

Induction of apoptosis on carcinoma cells by two synthetic cantharidin analogues

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Abstract. Cantharidin isolated from *Mylabris caraganae* and other insects has been used as an anti-cancer drug in China for many years. However, its toxicity on the renal system and suppression effect on bone marrow limits its usage clinically. Based on the core structure of cantharidin, we have chemically synthesized two cantharidin analogues (compounds 2 and 3). The cytotoxic activity of these analogues was demonstrated on the Hep3B hepatocellular carcinoma, MDA-MB231 breast cancer, A549 non-small cell lung carcinoma and KG1a acute myelogenous leukaemia (AML) cell lines by monitoring the intracellular adenosine triphosphate level. Morphological changes in these cancer cell lines, including cell shrinkage and loss of adherent potential, were readily observed. By making use of the KG1a AML cells as a test model, we further found that mitochondrial membrane potential depolarization and reduction of intracellular bcl-2 anti-apoptotic protein level were involved. These resulted in the activation of caspase 3 protease activity and oligonucleosomal length DNA fragment formation as detected by both time resolved fluorescence technology-based caspase activity assay and TdT-mediated dUTP nick end-labelling assay.

Introduction

Cancer treatment remains crucial in the 21st century and much effort has been made for drug discovery as chemotherapy is not only important during new case presentation but also critical during disease relapse. Cantharidin has been used for the treatment of cancer, including hepatocellular

carcinoma (HCC) and acute myelogenous leukaemia (AML), in China. The compound can be isolated naturally from many types of commonly found insect, including *Mylabris caraganae*. However, its highly toxic nature limits its usage (1,2). Thus, alternatives are searched for in anti-cancer treatment. One commonly recognized example is norcantharidin (demethylated form of cantharidin), which was shown to have anti-tumour potential but with less adverse effects (3). Norcantharidin is used in the treatment of hepatitis and hepatoma in China (4). Potential new anti-tumour agents from a combination of norcantharidin with an inorganic metallic atom, platinum moiety, were reported which were shown to bear cytotoxicity on a series of human cancer cell lines and nude mice xenograft (5). Furthermore, modified cantharidinimide derivatives with aliphatic, aryl and pyridyl groups also exhibited a certain degree of cytotoxic activity on HepG2 hepatoblastoma and HL-60 acute promyelocytic leukaemia cells (6). Therefore, it seems that a modification of cantharidin may provide a new direction for drug discovery in cancer therapy.

Based on the core structure of cantharidin, we have synthesized a compound (compound 2) by making a dimer of cantharidin analogue (compound 1). Although cantharidin analogue (compound 3) was reported to possess anti-cancer activity on two HCC cell lines, Hep3B and SK-Hep-1 (7), the possible mechanism involved has not been reported. We tested the *in vitro* cytotoxicity of compounds 2 and 3 on a panel of cancer cell lines including the Hep3B HCC, MDA-MB231 breast cancer, A549 non-small cell lung carcinoma and KG1a AML by monitoring the intracellular adenosine triphosphate (ATP) level and morphological investigation. Using KG1a as a demonstration model, we studied the possible apoptotic mechanism involved, including mitochondrial membrane potential depolarization, change of bcl-2 anti-apoptotic protein level in compound 2 and compound 3 induced cell death. Furthermore, caspase 3 activation and DNA fragmentation formation were investigated. The effect of these analogues on the regenerating potential of primary bone marrow culture obtained from patients with non-malignant haematological disorders and AML were studied by methylcellulose colony formation assay.

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Materials and methods

Synthesis of compound 1. Unless otherwise indicated, all reactions were carried out in a nitrogen atmosphere. NMR spectra were recorded on a Varian 500 MHz Fourier transform spectrometer. ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra were recorded relative to residual protiated solvent; a positive value of the chemical shift denotes a resonance downfield from TMS. Mass analyses were performed on a Finnigan model Mat 95 ST mass spectrometer. Cantharidin was purchased from Sigma-Aldrich. Piperazinium dichromate was synthesized according to the literature (8). All other chemicals were purchased from commercial suppliers and were used without further purification. Dichloromethane was freshly distilled from calcium hydride under nitrogen. Toluene and triethylamine were freshly distilled from sodium under nitrogen. All reactions were monitored by analytical thin-layer chromatography (TLC) on Merck aluminum-precoated plates of silica gel 60 F₂₅₄ with detection by spraying with 5% (w/v) dodecamolybdophosphoric acid in ethanol or 5% (w/v) ninhydrin in ethanol and subsequent heating. Merck silica gel 60 (230-400 mesh) was used for flash chromatography.

A mixture of cantharidin (31.2 mg, 0.16 mmol), 5-amino-1,3,4-thiadiazole-2-thiol (163.6 mg, 1.2 mmol), dried toluene and dried triethylamine (6 ml; 2:1, v/v) in a sealed tube was heated for 2.5 h at 180°C. Concentration followed by flash chromatography gave the product (49.8 mg) in quantitative yield; ^1H NMR (CDCl_3 , 500 MHz): δ 10.92 (brs, H), 4.72 (dd, $J=3$ Hz and 2 Hz, 2H), 1.88-1.72 (m, 4H), 1.27 (s, 6H); ^{13}C NMR (CD_3COCD_3): δ 205.7, 177.9, 84.8, 54.8, 23.8, 12.0; MS (ESI) m/z (%): 334 (M^+Na , 89), 117 (100), 149 (56).

Synthesis of compound 2. A mixture of compound 1 (30.9 mg, 99 μmol) and piperazinium dichromate (91 mg, 0.3 mmol) in dried dichloromethane was stirred at room temperature for 24 h. Concentration of the resulting mixture followed by flash chromatography gave the corresponding disulfide compound 2 (19.3 mg) in 31% yield; ^1H NMR (CDCl_3 , 500 MHz): δ 8.12 (d, $J=8.5$ Hz, 1H), 7.90-7.88 (m, 1H), 1.28 (s, 6H); ^{13}C NMR (CDCl_3): δ 176.8, 164.7, 155.4, 84.4, 54.5, 23.7, 13.0; MS (ESI) m/z (%): 643 (M^+Na , 100), 621 ($\text{M}^+\text{+1}$, 61), 381 (41).

Synthesis of compound 3. A mixture of cantharidin (10 mg, 51 μmol), 2-aminobenzothiazole (38 mg, 0.25 mmol), dried 4 Å MS (ca. 0.5 g), dried toluene and dried triethylamine (3 ml; 2:1, v/v) in a sealed tube was heated for 3 h at 150°C. Concentration followed by flash chromatography gave the product compound 3 (16 mg) in 96% yield; ^1H NMR (CDCl_3 , 500 MHz): δ 8.12 (d, $J=8.5$ Hz, 1H), 7.90-7.88 (m, 1H), 7.50 (dt, $J=8.0$ Hz and 1.0, 1H), 7.44-7.40 (m, 1H), 1.88-1.86 (m, 2H), 1.78-1.76 (m, 2H), 1.30 (s, 6H); ^{13}C NMR (CDCl_3): δ 178.2, 151.6, 149.1, 133.0, 126.4, 125.6, 123.6, 121.1, 84.4, 84.4, 54.2, 23.8, 13.0; MS (ESI) m/z (%): 329 ($\text{M}^+\text{+1}$, 100), 351 (M^+Na , 45).

Cell lines and cell culture. A total of four cell lines were used in this study; the hepatocellular carcinoma Hep3B, breast cancer MDA-MB231, non-small cell lung cancer A549, and acute myelogenous leukaemia KG1a. All of them were

obtained from American Type Culture Collection (ATCC) and maintained in Iscove's modified Dulbecco's medium (JRH BioSciences) supplemented with 5% fetal bovine serum (Hyclone) together with penicillin and streptomycin (Invitrogen). They were maintained in a 5% carbon dioxide humidified incubator at 37°C.

Anti-proliferative and cytotoxic tests. The different types of cancer cells were seeded in a 96-well microtitre plate on the first day. On the second day, the growth medium was changed and different concentrations of compounds 2 and 3 (6.25, 12.5 and 25 $\mu\text{g/ml}$) were added. In each case, the maximum volume of solvent (dimethylsulfoxide, DMSO) contribution was less than 0.05%. After 48 h, the intracellular ATP level was measured by using the ATPlite-1step kit (Perkin-Elmer, Life Sciences) for anti-proliferative and cytotoxic activity test. The resulting luminescence was recorded according to the instruction manual provided (9).

Morphological changes of cantharidin analogue-treated cells. Cancer cells were seeded as mentioned above and then cantharidin analogues at a concentration of 25 $\mu\text{g/ml}$ were added. Any morphological changes after 24 h were observed under an inverted microscope (9).

Mitochondrial membrane potential depolarization. JC-1 was used as the detection probe (Molecular Probes) for the change of mitochondrial membrane potential. Briefly, after incubating KG1a cells with compounds 2 and 3 for 48 h, they were stained with JC-1. Mitochondria with a high membrane potential retain JC-1 as J-aggregate and give a red signal. In the case of mitochondrial membrane potential depolarization, JC-1 is lost from mitochondria, resulting in a shift from red to green. Thus, a change in the green:red fluorescence intensity ratio from a low to high value indicates a collapse in mitochondrial membrane potential (10).

Change of bcl-2 protein level. After incubation with both cantharidin analogues for 48 h, KG1a cells were washed with PBS and then fixed with fixative. Afterwards, the cells were permeabilized and bcl-2 antibody (Dako) was added to stain the intracellular bcl-2 protein. Samples were subsequently analyzed by quantifying to their corresponding relative fluorescence units. The results were normalized with their corresponding protein contents and compared with the control.

Caspase 3 activation. To study whether the caspase 3 protease activity increased after compound 2 or 3 treatment of KG1a cells, we used a time resolved fluorescence technology-based caspase 3 activity kit from Trupoint, Perkin-Elmer (Finland). Cantharidin analogue-treated cells (48 h) were washed with PBS and the total cellular protein extract was prepared. Afterwards, caspase 3 specific substrate and detection buffer were added and the caspase 3 activity was analysed according to the instruction manual provided. The protein content was determined using the Bradford method. The results were normalized with their corresponding protein contents and compared with the control. Finally, the samples were analyzed by quantifying their corresponding relative fluorescence units. The results were normalized with their corresponding protein contents and compared with the control (10).

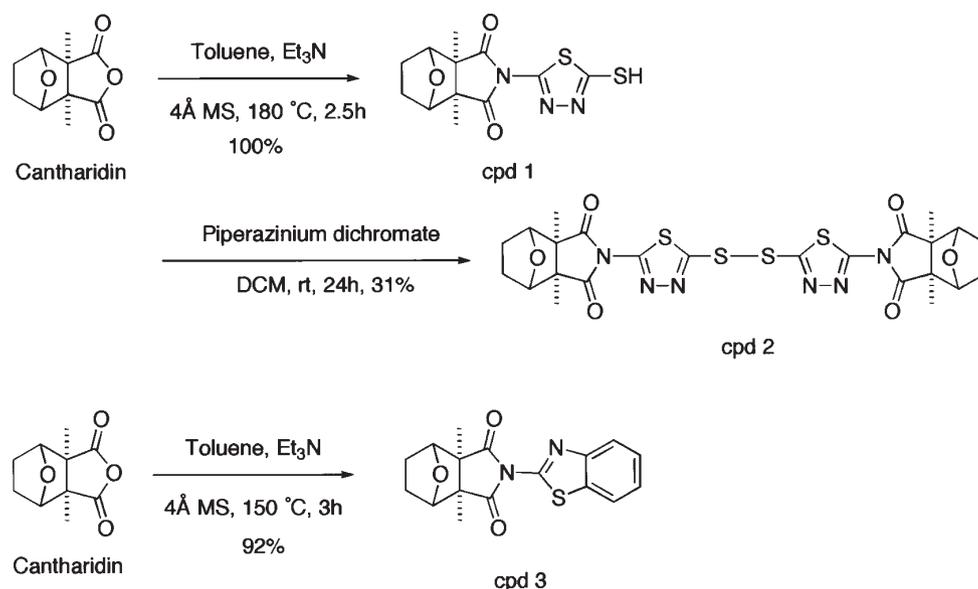


Figure 1. Synthesis scheme of compounds 1, 2 and 3.

In situ cell death detection. Cancer cells were seeded and the medium was changed as mentioned above. After incubation with cantharidin analogues for 48 h, KG1a cells were washed and collected. They were further incubated with 4% paraformaldehyde solution for 60 min. Then they were washed and permeabilized with 70% cold ethanol for 2 min on ice. Afterwards, the cells were washed and incubated with TdT-mediated dUTP nick end-labelling assay (TUNEL) reaction mixture (Roche) for 1 h at 37°C. Finally, the cells were washed and resuspended in phosphate-buffered saline (11). Samples were subsequently analyzed by quantitating their corresponding relative fluorescence units. The results were further normalized with their corresponding protein contents and compared with the control (10).

Bone marrow sample preparation. Bone marrow samples were obtained from 4 patients with their informed consent (2 with non-malignant haematological disorders and 2 patients with AML). Mononuclear cells were enriched and harvested immediately after Ficoll density centrifugation (Pharmacia L.K.B.) and washed with phosphate-buffered saline (PBS). Cell pellets were resuspended in RPMI-1640 culture medium and cell viability was determined by trypan blue exclusion assay as previously described (12,13).

Colony formation assay. Mononuclear cells isolated from the bone marrow aspirate of patients with non-malignant haematological disorders and AML were mixed with methylcellulose (Stem Cell, Canada) together with cantharidin analogues at a concentration of 12.5 µg/ml. Plates were incubated for 10-14 days. Finally, the total numbers of viable colonies stained with 3-(4,5-dimethylthiazol-2-yl) 2,3-diphenyl-tetrazolium bromide (MTT) were counted under an inverted microscope.

Results

Synthesis of cantharidin analogues. Based on the principal chemical structure of the mother compound, cantharidin, we

synthesized compounds 1-3, respectively. The details of the chemical reactions and the characterization involving the ¹H spectra are shown in Figs. 1 and 2 respectively. Both compound 2 and compound 3 were dissolved in DMSO to a concentration of 50 mg/ml and clear solutions were obtained. They were stored in aliquot at -20°C. For the biological assay, the maximum concentration used for both cantharidin analogues was 25 µg/ml; thus always making the contribution of solvent less than 0.05%.

Both cantharidin analogues induced apoptosis on carcinoma cell lines. We used a luminescence-based assay to monitor the intracellular ATP level in order to study the possible activity of compounds 2 and 3 on a series of carcinoma cell lines after 48 h of continuous incubation. Compound 2 showed more specific inhibitory and cytotoxic activities on both the Hep3B HCC and the KG1a AML cell lines. Their cytotoxic effect was weaker on both A549 non-small cell lung cancer and MDA-MB231 breast cancer (Fig. 3A). Interestingly, the cytotoxic pattern of compound 3 on carcinoma cell lines was similar to that of compound 2 (Fig. 3B). When comparing compound 2 with compound 3, both showed a similar potency towards Hep3B HCC as the survival percentage was dropped to less than 50% when both cantharidin analogues were at 6.25 µg/ml. The major difference between them was observed in KG1a, where compound 2 was still effective at 12.5 µg/ml but a higher concentration was required for that of compound 3.

Morphological change of carcinoma cells. After incubation with compounds 2 and 3 at 25 µg/ml for 24 h, the cellular morphology of all four cancer cell lines was observed under an inverted microscope and cell shrinkage was commonly found. All of the three solid tumour cell lines lost their adherent property and colony formation ability (Figs. 4 and 5).

Mitochondria was involved in both compound 2- and compound 3-induced apoptosis. By using the JC-1 fluorescence mitochondria specific probe, we examined whether the

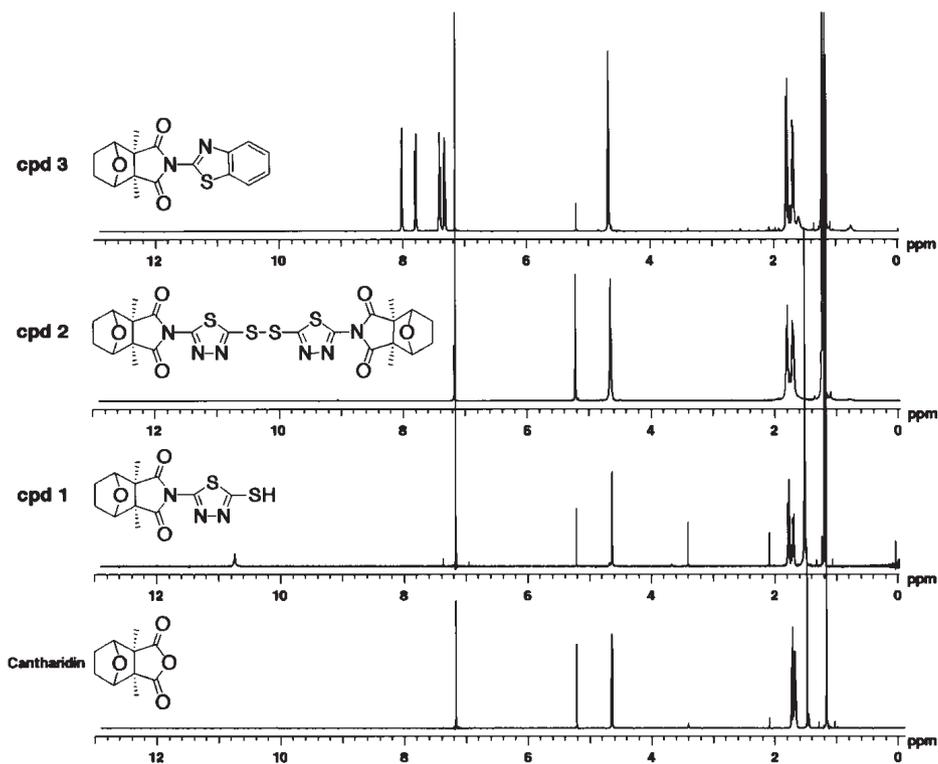


Figure 2. A comparison of the ¹H NMR spectra of compounds 1, 2 and 3 with cantharidin.

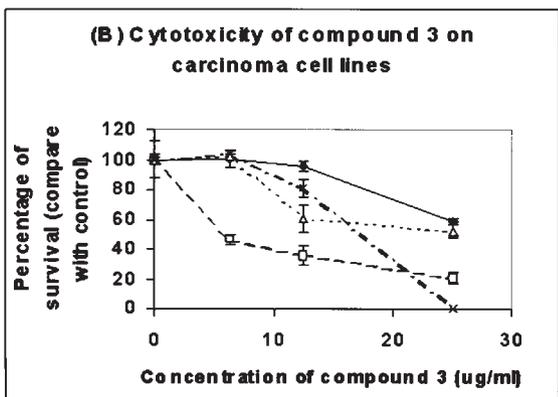
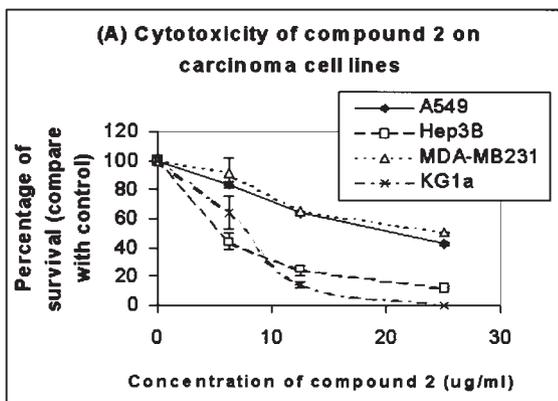


Figure 3. Intracellular ATP content assay of the carcinoma cell lines, A549 (non-small cell lung cancer), Hep3B (hepatocellular carcinoma), MDA-MB231 (breast cancer) and KG1a (acute myelogenous leukaemia), after 48 h of incubation with (A) compound 2 and (B) compound 3. The results are shown as the mean \pm standard deviation (SD) of triplicate experiments. This is one of the representative results of three independent experiments.

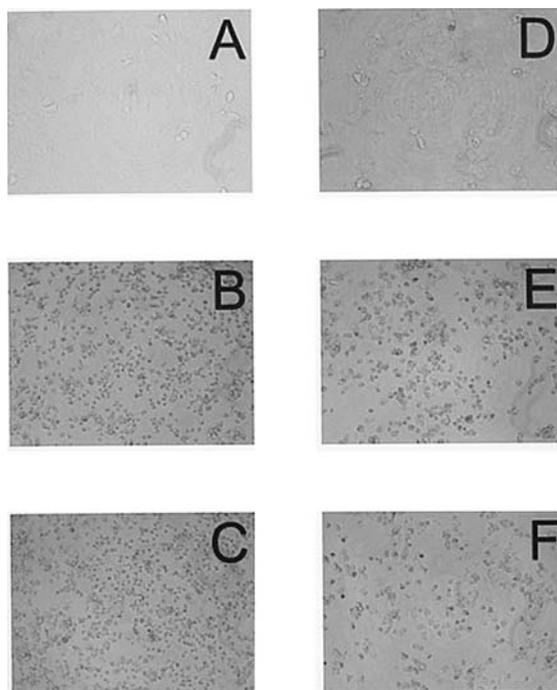


Figure 4. Morphological investigation for the action of compounds 2 and 3 (25 μ g/ml) on cancer cell lines after 24 h. (A-C) A549 non-small cell lung cancer. (D-F) Hep3B hepatocellular carcinoma. A and D were treated with vehicle, B and E were treated with compound 2 while C and F were treated with compound 3.

mitochondrial-dependent pathway was involved in cantharidin analogue-induced cell death. As shown in Fig. 6A, both cantharidin analogues were able to cause mitochondrial

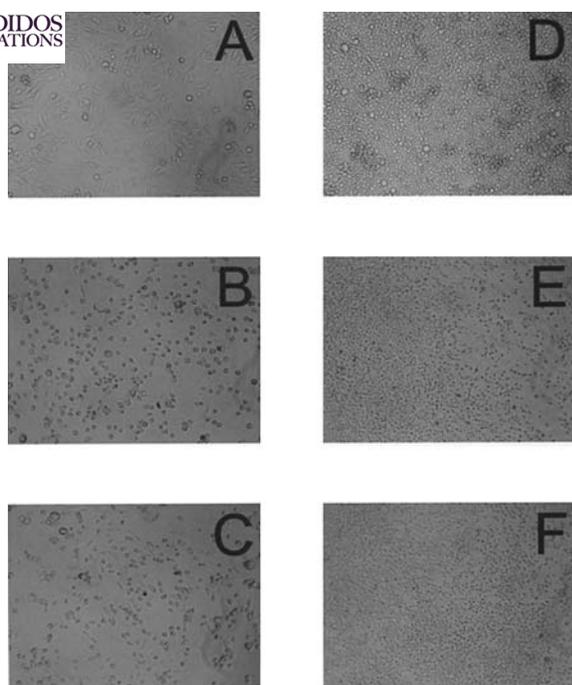


Figure 5. Morphological investigation for the action of compounds 2 and 3 ($25 \mu\text{g/ml}$) on cancer cell lines after 24 h. (A-C) MDA-MB231 breast cancer. (D-F) KG1a acute myelogenous leukaemia. A and D were treated with vehicle, B and E were treated with compound 2 while C and F were treated with compound 3.

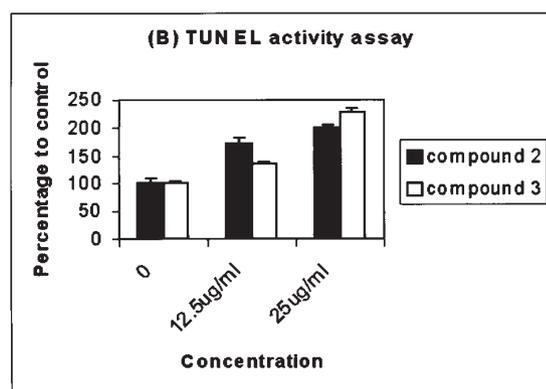
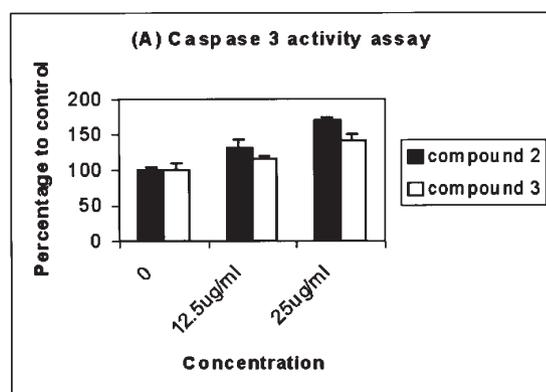


Figure 7. (A), caspase 3 activity assay for the action of cantharidin-related compounds on the KG1a cell after 48 h of incubation. (B), TUNEL activity assay for the action of cantharidin analogues on the KG1a cell after 48 h of incubation for the detection of oligonucleosomal length DNA fragment formation. In both (A) and (B), results are shown as mean \pm SD of triplicate experiments. This is one of the representative results of three independent experiments.

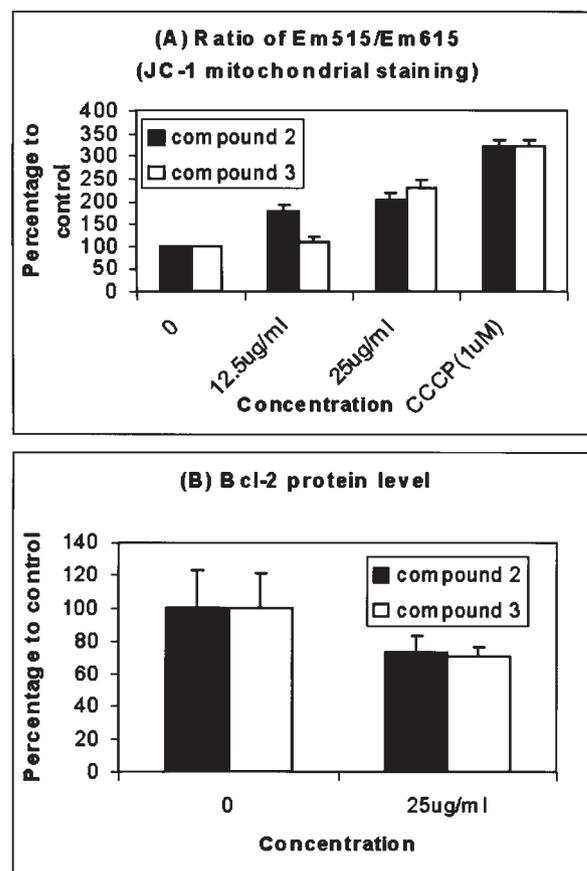


Figure 6. (A), synthetic cantharidin analogues induced a collapse of mitochondrial membrane potential in KG1a leukaemia cells by staining with JC-1 probe. An increment of emission 515 nm to 615 nm after an excitation at 485 nm indicated that there was a collapse in mitochondrial membrane potential. CCCP is an ionophore which will cause a dissipation of the proton gradient built up on the inner mitochondrial membrane that acts as a positive control. Each experiment was performed in triplicate and a mean was obtained. Results are shown as mean \pm SD of three independent experiments. (B), detection of intracellular bcl-2 protein in the KG1a cells after cantharidin analogue incubation. Each experiment was performed in triplicate and a mean was obtained. Results are shown as mean \pm SD of one representative experiment.

membrane potential depolarization in KG1a AML cells. This happened in a dose-dependent manner under the action of both cantharidin analogues. Furthermore, the anti-apoptotic protein bcl-2 level decreased after compound 2 and compound 3 incubation at $25 \mu\text{g/ml}$ (Fig. 6B).

Increment of caspase 3 activity and TUNEL activity. We further analyzed whether the central machinery of apoptosis, caspase 3 activity, was affected by compounds 2 and 3. The results from Fig. 7A clearly demonstrated that caspase 3 activity increased significantly. Simultaneously, TUNEL activity also was elevated after treating the KG1a cells with both cantharidin analogues (Fig. 7B) and both activity increments were dose-dependent.

Effects of compounds 2 and 3 on the regeneration of non-malignant and AML bone marrow. We attempted to use long-term incubation of both cantharidin analogues to test their effects on the regeneration potential of bone marrow cells isolated from 2 patients with non-malignant haematological

Table I. Inhibitory effects of compounds 2 and 3 towards the colony formation potential of non-malignant haematological disordered bone marrow and bone marrow aspirate isolated from patient with AML.^a

	BM1	BM2	AML1	AML2
Compound 2	17%	14%	0	0
Compound 3	13%	12%	0	0

^aThe results are shown as the mean percentage of total number of colony formation when compared with vehicle control from triplicate experiments. See Materials and methods for details.

disorders and 2 patients with AML. Table I showed the resulting regeneration efficiency as compared with the untreated control (with 0.025% DMSO). For both non-malignant bone marrow samples, 83%-88% of stem-cell regeneration suppression was observed after cantharidin analogue incubation at a concentration of 12.5 $\mu\text{g/ml}$ (Fig. 8) while a complete suppression of leukaemic blast progenitor cell regeneration was observed in both cases of AML.

Discussion

Compound 2 is a new compound derived from the dimerization of compound 1. Compound 3 was reported to bear cytotoxic effects on HCC cell lines but the underlying mechanism was uncertain (7). We showed the possible activity of both cantharidin analogues by measuring the intracellular ATP level as ATP monitoring was generally used to assess the potential cytotoxic, cytostatic and proliferative effects of a wide range of drugs and biological compounds. Compound 2 showed cytotoxic potential on all of the four cancer cell lines tested. As anticipated, compound 3 had strong cytotoxicity on Hep3B HCC cells but it was weaker for KG1a AML cells when compared with compound 2. Their anti-cancer property was further supported by morphological investigation as a characteristic feature of apoptosis, cell shrinkage, was readily observed on both solid tumour and leukaemia cell lines.

Mitochondrion is said to play an important role in apoptosis (14). Mitochondrial membrane potential collapse can result in the release of apoptotic inducing factor that can further enhance the downstream protease activating process where bcl-2 is an anti-apoptotic protein. Recently, it has been demonstrated that norcantharidin down-regulates the expression of bcl-2 protein in the Ca9-22 p53 mutant human oral cancer cell line (15). In this study, compounds 2 and 3 both depolarized the mitochondrial membrane potential and reduced the bcl-2 protein level of p53-deficient KG1a cells. Thus, the intrinsic mitochondrial-dependent pathway was postulated in the mechanism involved in cantharidin analogue-induced cell death.

Recent research demonstrated that elevated caspase 3 activity and genomic DNA fragmentation were involved in norcantharidin-induced apoptosis (16). In this study, compounds 2 and 3 both activated the caspase 3 activity and increased the TUNEL activity. Interestingly, both caspase 3

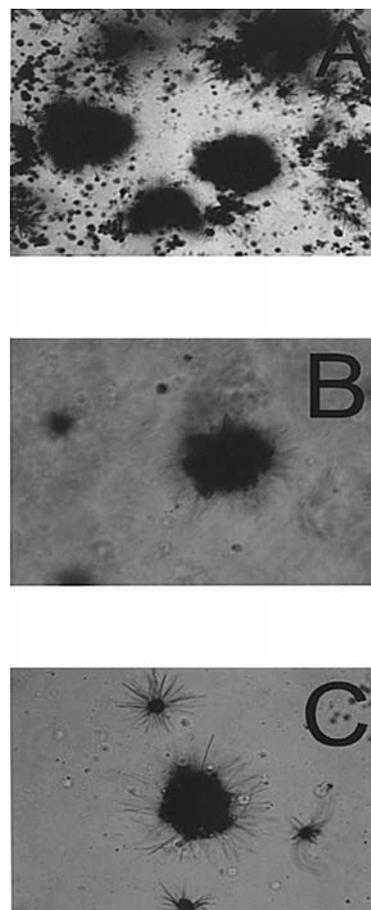


Figure 8. Representative results of colony formation assay from non-malignant haematological disorder bone marrow samples after (A) vehicle incubation, (B) compound 2 incubation and (C) compound 3 incubation.

activation and TUNEL activity patterns were similar to their cytotoxic ability on KG1a cells, which suggested that these apoptotic descriptions were closely related to the cytotoxic ability of compounds 2 and 3 respectively.

Bone marrow suppression is a common clinical limitation of most chemotherapeutic regimens, which is also a limiting factor for deciding the further usage of any cantharidin-related compounds. As shown in Table I, both synthetic cantharidin analogs showed certain degrees of haematopoietic stem cell regeneration inhibition at 12.5 $\mu\text{g/ml}$ while complete colony growth inhibition from primary culture of leukaemic blast was observed. Although both compounds were considerably toxic towards primary cultured non-malignant bone marrow cells at this concentration, there was still a differentiation between their effects on the colony regeneration potential of non-malignant bone marrow cells and leukaemia cells *in vitro*. Further chemical modification to balance the cytotoxicity towards cancer cells and the toxic effect towards non-malignant bone marrow cells is in progress.

In conclusion, we have demonstrated that a novel cantharidin analogue, compound 2, and a previously reported cantharidin analogue, compound 3, were active in carcinoma cell lines, especially Hep3B HCC and KG1a AML cells, and mitochondrial dependent apoptosis was involved. These results shed light on the discovery of anti-cancer drugs for hepatoma and leukaemia in future studies.

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