAR of cantharidin analogues with inhibitory activity identifies bridging oxygen on the ring and anhydride and dicarboxylate structures as critical function groups [10]. In addition, the substitution of sulfur for oxygen in anhydride modified cantharidin analogues enhances the inhibition of PP2A, as the bridging oxygen on the ring structure creates a hydrogen-bonding site for PP2A [11]. Other studies consider PP2A to be a tumor suppressor protein [7].

A recent report shows that the antibiotic thiazole not only targets FoxM1 and efficiently inhibits its growth, but also induces apoptosis in human cancer cell lines A549 and SW480 [12]. FoxM1 inhibitors are potential anticancer drugs [13]. It is theoretically possible to synthesize thiazole as a functional group for anticancer activity with anhydrides and aminothiazole analogues **3**–**9**. These chemically synthesized compounds are comparable to cantharidin

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Table 1
The cytotoxicity of all tested compounds **1**–**12** in terms of their IC_{50} (μM) for different HCC cell lines or rat hepatocytes.

Compound	HepG2	SK-Hep1	Rat hepatocyte
1	11	34	21
2	74	126	75
3	>200	>200	>200
4	>200	>200	>200
5	>200	>200	>200
6	>200	>200	>200
7	>200	>200	>200
8	>200	>200	>200
9	>200	>200	>200
10	62	151	>200
11	121	199	>200
12	92	>200	>200

derivatives. In an attempt to decrease the toxicity of cantharidin, we synthesized three derivatives without bridging oxygen on the ring in this study. These derivatives were synthesized according to previously described methods and investigated for their influence on the protein phosphatase family and apoptosis in human hepatocellular carcinoma cells. We also examined cantharidin, cis-5-norbornene-endo-2,3-dicarboxylic anhydride (compound **10**), 4,4′-oxydiphthalic anhydride (compound **11**) and 5-(5-methyl-thiazol-2-ylimino)-4-oxa-tricyclo [5.2.2.02,6] undec-8-en-3-one (compound **12**) for cellular cytotoxicity in HepG2, SK-Hep1 and primary cultured rat hepatocytes [14]. The study aimed to identify compounds that decrease cellular cytotoxicity in normal rat hepatocytes and increase apoptotic activity in human hepatocellular carcinoma cells.

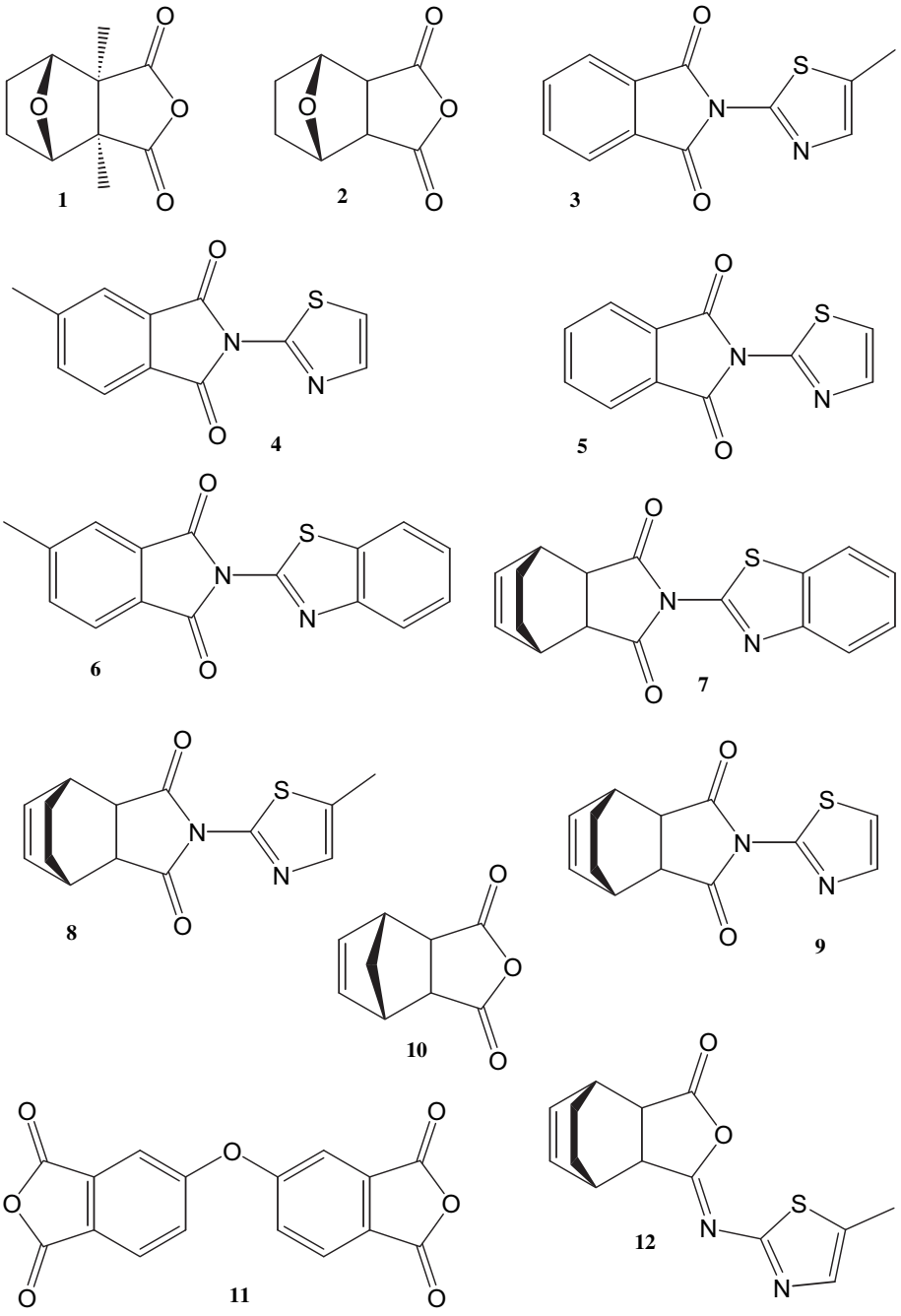


Fig. 1. Structures of cantharidin (compound **1**), norcantharidin (compound **2**), and compounds **3**–**12**.

2. Results and discussion

IC_{50} values were calculated (Table 1) for all 12 synthetic compounds (Fig. 1) in SK-Hep1, HepG2, and primary rat hepatocytes. Compounds **1**, **2**, **10**, **11**, and **12** had the lowest IC_{50} values in the HCC cell lines. Compounds **10**, **11**, and **12** were chemically modified after 48 h of exposure to anhydride and exerted cytotoxicity [15] in a dose-dependent manner in the HCC, SK-Hep1, and HepG2 cell lines, but not in the primary cultured rat hepatocytes (Fig. 2). The primary rat hepatocytes and HCC cell lines treated with compounds **1** and **2**, induced cytotoxicity at low concentrations (10–25 μM). However, the incubation of primary rat hepatocytes with compounds **10**, **11,** or **12** at a concentration of 200 μM for 48 h resulted in a nearly 80% survival rate. Fig. 2 shows the viability curve of these chemicals in the HepG2, SK-Hep1, and primary hepatocytes.

The influence of compounds **1**, **2**, **10**, **11**, and **12**, at a concentration of 50 μM for 48 h, on rat hepatocytes, HepG2 and SK-Hep1 cells was determined by examining morphological changes by light microscopy. DAPI staining [16] showed that treated HCC cell lines had chromatin condensation and nucleolus condensation, whereas the chromatin in the nuclei of untreated cells was homogeneously distributed. These results suggest that cancer cells create an apoptotic pathway in the presence of cantharidin analogues. Nucleolar condensation appeared in both cancer cell lines and primary cell cultures after treatment with cantharidin (50 μM) or norcantharidin (50 μM). Interestingly, chromatin was homogeneously distributed within the nuclei of rat hepatocytes treated with compounds **10**, **11**, or **12** at a concentration of 50 μM for 48 h implying that these analogues are toxic to cancer cells, but not to primary rat hepatocytes.

Effective anti-tumor agents such as 5-fluorouracil (5-FU) often induce neutropenia, the most common side effect, followed by gastrointestinal toxicity [17], especially at the high dosage necessary to achieve anti-tumor efficacy. A series of cantharidin analogues were synthesized and screened for their anticancer potential in an attempt to achieve relatively low toxicity to normal cells [18,19].

This study hypothesizes that the chemical structure of thiazole can be modified into cantharidin. This approach effectively enhances the apoptosis of cancer cells using a thiazole functional group. The IC_{50} values of compounds **3**–**9** initially seemed to show a decrease in anticancer activity and exhibited low cytotoxicity in primary rat hepatocytes. Motivated by these results, we redesigned compounds **10**–**12** using an anhydride structure. These compounds were easily synthesized by a solvothermal method.

The effects of the cantharidin analogues **10**–**12** were evaluated using HepG2 and SK-Hep1 and then compared with primary cultured rat hepatocytes as the normal cells. After 48 h, cell survival curves showed that compounds **10** ($IC_{50} = 253$ μM), **11** ($IC_{50} = 407$ μM), and **12** ($IC_{50} = 262$ μM) generated low cytotoxicity in rat hepatocytes whereas rat hepatocytes treated with cantharidin ($IC_{50} = 21$ μM) and norcantharidin ($IC_{50} = 75$ μM) manifested the highest cytotoxicity.

Previous research indicates that removing the methyl group from cantharidin to form norcantharidin decreases irritation and toxicity [3]. This study shows that norcantharidin exhibited less cellular toxicity than cantharidin (Fig. 2A), which is consistent with the previous study. Nevertheless, norcantharidin remained toxic to primary cultured rat hepatocytes. Compounds **1**, **2**, **10**, **11**, and **12** had different degrees of cytotoxicity on the HepG2 cell line (Fig. 2B), suggesting that these analogues modulate apoptosis. A possible explanation for this is that the selective suppression of protein phosphatases causes treated liver cancer cells to undergo apoptosis via different pathways. Treating SK-Hep1 with cantharidin analogues including compounds **1**, **2**, **10**, **11,** and **12** at a concentration of 25 μM for 48 h resulted in cytotoxicity (Fig. 2C). DAPI staining and fluorescence microscopy detected induced apoptosis

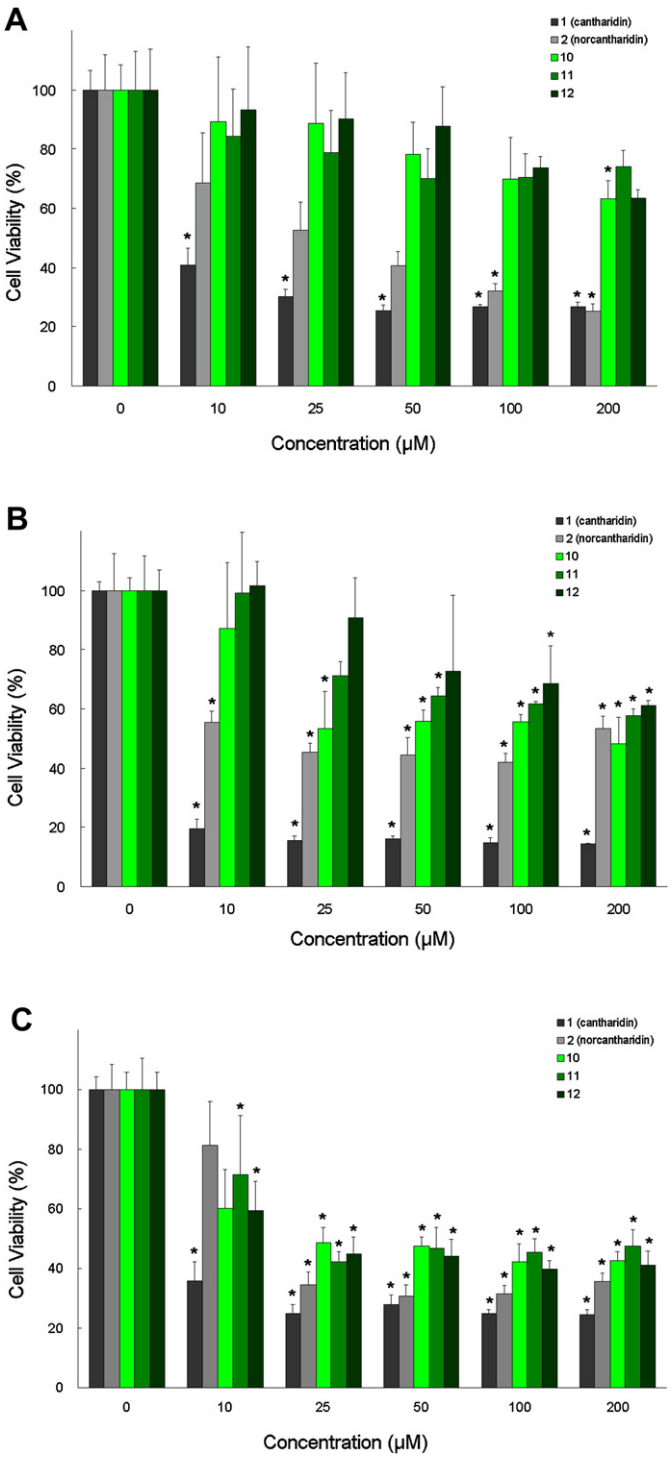
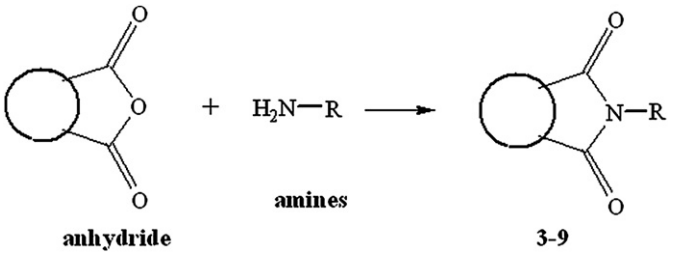


Fig. 2. Cell cytotoxicity assay of cantharidin (**1**), norcantharidin (**2**), and compounds **10**, **11,** and **12** on (A) primary cultured rat hepatocytes, (B) HepG2, and (C) SK-Hep1 cell lines. (*$P < 0.01$, compared with the control).

in human hepatocellular carcinoma cells by the anhydride modified cantharidin analogues [20]. These results shown that the bridging ether oxygen on the ring and the anhydride structure of the cantharidin analogues are both crucial for the inhibitory activity on protein phosphatases [9]. Even though cantharidin analogues are PP1, PP2A, and PP2B inhibitors, an anhydride or dicarboxylate structure is crucial for inhibitory activity [10]. Cantharidin is a potent PP1 and PP2A inhibitor, but not an efficient PP2B inhibitor.



Scheme 1. Compounds **3**–**9** were synthesized in a high-pressure sealed tube.

Although PP2A might be a tumor suppressor, it is difficult to manipulate with pharmaceutical agents because it acts via various signaling pathways [21].

Cantharidin and norcantharidin are promising PP1, PP2A, and PP2B selective inhibitors, and induce serious cytotoxicity in primary cultured rat hepatocytes. We modified compounds **3**–**9** for examination. To confirm that the anhydride structure is a critical structure for selective anticancer activity, IC_{50} values were used to compare synthetic compounds **3**–**9**, which have a thiazole structure, with compounds **10**–**12**, which have an anhydride structure. Table 1 shows that compounds **10**–**12** were cytotoxic to HCC, while compounds **3**–**9** were not. Compounds **3**, **8**, and **12** were screened with the same thiazole functional group, but only compound **12** inhibited the growth of HepG2 (Fig. 2B) and SK-Hep1 (Fig. 2C) and induced apoptosis. These results suggest that the anhydride structure is crucial for anticancer activity.

When observing the cytotoxicity of cantharidin and norcantharidin in primary cultured rat hepatocytes, the bridging ether oxygen differed between compounds **3**–**12**, but none of these compounds were cytotoxic in primary hepatocytes using IC_{50} values for comparison (Table 1). This indicates that bridging ether oxygen on the ring of cantharidin analogues is a critical factor in inducing or increasing cytotoxicity in primary cultured rat hepatocytes. Based on the SAR of these cantharidin analogues and aminothiazole derivates, deleting the bridging ether oxygen on the ring decreased the hepatocyte cytotoxicity of these derivates, and the anhydride modified structure promoted apoptosis in human hepatocellular carcinoma cells.

3. Conclusion

The results of this study show that cantharidin (compound **1**) and norcantharidin (compound **2**) are potent serine/threonine protein phosphatases inhibitors due to their anhydride structure. The anhydride modified cantharidin analogues (compounds **10** and **11)** and aminothiazole derivative (compound **12)** reduced toxicity in rat hepatocytes compared to their parent compounds. These results suggest that removing the bridging ether oxygen on the ring can decrease their cytotoxicity and preserving the anhydride structure may provide the necessary structure for HCC apoptosis.

4. Experimental

^1H NMR spectra were recorded on a Bruker DRX-500 spectrometer in a $CDCl_3$ solution using the corresponding solvent as the internal reference. Compound purity was >95% determined by ^1H NMR spectral analysis.

4.1. Chemistry

Cantharidin was extracted from Chinese blister beetles using a previously described method [1] and was designated as compound **1**. Norcantharidin, cis-5-norbornene-endo-2,3-dicarboxylic anhydride, and 4,4′-oxydiphthalic anhydride (compounds **2**, **10**, and **11** respectively) were purchased from Sigma–Aldrich (USA) and were used without further purification. Endo-bicyclo[2,2,2]oct-5-ene-2, 3-dicarboxylic anhydride and 2-amino-5-methylthiazole were purchased from Acros (USA). Toluene and triethylamine were freshly distilled from sodium under nitrogen. 5-(5-methyl-thiazol-2-ylimino)-4-oxa-tricyclo [5.2.2.02,6] undec-8-en-3-one (compound **12**) and compounds **3**–**9** were synthesized by heating various anhydrides and aminothiazole derivatives to 200 °C with 3 mL of dry toluene and 1–2 mL of triethylamine in a high-pressure sealed tube (Büchi glasuster 0032) to obtain a good yield of phenylphthalimide derivatives (Scheme 1).

4.2. General procedure for preparing imides

Scheme 1 shows the synthesis of the desired imide derivatives of anhydrides and related compounds with primary aminothiazole. Compounds **3**–**9** (at 0.2 mM) were dissolved in 3 mL of dry toluene and 1–2 mL of triethylamine (TEA), after primary benzylamine and various aromatic amines (1.2 eq. and 1.0 eq. respectively) were added. The mixtures were heated to ca. 200 °C for 2 h and then allowed to cool to room temperature. The mixtures were heated a second time to remove the solvent, and the residues were purified by silica gel column chromatography.

4.2.1. 2-(5-Methyl-thiazol-2-yl)-isoindole-1,3-dione (**3**)

Yield 91%; m.p. 173–175 °C. ^1H NMR ($CDCl_3$):δ 2.26 (3H, s, CH_3), 7.82 (2H, d, J = 6.8 Hz, phenyl H-5, 6), 7.97 (2H, d, J = 6.8 Hz, phenyl H-4, 7), 8.02 (1H,s, thiazolyl H-3) ^{13}C NMR: 11.53 (CH_3), 124.36 (C-5″), 128.06 (C-4, C-7), 130.42 (C-3, C-8), 132.02 (C-5, C-6), 135.35 (C-4″),137.55 (C-2″), 166.26 (C-2), 166.84 (C-9).

4.2.2. 5-Methyl-2-thiazol-2-yl-isoindole-1,3-dione (**4**)

Yield 90%; m.p. 137–142 °C. ^1H NMR ($CDCl_3$): δ 2.42 (3H, s, CH_3), 6.23 (1H, d, J = 3.6 Hz, thiazolyl H-4), 6.78 (1H, d, J = 3.5 Hz, thiazolyl H-3), 7.49 (1H, d, J = 6.8 Hz, phenyl H-5), 7.81 (1H, d, J = 6.8 Hz, phenyl H-7), 7.95 (1H, d, J = 6.9 Hz, phenyl H-4) ^{13}C NMR: 21.43 (CH_3), 124.36 (C-5″), 128.08 (C-4,C-7), 130.82 (C-3, C-8), 132.65 (C-5, C-6), 135.93 (C-4″), 139.90 (C-2″), 160.64 (C-2), 167.0 (C-9).

4.2.3. 2-(3-Phenyl-[1,2,4]thiadiazol-5-yl)-isoindole-1,3-dione (**5**)

Yield 93%; m.p. > 300 °C. ^1H NMR ($CDCl_3$): δ 2.42 (3H, s, CH_3), 6.23 (1H. d, J = 3.6 Hz, thiazolyl H-4), 6.78 (1H, d, J = 3.5 Hz, thiazolyl H-3), 7.45 (1H, d, J = 6.9 Hz, phenyl H-5), 7.82 (1H, d, J = 6.9 Hz, phenyl H-7), 7.95 (1H, d, J = 6.9 Hz phenyl H-4) ^{13}C NMR: 124.36 (C-5″), 128.08 (C-4, C-7), 130.82 (C-3, C-8), 132.65 (C-5, C-6), 135.93 (C-4″), 139.90 (C-2″), 160.64 (C-2), 167.0 (C-9).

4.2.4. 2-Benzothiazol-2-yl-5-methyl-isoindole-1,3-dione (**6**)

Yield 62%; m.p. 160–163 °C.^1H NMR ($CDCl_3$): δ 1.27 (3H, s, CH_3), 7.55 (1H, d, J = 6.8 Hz, phenyl H-5), 7.61 (1H,d, phenyl H-2′), 7.74 (1H, d, J = 6.9 Hz, phenyl H-7), 7.8 (1H, d, J = 6.9 Hz, phenyl H-3′), 7.81 (1H, d, J = 6.9 Hz, phenyl H-1′), 7.89 (1H, d, phenyl H-4′), 8.11 (1H, d, phenyl H-4) ^{13}C NMR: 21.28 (CH_3), 121.1(C-5″), 121.57 (C-8″), 125.32 (C-6″), 126.37 (C-7″), 128.10 (C-4, C-7), 130.57 (C-9″), 130.95 (C-3, C-8), 132.78 (C-4″), 136.05 (C-5,C-6), 166.42 (C-2), 166.88 (C-9).

4.2.5. 4-Benzothiazol-2-yl-4-aza-tricyclo [5.2.2.02,6]undec-8-ene-3,5-dione (**7**)

Yield 60%; m.p. 193–195 °C. ^1H NMR ($CDCl_3$): δ 1.45 (2H, d, J = 7.6 Hz, ethenyl H-8), 1.68 (2H, d, J = 7.6 Hz, ethenyl H-9), 3.10 (2H, s, H-4, 7), 3.45 (2H, s, H-7a, 3a), 6.28 (2H, d, H-5, 6), 7.40 (1H, d, phenyl H-2′), 7.46 (1H, d, J = 6.8 Hz, phenyl H-3′), 7.84 (1H, d, J = 6.8 Hz, phenyl H-1′), 8.08 (1H, d, phenyl H-4′) ^{13}C NMR: 23.54

(C-3, C-10), 32.11 (C-4, C-7), 44.16 (C-8, C-9), 121.13(C-5″), 123.63(C-8″), 125.63(C-6″), 126.37(C-7″).

4.2.6. 4-(5-Methyl-thiazol-2-yl)-4-aza-tricyclo[5.2.2.02,6]undec-8-ene-3,5-dione (**8**)

Yield 90%; m.p. 165–168 °C. ^{1}H NMR ($CDCl_3$): δ 1.44 (2H, d, J = 7.4 Hz, ethenyl H-8), 1.56 (3H, s, CH_3), 1.66 (2H, d, J = 7.6 Hz, ethenyl H-9), 3.03 (2H, s, H-4, 7), 3.25 (2H, s, H-7a, 3a), 6.31 (1H, d, H-5, 6), 7.54 (1H, s, thiazolyl H-3).

4.2.7. 4-Thiazol-2-yl-4-aza-tricyclo [5.2.2.02,6] undec-8-ene-3,5-dione (**9**)

Yield 88%, m.p. 244–245 °C. ^{1}H NMR ($CDCl_3$): δ 1.35 (2H, d, J = 7.5 Hz, ethenyl H-8), 1.64 (2H, d, J = 7.5 Hz, ethenyl H-9), 3.05 (2H, s, H-4, 7), 3.26 (2H, s, H-7a, 3a), 6.24 (2H, m, H-5, 6), 7.27 (1H, d, thiazolyl H-4), 7.73 (1H, d, thiazolyl H-3) ^{13}C NMR: 121.46(C-5″), 126.73(C-6, C-8), 131.20(C-5, C-9), 131.38(C-7), 134.80 (C-4, C-10), 140.48(C-3, C-11), 152.21 (C-2″), 163.22 (C-2, C-13).

4.3. Cell cultures

The hepatocellular carcinoma cell lines HepG2 and SK-Hep1 were obtained from the American Tissue Culture Collection (ATCC, USA) and grown to confluence in Dulbecco's minimum essential medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 2 mM Glutamine, 1% non essential amino acids (NEAA) and a 1% antibiotic mixture (50 μg/mL of penicillin, 50 μg/mL of streptomycin, and 10 μg/mL of neomycin/mL) at 37 °C in a humidified atmosphere of 5% CO_2. The primary rat hepatocytes were isolated from Wistar rats as previously described [14]. The isolated hepatocytes (viability > 90%) were prepared by liver perfusion with collagenase IV (Sigma) and separated by density centrifugation in Percoll solution (GE Healthcare Bio-Sciences AB). Cells were cultured in William's E Medium (Sigma) supplemented with 10% fetal bovine serum (Gibco), 2 mM Glutamine, 1% non essential amino acids (NEAA) and a 1% antibiotic mixture (50 μg/mL of penicillin, 50 μg/mL of streptomycin, and 10 μg/mL of neomycin/mL) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. HepG2, SK-Hep1 cells and primary cultured rat hepatocytes were seeded in 24-well plates or in 100-mm culture dishes. After the cells adhered overnight, they were treated with reagents or vehicle only (control samples) in triplicate.

4.4. Determination of vitro cytotoxicity

The 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used for measurement of cytotoxicity [16]. Primary rat hepatocytes, SK-Hep1, and HepG2 were incubated in 24-well plates at a density of 1×10^5 cells/well for 12 h and treated with test chemicals or vehicle for 48 h. The culture supernatants were removed and MTT (Sigma, USA) in phosphate buffered saline (PBS; pH 7.4) was added to each well. After 4 h of incubation at 37 °C, the MTT solution was removed and DMSO was added to dissolve the formazan crystals. The absorbance was determined at 540 nm in a Flexstation 3 device (MDS Analytical Technologies, Canada). The percentage of viability was calculated using the following equation: (test-background)/(control-background) × 100.

4.5. Statistical analysis

The results are shown as the means ± SD from at least three independent experiments (involving three to six observations for each treatment). Only primary rat hepatocytes freshly isolated with a viability > 90% were used. Statistical analysis was performed using one-way analysis of variance, while the Student's t test was applied to paired samples.

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