Effects of Cantharidinimides on Human Carcinoma Cells

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Modification of the cantharidinimide structure led to the discovery of a novel class of antitumor compounds. These cantharidinimide derivatives containing aliphartic, aryl, and pyridyl groups showed some effect *in vitro* against HepG2 and HL-60 cells.

Key words cantharidin; cantharidinimide; HepG2; HL-60

Cantharidin is found in *Mylabris caraganae* and various other insects and shows extremely high vesicant potency and toxic properties.[1—4] In a pharmacologic study, cantharidin increased the force of contraction in isolated guinea pig papillary muscles.[5] It can also cause severe congestion and edema of the liver.[6] Recent reports have indicated that the action of cantharidin in mice is associated with binding to protein phosphatase 2A in liver cytosol and inhibition of its phosphorylase phosphatase activity.[7,8] In clinical studies it had significant activity against liver tumors and the KB cell line in tissue culture at low concentrations.[9—12] In our previous studies,[2] increasing the solubility of the title compounds decreased the toxicity while maintaining the biological activities. *N*-Thiazolyl and thiadiazolyl cantharidinimides with better solubility showed cytotoxicity against human hepatocellular carcinoma cell lines, which encouraged us to prepare cantharidinimide derivatives. These compounds were synthesized from cantharidin and primary amines in triethylamine by heating to *ca.* 200 °C. *N*-Methylcantharidinimide has shown tumor-inhibitory action in animals, and some cantharidinimides were tested for their effects on xanthine oxidase; those tests showed inhibitory effects on xanthine oxidase.[13] Some cantharidinimides also show antiplatelet effects on thrombin, arachidonic acid, collagen, and platelet-activating factor-induced aggregation.

Chemistry We prepared effective cantharidinimides by heating the reactants cantharidin **1** and primary amines, aniline derivatives, and aminopyridines to *ca*. 200 °C with 3 ml of dry toluene and 1—2 ml of triethylamine in a high-pressure sealed tube (Buchi glasuster 0032) to provide cantharidinimides (compounds **2**—**22**, Chart 1) in good yields. In further studies, all of the synthetic compounds were tested for their cytotoxicity in suppressing the growth of the human carcinoma cell lines HL-60 and Hep G2.

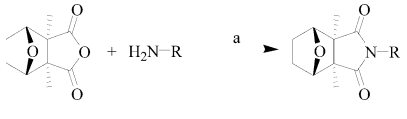
Results and Discussion

With the high-pressure technique, we obtained cantharidinimides (after recrystallization from methanol) in yields of 29—96%, as listed in Table 1. The highest yields were obtained from aliphatic primary amines, (**2**—**4**) as well as phenetyl amines **18**—**22**. The variable yields of compounds **5**—**16**, which had parent aniline and pyridine components, might reflect the inductive electron negative effect influencing the yield. The results of these yields strongly confirm the influence of amine nucleophilicity and basicity. The potential cytotoxicities of the prepared cantharidinimides were investigated against hepatocellular carcinoma cell (Hep G2) and human myeloid leukemia cell (HL-60) lines and evaluated

Table 1. Cytotoxicity of Cantharidin **1** and Cantharidinimides in Human Hepatocellular Carcinoma (Hep G2) and Myeloid Leukemia Cell Lines (HL-60)

Compd.	R	IC_{50} (μM)[a]		Yield (%)[b]
		HL-60	Hep G2	
1		5.4	4.2	
2	Cyclopropyl	>12.5[c]	>12.5[c]	96
3	Allyl	>5[c]	>5[c]	90
4	4-Aminobutyl	>10[c]	>10[c]	74
5	4-Pyridyl	72.7	32.5	50
6	3-Pyridyl	>500[c]	>500[c]	29
7	2-(4-Methyl)pyridyl	>500[c]	>500[c]	57
8	2-(5-Nitro)pyridyl	>500[c]	>500[c]	47
9	5-(3-Chloro)pyridyl	72.2	53.7	28
10	2-(4,6-Dimethyl)pyridyl	>500[c]	442.8	62
11	2-(5-Nitro)pyridyl	5.1	4.6	29
12	2-(3-Hydroxy)pyridyl	>500[c]	>500[c]	52
13	3,4-Diethylenedioxyphenyl	30.4	27.6	76
14	4-(1-Hydroxy)phenyl	341.3	396.3	67
15	4-Biphenyl	27.2	32.5	66
16	3-(1-Hydroxy)phenyl	>375[c]	>375[c]	52
17	4-(1-Nitro)phenyl	8.4	6.9	47
18	1-(4-Hydroxyphenyl)ethyl	224.3	>250[c]	88
19	1-(4-Methoxyphenyl)ethyl	100.2	113.6	68
20	1-(3,4-Dimethoxyphenyl)ethyl	376	>500[c]	93
21	2-Naphthylethyl	45.3	60.6	48
22	3-Indolylethyl	>12.5[c]	>12.5[c]	77

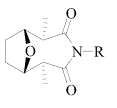
a) IC_{50} was calculated after 48 h of continuous drug exposure, values are the mean of three to four experiments with coefficients of variation of 5—10%. *b*) Obtained after purification by chromatography on silica gel. *c*) No indication of the cytotoxicity was considered indicative of IC_{50} values substantially rather than the highest doses assayed.



1 **2-22** (29-96%)

(a): TEA, Toluene, c.a. 200°C reflux
R is defined in Table 1.

Chart 1



cantharidinimide

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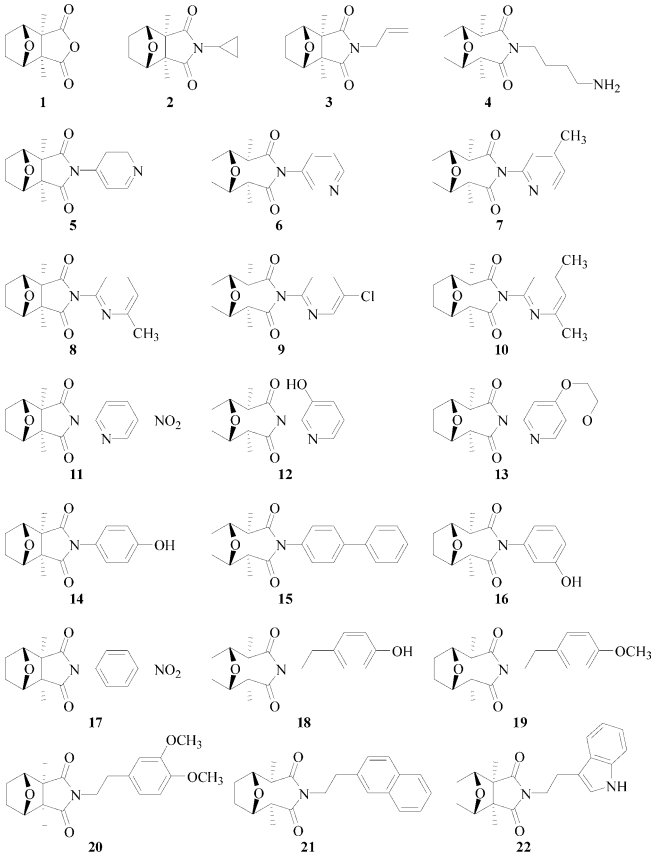

Fig. 1. Structures of the Compounds Tested

using MTT cell viability assays (Table 1). Cytotoxicity tests showed that cantharidin and its cantharidinimide analogues, listed in Fig. 1, and some inhibitory effects on HepG2 and HL-60. Cantharidin **1** was more toxic and exhibited greater cytotoxicity, while synthesized cantharidinimides were less toxic but also exhibited inhibitory effects. In this study, compounds **11** and **17**, which had electron-withdrawing NO_2 groups on the pyridyl and benzene rings, displayed strong inhibitory effects on both cell lines as did cantharidin. This suggests that the presence of a nitropyridyl or nitrophenyl moiety is probably important for the cytotoxic properties of this series. *N*-Pyridylcantharidinimides displayed bioactivity depending on the functional groups on the ring. Methyl-containing compounds **7**, **8**, and **10** had less effect, and the halogen-containing compound **9** had a medium effect. A hydroxy group (electron-donating group) on the pyridine ring result in less effect. In contrast, compound **11** with a 2-(3-nitropyridyl) group had stronger cytotoxicity. *para*-Pyridyl imide **5** had a greater effect than did *ortho*- or *meta*-pyridyl imide, and compound **6** and *N*-phenyl imides **14** and **16** gave similar results. Compounds **13**, **15**, and **21** with a planar side chain exhibited medium cytotoxicity on both cell lines, and aliphatic chain imides **2**, **3**, and **4** displayed very low effects against the cell lines. *N*-Azaethyl or *N*-aryl imides **18**, **19**, **20**, and **22** also showed medium effects against both cell lines.

Experimental

Test Samples Chinese blister beetles were extracted with a water : ethanol 1 : 1 solution, filtered with celite, purified by chromatography on silica gel, and then recrystallized with ethanol to give cantharidin **1**. Compounds **2**—**22** were prepared from cantharidin and primary amines in the presence of triethylamine in toluene in a high-pressure tube; the mass spectra of all compounds were measured and proton NMR, was also used for testing (Fig. 1).

Preparation of Test Solutions and Assay Procedure for Cytotoxicity Media and sera for cell culture were purchased from Gibco/BRL (Grand Island, NY, U.S.A.). Most chemicals were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). The human hepatocarcinoma cell line (Hep G2) and the human myeloid leukemia cell line (HL-60) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, U.S.A.). Hep G2 cells were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin 100 units/ml, streptomycin 100 μg/ml, nonessential amino acids 100 μM, and 1 mM glutamine in a controlled atmosphere of 5% CO_2 and 95% air at 37 °C. Hep G2 and HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, pencillin 100 units/ml, streptomycin 100 μg/ml, and 1 mM L-glutamine.

MTT Assay for Cellular Viability Cells were seeded into 96-well plates and allowed to adhere for 24 h before drugs were introduced. Following 48-h incubation, drugs and media were removed and each well was treated with 100 μl of 500 μg/ml 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) in culture medium. Following a 4-h incubation period to allow the metabolism of MTT by mitochondrial dehydrogenases of viable cells to form an insoluble formazan product, the plates were centrifuged at 450×***g*** for 10 min and supernatants were removed and replaced with DMSO 100 μl. The plates were shaken to maximize solubilization of the formazan crystals. Absorbance, as a measure of viable cell number, was read the following day in a model MA310 automated EIA plate reader at a wavelength of 550 nm. IC_{50} values were obtained by a linear regression analysis of percent absorbance versus log of drug concentration. It has been shown previously that viable cell numbers correlate with optical density as determined in the MTT assay.[14,15]

All temperature are reported in degrees centigrade. Melting points were determined with a Buchi B-545 melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer Model 882 and a Nicolet 510 Pet spectrophotometers. ^{1}H-NMR spectra (in $CDCl_3$ unless otherwise stated) were recorded at 300 MHz and 400 MHz on a Bruker AC and at 500 MHz on a Bruker Advance DRX. Mass spectra were obtained on a JOEL JMSHX 110 FAB-MS spectrometer. Elemental analysis spectra were obtained on a Perkin-Elmer 2400. The tube was Buchi glasuster (Bursting disc, 0032). General procedures were followed for the reaction of primary amines with cantharidin. These compounds were prepared according to similar procedures and reactions took place in high-pressure tubes. Cantharidin was added to a tube containing 3 ml of dry toluene and triethylamine, and the solution was stirred and heated to *ca.* 200 °C. After being stirred for 2 h, the mixture was evaporated, and the residue mass was purified by column chromatography and recrystallized from methanol.

3,4-Diethylenoxyphenylcantharidinimide (**13**): 76% yield, mp 186—189.1 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.20 (6H, s), 1.67—1.82 (4H, m), 4.22 (4H, s), 4.64 (2H, s), 6,71 (1H, dd, J=2.0, 8.7 Hz), 6.78 (1H, d, J=2.0 Hz), 6.89 (1H, d, J=8.7 Hz). MS *m/z* (rel. int.): 329 (M^+) , 260, 96. IR (KBr) cm^{-1}: 1704. HR-MS *m/z*: 329.1270 (Calcd for $C_{18}H_{19}O_5N$: 329.1263).

N-(4-Hydroxyphenyl)cantharidinimide (**14**): 67% yield, mp 128—132.1 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.13 (6H, s), 1.60—1.78 (4H, m), 4.50 (2H, t, J=2.8 Hz), 6.67 (2H, dd, J=2.0, 6.8 Hz), 6.92 (2H, dd, J=2.0, 6.8 Hz), 7.69 (1H, s). MS *m/z* (rel. int.): 288 (M^+), 217, 91. IR (KBr) cm^{-1}: 1690, 3238. HR-MS *m/z*: 287.1175 (Calcd for $C_{16}H_{17}O_4N$: 287.1158).

N-(4-Biphenyl)cantharidinimide (**15**): 66% yield, mp 154.4—156.7 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.22 (3H, s), 1.25 (3H, s), 1.74—1.87 (4H, m), 4.70 (2H, d, J=2.1 Hz), 7.34 (1H, d, J=2.6 Hz), 7.36 (2H, dd, J=2.6, 7.5 Hz), 7.42 (2H, d, J=7.5 Hz), 7.58 (2H, d, J=8.3 Hz), MS *m/z* (rel. int.) 347 (M^+), 278, 128, 96. IR (KBr) cm^{-1}: 1705, HR-MS *m/z*: 347.1536 (Calcd for $C_{22}H_{20}O_3N$: 347.1521).

N-3-(1-Hydroxy)phenylcantharidinimide (**16**): 52% yield, mp 174—176.3 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.20 (6H, s), 1.60—1.90 (4H, m), 4.55 (2H, s), 6.63 (1H, s), 6.64 (1H, d, J=10.8 Hz), 6.80 (1H, d, J=8.0 Hz), 7.22 (1H, dd, J=8.0, 10.8 Hz), MS *m/z*: (rel. int.) 288 (M^+), 218. IR (KBr) cm^{-1}: 1693, 3327, HR-MS *m/z*: 287.1158 (Calcd for $C_{16}H_{17}O_4N$: 287.1158).

N-4-(1-Nitro)phenylcantharidinimide (**17**): 47% yield, mp 135.8—139.3 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.22 (3H, s), 1.25 (3H, s), 1.74—1.76 (4H, m), 4.69 (2H, t, J=4.7 Hz), 7.58 (2H, d, J=9.1 Hz), 8.29 (2H, d, J=9.1 Hz), MS *m/z*: (rel. int.) 316 (M^+), 306, 91. IR (KBr) cm^{-1}: 1715. HR-MS *m/z*: 316.1050 (Calcd for $C_{16}H_{16}O_5N_2$: 316.1059).

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