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. A synthetic analogue of cantharidin (CAN 037) has been shown to have cytotoxic effect on the SK-Hep 1 hepatoma cell line but its underlying working principle remains undefined. Here we further report the action of CAN 037 on an acute myelogenous leukaemia (AML) cell line, KG1a. [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) assay was used to demonstrate the cytotoxicity of CAN 037 on KG1a cells. Morphological changes of CAN 037-treated leukaemia cells were recorded under an inverted microscope. Possible activation of caspase 3, 8 and 9 from KG1a cells was also investigated. KG1a AML cells were sensitive to CAN 037. Morphological changes including cell shrinkage and loss of colony formation ability were observed. Caspase 3, 8 and 9 activity was elevated, whereas pre-incubating the KG1a cells with the generic caspase inhibitor z-VAD-fmk could only partially reverse the CAN 037-induced cell death. In addition to the SK-Hep-1 hepatoma cell line, CAN 037 is also effective in inducing the death of KG1a AML cells in vitro. Apoptosis is involved in the action of CAN 037 including the activation of the caspase family. Caspase-dependent cell death pathway may be necessary but not essential in CAN 037-induced apoptosis of KG1a cells. Further consideration of the structural activity relationship of CAN 037 may provide opportunities to improve its therapeutic value.

Introduction

Plants are very important and useful sources of molecules of great interest for the development of new pharmaceutical products (1). Many of them are found to be effective in cancer therapy but are considerably expensive. Examples include the isolation of taxol from *Taxus brevifolia* and both vincristine and vinblastin from *Catharenthus roseus*. It is estimated that the unit price of taxol is now approximately US$ 600 000 per kg while the unit price of both vincristine and vinblastin could be up to US$ 1 000 000-2 000 000 per kg (2).

Recently, we have demonstrated the possible in vitro underlying working principles of many Traditional Chinese medicines (TCMs) in herbal form extract that have been claimed to yield anti-cancer potential (3), including the fruit of *Gleditsia sinensis* (4), *Scutellaria barbata* and *Radix Sophorae Tonkinensis* (5) as well as *Brucea javanica* (6). Our results bring TCMs closer to a scientific approach.

Cantharidin has been used for the clinical treatment of tumours including hepatocellular carcinoma and leukaemia. It can be isolated naturally from many types of commonly found insects including the dried body of *Mylabris caraganae*. However, cantharidin is highly toxic (7). Therefore, many modified cantharidin analogues are synthesized chemically in order to achieve a comparable anti-tumour property to cantharidin but with a less toxic effect on non-tumour cells.

Recently, Lin et al published a family of cantharidin analogues which showed cytotoxic effect on hepatoma cells but no further mechanistic work followed (8). We have studied the underlying working principle of one of them [compound 3e (8), which we name CAN 037: Fig. 1] using the KG1a acute myelogenous leukaemia (AML) model. Our results demonstrated that CAN 037 showed certain selectivity between the KG1a AML cell line and non-malignant haematological disordered bone marrow. Furthermore, its chemical structure provides a skeleton for chemical modification.
treated cells were washed with PBS and then total cellular assay was performed using the Promega luminescence kit. Briefly, CAN 037-Lance Perkin Elmer (Finland) while caspase 9 activity was determined using caspase 3 and 8 activity kits purchased from R&D Systems.

To study whether caspase 3, 8 and 9 were activated after 24 h of CAN 037 treatment, we used timeresolved fluorescence technology based caspase activity kits purchased from Lance Perkin Elmer (Finland) while caspase 9 activity was assayed by the Promega luminescence kit. Briefly, CAN 037-treated cells were washed with PBS and then total cellular protein extracts were prepared and quantitated by the Bradford method using bovine serum albumin as calibration. Afterwards, caspase 3, 8 or 9 specific substrate and detection buffer were added to an equal amount of total cancer cell protein extract and individual caspase activity was analysed according to the instruction manuals provided. Finally, the resulting relative fluorescence units or relative luminescence units were compared with the untreated control.

Caspase dependency cytotoxicity assay. To determine whether CAN 037-mediated induction of apoptosis involved also caspase-independent pathways, KG1a AML cells were pre-treated with caspase inhibitor z-VAD-fmk, at a concentration of 20 μM. Afterwards, CAN 037 was added and cancer cells were further incubated for 48 h. The resulting cellular viability was then quantified by using the MTS assay as mentioned previously.

Results

Synthesis of CAN 037. The reactions were performed as previously described. The compounds were purified using silica gel column chromatography. The purity of the synthesized CAN 037 was confirmed by elemental analysis, NMR, and mass spectrometry.

[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) assay. Changes in the cellular viability of CAN 037-treated cells were monitored using the MTS activity assay as reported previously. Briefly, KG1a and non-malignant haematological disorder bone marrow cells were seeded at day 0. After 6 h, CAN 037 was added at different concentrations. After 48 h of incubation, the medium was removed and MTS/PMS solution was added and the cells were incubated for a further 30 min exactly. Afterwards, optical absorbance was determined at 490 nm according to the user manual (Promega).

Cytotoxicity of CAN 037 on the KG1a AML cell line and non-malignant haematological disorder bone marrow. To see whether CAN 037 could be also cytotoxic to KG1a AML cells and primary culture of human bone marrow cells, we tested it against several cancer cell lines including non-malignant haematological disorder bone marrow. The cell lines were treated with different concentrations of CAN 037 and the resulting cellular viability was determined using the MTS assay as mentioned previously.

Mechanistic action of CAN 037 on KG1a AML cells. To demonstrate whether caspase-dependent or –independent pathway is involved in CAN 037-induced KG1a apoptosis, we examined the caspase 3, 8 and 9 activity of KG1a protein extract after CAN 037 incubation. Fig. 4 demonstrates that the activation of all of these caspases was recruited but to different extents. Therefore, we conclude that intrinsic programmed cell death pathway is involved in CAN 037-induced KG1a apoptosis.
death pathway is highly likely to be involved in CAN 037-induced apoptosis of KG1a cells.

Is caspase dependent pathway exclusively required in CAN 037-dependent cell death? If caspase 3, 8 and 9 are activated during CAN 037-induced apoptosis, we questioned whether inhibition of all of these caspases could completely inhibit the cytotoxic action of CAN 037. To approach this hypothesis, the KG1a cells were pre-incubated with the generic caspase inhibitor z-VAD-fmk so that caspase activity could be significantly inhibited in the presence of CAN 037 (Fig. 4). As shown in Fig. 5, the MTS activity of KG1a cells increased only slightly in the presence of CAN 037 when they were pre-incubated with z-VAD-fmk. This phenomenon showed...
that caspase activity seemed to be necessary but might not be essential for CAN 037-induced apoptosis of KG1a AML cells and it is speculated that both caspase-dependent and -independent pathways are active in vitro.

Discussion

Based on the principle chemical structure of cantharidin, we have synthesized a number of its family analogues and named them the CAN series. After obtaining a family of cantharidin analogues, we further screened its possible cytotoxic activity using a panel of carcinoma cell lines. Two of these synthetic samples (CAN 029 and CAN 030) showed a significant cytotoxic response on cancer cell lines. However, they also exhibited similar cytotoxicity on non-malignant haematological disorder bone marrow (9) in vitro.

We then further moved to synthesize another cantharidin analogue named CAN 032 (10). CAN 032 showed a certain level of selectivity for the primary culture of non-malignant haematological disorder bone marrow and hepatoma cells. We also demonstrated the underlying working principle of CAN 032 by using KG1a AML cells as the demonstration model (11). CAN 032 thus provided a milestone for further chemical synthesis of cantharidin analogues.

When referring back to the chemical structure of CAN 032, we noticed that there is an interesting electron distribution of the molecule (Fig. 6). As the core structure of cantharidimide with diimide and the 6-position trifluoromethoxy group are both electron withdrawing groups, this would make the benzothiazole moiety of CAN 032 under a high electron deficiency state. We speculate that this special electron distribution status may exert a crucial effect on its cytotoxicity to carcinoma cells.

Similarly, Lin et al also stated that the electronegativity of the substituent group plays an important role in the cytotoxicity (8). However, they assumed that the increase of electronegativity of the substituent group would decrease the cytotoxic nature for the benzothiazole derivatives. The chemical structure of CAN 037 opens a new modification opportunity by modifying the methyl group at the 6-position. We hypothesize that, based on the results of CAN 032 and CAN 037, one important characteristic of cantharidin is that its relative reactivity toward anti-cancer activity may be controlled by the introduction of an electronic withdrawing or donating group at the 6-position. Currently, we are studying both hypotheses by either changing the methyl group at the 6-position of CAN 037 into a more electron donating group or a more electron withdrawing group (Fig. 7). The resulting cytotoxicity on cancer cells will clarify which assumption is applicable.
Recently, there have been many reports on the novel synthesis of cantharidin-related compounds (12) as well as their possible molecular action in cancer cells (13). Here we demonstrated that the activated biological pathways of CAN 037-stimulated cancer cells \textit{in vitro} are similar to those of CAN 029, CAN 030 and CAN 032. The cross pollination of biological and chemical concept allows us to have a better understanding of the structural activity relationship of cantharidin analogues during anti-cancer drug synthesis. This could be implicated in synthesizing a novel anti-tumour drug under a cost-effective pathway in the future.

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