

Apoptotic activity of a novel synthetic cantharidin analogue on hepatoma cell lines

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Abstract. Cantharidin isolated from *Mylabris caraganae* and other insects is used traditionally as an anti-cancer drug. However, its toxicity on the renal system and suppression effect on bone marrow limits its clinical usage. Recently, we have synthesized two cantharidin analogues, CAN 029 (compound 2) and CAN 030 (compound 3). Although both showed an apoptotic induction ability on cancer cells, they were still relatively toxic towards non-malignant haematological disordered bone marrow. Based on the principle structure of cantharidin, we have further chemically synthesized another analogue, CAN 032. The cytotoxic activity of this analogue was screened on both Hep3B hepatocellular carcinoma and SK-Hep-1 liver adenocarcinoma cell lines by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) assay. Morphological changes of hepatoma cell lines were recorded under an inverted microscope. The possible tolerance of these analogues was further investigated using non-malignant haematological bone marrow primary culture. CAN 032 showed a significant cytotoxic response on both hepatoma cell lines in which the potencies were comparable to that of cantharidin. Further screening on the bone marrow tolerance revealed that compound CAN 032 showed a relatively less toxic effect. Phase contrast microscopy demonstrated that cell shrinkage, rounding, loss of adherent property and loss of colony-formation ability were induced. The dose-dependence of the

response of CAN 032 on Hep3B was further assayed by DNA fragmentation gel electrophoresis. The G₁ peak of Hep3B cells was reduced. Chemically synthesized CAN 032 may provide an improved therapeutic advantage over traditional cantharidin.

Introduction

Hepatocellular carcinoma is commonly observed in East Asia. Even though tumour resection could enhance disease-free survival, some hepatocellular carcinoma remain unresectable due to the tumour size/volume and their complicated location. Thus, chemotherapy remains a major therapeutic pathway which could either reduce the tumour size or achieve complete remission (1).

Cantharidin has been used for the treatment of tumours, including hepatocellular carcinoma, clinically. It can be isolated naturally from many types of commonly found insects, including *Mylabris caraganae*. However, the highly toxic nature limits its usage (2,3). Therefore, many modified cantharidin analogues are synthesized chemically in order to achieve a comparable antitumour property to the mother compound but simultaneously produce a less toxic effect on non-cancer cells.

Recently, we synthesized two cantharidin analogues and, although both of them showed apoptotic induction potential on cancer cells, they were still relatively toxic towards non-malignant haematological disordered bone marrow (4). Based on the principle structure of cantharidin, we have synthesized another analogue (CAN 032) by further chemical modifications. We have tested its *in vitro* cytotoxicity on two hepatoma cell lines, Hep3B and SK-Hep-1. CAN 032 showed a potential cytotoxic effect on these hepatoma cell lines similar to that of cantharidin. The tolerance test of these potential antitumour compounds was carried out using primary culture of non-malignant haematological bone marrow samples. Cell cycle arrest was investigated. Both the morphological investigation and DNA fragmentation formation were studied by inverted microscopy and DNA electrophoretic analysis. Our results

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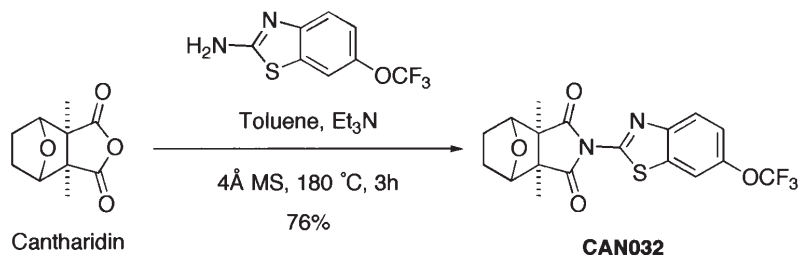
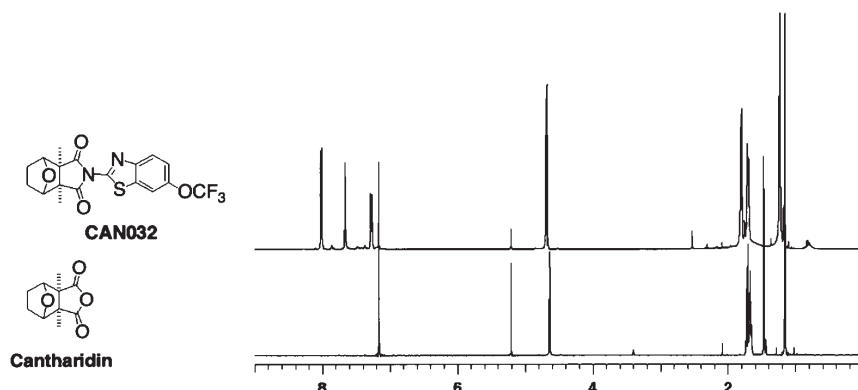


Figure 1. Chemical synthesis scheme of CAN 032.

Figure 2. ¹H spectra comparison of CAN 032 with the original cantharidin.

suggested that CAN 032 is a novel cantharidin analogue that could possess comparable activity to its mother compound but with a relatively less toxic effect *in vitro*.

Materials and methods

Synthesis of CAN 032. Unless otherwise indicated, all reactions were carried out under a nitrogen atmosphere. NMR spectra were recorded on a Varian 500 MHz Fourier transform spectrometer. ¹H and ¹³C[¹H] 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 and 2-amino-6-(trifluoromethoxy)-benzothiazole were purchased from Sigma-Aldrich. All other chemicals were purchased from commercial suppliers and were used without further purification. 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 (10 mg, 0.051 mmol), 2-amino-6-(trifluoromethoxy)-benzothiazole (59.6 mg, 0.26 mmol), dried toluene and dried triethylamine (4 ml; 2:1, v/v) in a sealed tube was heated for 2.5 h at 180°C. Concentration followed by flash chromatograph gave the product (16 mg) in 76% yield: ¹H NMR (CDCl₃, 500 MHz), δ 1.31 (brs, 6H), 1.75-1.81 (m, 2H), 1.87-1.91 (m, 2H), 4.77-4.78 (m, 2H); 7.36

(dd, J=8.5 and 1 Hz, 1H), 7.76 (s, 1H), 8.11 (d, J=9.5 Hz, 1H); ¹³C NMR (CDCl₃), δ 13.0, 23.8, 54.3, 84.5, 113.7, 119.4, 120.4, 124.5, 146.7, 147.5, 152.7, 178.0; and MS (ESI) m/z (%), 413 (M⁺ + 1, 100), 435 (76).

Cell lines and cell culture. Two hepatoma cell lines, Hep3B and SK-Hep-1, were obtained from American Type Culture Collection. Both hepatoma cell lines were maintained in minimum essential medium (JRH Biosciences) supplemented with 10% of heat inactivated fetal bovine serum (Hyclone) together with antibiotics involving penicillin and streptomycin. Cells were allowed to grow in a humidified cell culture incubator maintained at 5% carbon dioxide.

Human bone marrow cell collection and isolation. Non-malignant haematological disorder bone marrow cells were collected from consenting patients by bone marrow aspirate method. A total of three adult subjects were recruited; one male and two females. Immediately after collection, mononuclear cells were enriched by Ficoll-Plaque (General Electricity, Amersham) gradient centrifugation. Cells were washed twice with phosphate-buffered saline and resuspended in complete medium (5,6). Viable cell percentage was then estimated by trypan blue exclusion assay and counted using a haemocytometer as mentioned (7).

[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) assay. Changes in the cellular viability of cantharidin- and its analogue-treated cells were monitored using the MTS activity assay as reported previously (8-10). Briefly, hepatoma cells (Hep3B and SK-Hep-1) were seeded at day 0. After 24 h, the medium was

changed and various compounds were added at different concentrations while, for bone marrow cells, compounds were added on the same day as seeding. After 48 h of incubation, the medium was removed, MTS/PMS solution was added and they were incubated for a further 30 min. Afterwards, optical absorbance was determined at 490 nm according to the user's manual (Promega).

Morphological monitoring of cantharidin- and its analogue-treated cells. Any morphological changes from cantharidin- and CAN 032-treated Hep3B and SK-Hep-1 hepatoma cells were recorded by investigation under an inverted microscope at the scheduled time point (11).

DNA fragmentation analysis. After treating Hep3B cells with cantharidin and its analogue, genomic DNA was isolated as mentioned before (12). Genomic DNA was isolated by DNAzol (Invitrogen) according to the manufacturer's instructions. Purified DNA was then separated by running in a 1% agarose gel stained with ethidium bromide and photographed under ultraviolet illumination.

Cell cycle study. Hep3B hepatocellular carcinoma cells were either treated with vehicle (0.1% DMSO) or 5 $\mu\text{g/ml}$ of CAN 032 for 12 h and then stained with propidium iodide and analysed by using the CellQuest software of a Becton-Dickinson FACS flow cytometre (13).

Results

Chemical synthesis of cantharidin analogue. Based on the principal chemical structure of the mother compound, cantharidin, we synthesized CAN 032. Details of the chemical reactions and characterization including the ^1H spectra comparison of CAN 032 with the original cantharidin are shown in Figs. 1 and 2 respectively. The CAN 032 was further dissolved in DMSO to a concentration of 50 mg/ml and a clear solution was obtained. It was stored in aliquot at -20°C . For the biological assay, the maximum concentration used for CAN 032 was 25 $\mu\text{g/ml}$, thus making the contribution of solvent always $<0.05\%$.

Potency of cantharidin and its analogue on hepatoma cell lines. We studied the possible cytotoxic activity of cantharidin analogue by means of MTS assay. As shown in Table I, CAN 032 showed comparable MTS_{50} activity (50% of MTS reduction ability by the chemically treated cell as compared with control) to cantharidin and cisplatinum on both Hep3B and SK-Hep-1 hepatoma cell lines (Fig. 3).

Toxicity on primary bone marrow culture and morphological investigation. A tolerance test using non-malignant haematological disorder bone marrow was performed for CAN 032 together with the mother compound cantharidin. Bone marrow samples seemed to be less sensitive to CAN 032 than cantharidin (Table II). Cantharidin and CAN 032 began to induce Hep3B cell shrinkage, loss of colony-formation ability and adherent property at a dose of 12.5 $\mu\text{g/ml}$ after a 48-h incubation (Fig. 4). Approximately half of the cells showed such characteristics at 6.25 $\mu\text{g/ml}$. A similar dose-dependent

Table I. MTS_{50} activity test of a chemically synthetic cantharidin analogue (CAN 032) on Hep3B and SK-Hep-1 hepatoma cell lines.

$12.5 \mu\text{g/ml} > \text{MTS}_{50} > 6.25 \mu\text{g/ml}$	
CAN 032	* #
Cantharidin	* #
Cisplatinum	* #

* is the mean MTS_{50} for Hep3B and # is the mean MTS_{50} for SK-Hep-1. Each experiment was performed in triplicate. Results are the mean of three independent experiments.

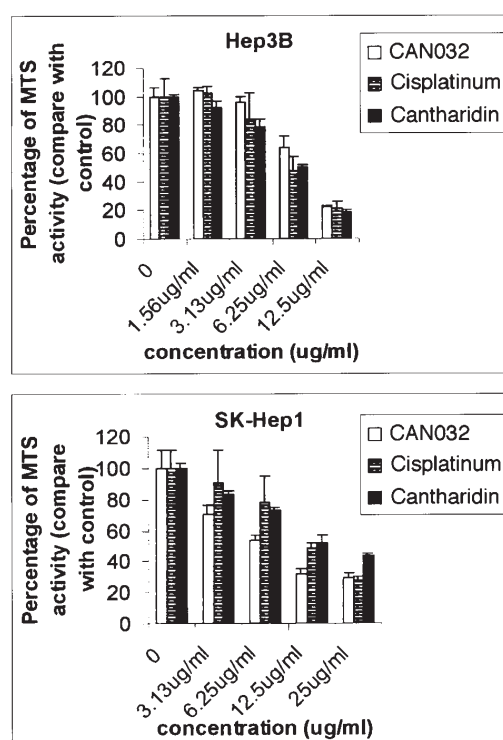


Figure 3. Cytotoxicity assay of cantharidin and CAN 032 on Hep3B and SK-Hep-1 hepatoma cell lines. Hepatoma cells were incubated with different compounds for 48 h and then assayed. Each experiment was performed in triplicate and a representative result of mean \pm SD is shown. Similar results were obtained.

Table II. MTS_{50} activity test of a chemically synthetic cantharidin analogue (CAN 032) on three non-malignant haematological bone marrow cells.

$\text{MTS}_{50} > 12.5 \mu\text{g/ml}$ $12.5 \mu\text{g/ml} > \text{MTS}_{50} > 6.25 \mu\text{g/ml}$	
CAN 032	BM1, BM2, BM3
Cantharidin	BM1, BM2, BM3

Each experiment was performed in triplicate. Results are the means of three independent experiments. BM 1 to 3, three non-malignant haematological bone marrow samples.

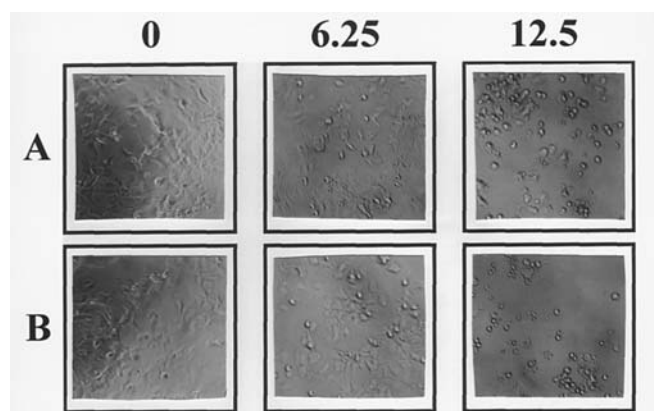


Figure 4. Morphological changes involving cell shrinkage, rounding, and loss of adherent ability and colony-formation property were observed for (A) cantharidin and (B) CAN 032 in a dose-dependent manner in hepatocellular carcinoma Hep3B cells (6.25 $\mu\text{g/ml}$ and 12.5 $\mu\text{g/ml}$).

relationship of cantharidin and CAN 032 on SK-Hep-1 was also observed (data not shown).

Induced-DNA laddering formation and cell cycle study. As shown in Fig. 5A, CAN 032 induced a dose-dependent formation of DNA fragmentation on the Hep3B hepatocellular carcinoma cells after 48 h of incubation. CAN 032 also reduced the G_1 peak of Hep3B cells (Fig. 5B).

Discussion

Naturally occurring products provide us with numerous of medicinal resources (14). Many drugs that are currently clinically used are actually synthesized from compounds that could be isolated from animal or herbal extracts. Based on the principle chemical structure of cantharidin, we have synthesized a new cantharidin analogue, CAN 032. We further screened its possible cytotoxic activity using two hepatoma cell lines, Hep3B and SK-Hep-1. CAN 032 showed a significant cytotoxic response on both hepatoma cell lines in which the potencies were comparable to that of cantharidin and cisplatin. Morphologically, cantharidin and CAN 032 showed cytotoxic action on Hep3B cells by inducing loss of colony-formation potential and cell shrinkage.

Since bone marrow suppression and gastrointestinal and urinary tract toxicity are important side effects of cantharidin, we further tested their toxicity on non-malignant haematological bone marrow samples. Bone marrow tolerance analysis suggested that the toxicity of CAN 032 is lower than that of cantharidin.

Induction of DNA fragmentation from intact genomic DNA is a description of apoptosis. Here, we demonstrated that CAN 032 can induce apoptosis in a dose-dependent manner. However, investigation for the mechanism of a potential drug, including the understanding of its signal transduction pathway, is considered to be clinically significant (15). Our preliminary data demonstrated that CAN 032 significantly reduces the G_1 phase of Hep3B HCC cells. Therefore, we speculated that those cell cycle-related checkpoints would be significant for CAN 032-induced apoptosis and this provided us with an interesting issue for further investigation.

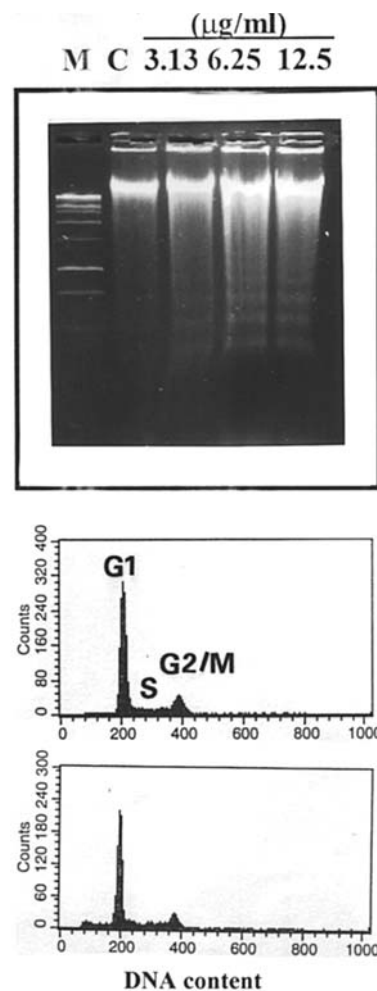


Figure 5. (A) DNA fragmentation analysis further demonstrated that programmed cell death might be a possible description for the activity of CAN 032 on hepatocellular carcinoma Hep3B cells, in a dose-dependent manner. (B) Cell cycle study for the effect of CAN 032 on Hep3B cells (lower) when compared with vehicle (0.1% DMSO, upper).

We chose Hep3B and SK-Hep-1 hepatoma cell lines as models to study the actions of cantharidin analogues because Hep3B lacks the functional p53 tumour suppressor gene while functional p53 is found in SK-Hep-1. Interestingly, both cell lines seemed to have a similar sensitivity to our cantharidin analogue. It is highly possible that this cantharidin analogue is effective on a large spectrum of p53-positive and -negative hepatoma cells and its anti-cancer activity is independent of p53 status.

Hepatocellular carcinoma remains to be one of the most important solid tumours and it is especially common in sub-Africa and East Asia (16,17). The intake of aflatoxin from fungal contaminated crop, infection of hepatitis B virus and cirrhosis may account for a significant proportion of the causes of hepatocellular carcinoma. Owing to the size and location of the tumours, hepatic resection may not be possible under certain conditions. Thus, any potential chemotherapeutic regimen which could be cytotoxic to the tumour but relatively less toxic to the haemopoietic system would be considered to be beneficial to those patients. As cantharidin is also suggested to be active on leukaemia, further study of the pharmaceutical activity of CAN 032, using leukaemia as a

demonstration model, is in process in order to elucidate the underlying molecular mechanisms involved.

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