A Simple Procedure for Preparation of N-Thiazol, Thiadiazol, Pyridyl and Sulfanylcantharidinimides Analouges and Evaluation of Their Cytotoxicities against Human HL-60, MCF7, Neuro-2a and A549 Carcinoma Cell

Ing-Jy Tseng, a Shiow-Yunn Sheu, b Ying-Tzu Chen, c Chu-Yun Huang, c Ching-Tung Lin, c and Pen-Yuan Lin a,d

a School of Geriatric Nursing and Care Management, Taipei Medical University; b Department of Pharmaceutical Analysis, School of Clinical Pharmacy, Taipei Medical University; c Department of Clinical Pharmacy, School of Pharmacy, Taipei Medical University; d Department of Pharmaceutical Sciences, School of Pharmacy, Taipei Medical University; 250 Wu-Hsing St., 11031 Taipei, Taiwan; and e Department of Chemistry, Tam-Kang University; 151 Yingzhuan Rd. Danshui Dist, 25137 New Taipei City, Taiwan. Received May 7, 2012; accepted August 3, 2012

The lab made an effort to prepare some biological active cantharidinimides by heating the reactant 1 and 2a–g, 5h–i and 7j–r amines to suitable temperature with ethanol to provide 18 N-thiazolyl-, sulfanyl-, aminopyridyl-, bromopyridyl-, alkylpyridyl- and hydroxy(3-pyridyl)cantharidinimines 3a–g, 4a–c, 6h–i and 8j–r in yield of 4–77% (Chart 1). These cantharidinimine derivatives were tested for their capabilities to suppress the growth of the human carcinoma cell lines, HL-60, MCF7, Neuro-2a and A549, because the incidence rate is more prominent in Asian countries than western countries. Compounds 3c–d and 6h–i were found to have some antitumor activity in HL-60 but less activity in MCF cell and compounds 8j–l displayed some inhibitory effects to A549 cell line, but less effect to Neuro-2a cell line. Compounds 8m–r had no cytotoxic effect against both cell lines. The cytotoxic effects of these cantharidinimine compounds seemed to be better than the cantharidinimide compounds which we had mentioned several years ago.

Key words cantharidin; N-azayl-thiadiazolylcantharidinimine; cytotoxicity; sulfanyl-thiadiazolylcantharidinimine; thiazolyl-thiadiazolylcantharidinimine

Cantharidin 1 is found in Mylabris caraganae and various other insects and shows extremely high vesicant potency and toxic properties.1–4) Recent reports have indicated that the action of cantharidin in mice is associated with binding to phosphorylase 2A in liver cytosol and inhibition of its phosphorylase phosphatase activity.5–9) In our previous studies,10) increasing the solubility of the title compounds decreased the toxicity while maintaining the biological activities. N-Thiazolyl, sulfanylcantharidinimides with a better solubility showed high cytotoxicity against human carcinoma cell lines. The present study shows that characters of amine basicity and the chosen temperature are crucial. Furthermore the characters of the groups and their position on the aromatic or azalyl ring also influence yields. In order to obtain novel types of related imines and study the scope of these synthetic reactions, the same technique was applied to the reaction of compounds 1 with thiazolyl, thiadiazolyl, sulfanyl, or azalylamines in high-pressure tube with dry ethanol and heated to ca. 140°C. This temperature and reaction time influence the product yields. The method might give good yields after evaporated and recrystallization in methanol (Table 1). In our previous research,11) we synthesized many cantharidinimide derivatives by modification with aliphatic, azalyl, thiazolyl, thiadiazolyl and pyridyl groups. Those compounds exhibited a certain degree of cytotoxic activity on HL-60, Hep3B and HepG2.1–5) NUGC (Human Gastric carcinoma cells), HONZ-1 (Human Nasopharyngeal carcinoma cells)11–16) All of these compounds contained an imide group. Thus, we use this feature to synthesize different imine derivatives to study effects of antitumor therapy and cytotoxicity as carcinoma cells inhibitors and therapy sources.

Results and Discussion

With the high-pressure technique, we obtained cantharidinimines (after recrystallization from methanol) in yields of 4–77%, as listed in Table 1. The highest yields were obtained from dimethylpyridyl 7q as well as thiazoylamines 2a–e. The NH2 basicities of aminothiazolyl-, aminosulfanyl- and pyridylcantharidinimide are unknown but will be slightly different between one of corresponding aminothiazoyl-, aminosulfanyl- and pyridylamine. There is an electron deficiency of the thiazol, sulfanylamide, azad and aminopyridine rings. Compounds 2h–i which had the sulfanyl and pyridyl ring exerted the most electron withdrawing capability with resonance and induction effects. The formation of cantharidinimide appeared to become more difficult hence showed the very low yields, 6h and 6i. We have noted in our previous works that the more conjugated character, the higher the yield obtained. Withdrawing functional groups on the azad ring always gave a lower yield than the electron donating group. Compounds 4a–c were obtained from the reduction of compounds 3a–e by using reductant reagent, NaBH4 with ethanol at room temperature and refluxed to give yields 28%, 14% and 2%. Their NMR values displayed the singlet signal at δ 5.78, 5.80 and 5.87 for one δ 4b, and all of their mass spectra were obtained on a Joel JMX-DX300 for high resolution spectrometer. The variable yields of compounds 2d–g, 2h–i, 8j–r, which had parent aryl, azalyl, sulfanyl and pyridyl compounds might reflect the inductive electron negative, bulkyl steric, or the anhydride oxygen linkage electrical

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: lpy0620@tmu.edu.tw © 2012 The Pharmaceutical Society of Japan
density effect influencing the yields.

The results of these yields strongly confirm the influence of amine nucleophilicity and basicity. The potential cytotoxicities of prepared cantharidinimines were investigated against human myeloid leukemia cell (HL-60), human breast adenocarcinoma (MCF7, from ATCC), rat brain neuroblastoma (Neuro-2a, N 2a, from ATCC) and human lung carcinoma (A549, from ATCC) lines and evaluated. Using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (Table 2) for cytotoxicity tests to show cantharidin and its cantharidinimine analogues listed in Table 1, 3a–g, 6h–i, 8j–r. Cantharidin 1 was more toxic and exhibited greater cytotoxicity. While synthesized cantharidinimines were less toxic but also exhibited inhibitory effects. Being comparable in cytotoxicity to cantharidin, the IC\textsubscript{50} values of all of the cantharidinimine derivatives 3a–8r were 6.0 to 100 \( \mu \)M, and of cantharidin were 4.6–82.8 \( \mu \)M and of 3a–c and 3g were completely inactive up to the high concentration tested (100 \( \mu \)M). Compounds 3d–f have thiadiazolyl functional group showed a higher cytotoxicity than 3a–c and 3g. Compounds 3d and 3f had azoyl ring structure that gave the same cytotoxicities. Sulfanylamine derivatives with two methyl substituents, 6h–i. showed high cytotoxicities to HL-60 cell, 6h displayed more cytotoxicity than 6i, with a different position of pyridyl ring. Unfortunately they all showed inactivities up to the MCF7 cell line. Compounds 8j–r had amino substituents and pyridyl group which might show that the presence of electron donating substitute, 8l, 8m, 8n and 8r enhanced cytotoxicities remarkably. Compounds 8j–l showed cytotoxic activity to A549 cell line but displayed no activity toward Neuro-2a cell line. It might have some effects for other cell lines and the less cytotoxicity might be used to become as prodrug references. Compared to our previous works, we noticed that these cantharidinimine derivatives had more cytotoxic effects than cantharidinimides, especially in the presence of sulfanlylamine groups, and the diamino derivatives of cantharidinimine also showed the activities better than its cantharidinimide derivatives. We will continue this works in the future.

**Experimental**

**Test Samples** Chinese blister beetles were extracted with a water–ethanol ratio of 1:1 solution, filtered with celite, purified by chromatography on silica gel, and then recrystallized with ethanol to give cantharidin 1. Compound 3–8 were prepared from cantharidin and primary amines in the presence of triethylamine in ethanol in a high-pressure tube. IR spectra were recorded on a Thermo Mattson IR300 spectrometer. NMR spectra were recorded on a Bruker DRX-500 spectrometer in pyridin-\( d_5 \) solution with respect to the corresponding solvent as the internal standard. \( ^1 \text{H} \)- and \( ^{13} \text{C} \)-NMR spectra were measured at 500 and 125 MHz, respectively. NMR experiments included \( ^1 \text{H}–^1 \text{H} \) correlation spectroscopy (COSY), distortionless enchancement by polarization transfer (DEPT), \( ^1 \text{H} \)-detected heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC). All chemical shifts were expressed in ppm (\( \delta \)). Multiplicities of all carbon signals were verified through DEPT experiments. Mass spectra were measured on a Finnigan/Thermo Quest MAT 95XL, JEOL JMX-HX 110 and JEOL JMX-DX 300 for high or low resolution spectrometers.\textsuperscript{14} For the chromatographic analysis Merck Silica Gel 60 (230–400 mesh ASTM) was used. The chemical reagents used in synthesis were purchased.
Table 1. Preparation of Cantharidinimines

<table>
<thead>
<tr>
<th></th>
<th>Structure</th>
<th>Yield (%)</th>
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<tbody>
<tr>
<td>1</td>
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<td>3a (36)</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2" alt="Structure 2" /></td>
<td>3b (39)</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3" alt="Structure 3" /></td>
<td>3c (20)</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4" alt="Structure 4" /></td>
<td>3d (6)</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5" alt="Structure 5" /></td>
<td>3e (4)</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="Structure 6" /></td>
<td>3f (47)</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7" alt="Structure 7" /></td>
<td>3g (10)</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8" alt="Structure 8" /></td>
<td>3h (6)</td>
</tr>
<tr>
<td>9</td>
<td><img src="image9" alt="Structure 9" /></td>
<td>3i (3)</td>
</tr>
</tbody>
</table>

The yields obtained after purification by chromatography on silica gel.

Imine linkage position.

Cantharidinimine

Table 2. Cytotoxicity of Cantharidin 1, N-Thiazol-, N-Thiadiazol-, and Sulfanylcantharidinimines in Human HL-60, MCF7, Nuro-2a, A549 Carcinoma Cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>1 (µM)</th>
<th>3a</th>
<th>3b</th>
<th>3c</th>
<th>3d</th>
<th>3e</th>
<th>3f</th>
<th>3g</th>
<th>6h</th>
<th>6i</th>
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<tr>
<td>HL-60</td>
<td>7.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>17.7</td>
<td>41.9</td>
<td>18.1</td>
<td>&gt;100</td>
<td>6</td>
<td>35.9</td>
</tr>
<tr>
<td>MCF7</td>
<td>82.8</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Nuro-2a</td>
<td>70.4</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>A549</td>
<td>4.57</td>
<td>21.6</td>
<td>21.1</td>
<td>31.8</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*IC₅₀ was calculated after 48h of continuous drug exposure. Values are the mean of three to four experiments with coefficients of variation of 5–10%. b) No indication of the cytotoxicity was considered indication of IC₅₀ values substantially rather than the highest doses assayed.
from Sigma-Aldrich Co. and E. Merck.

General Procedures  General procedures were followed for the reaction of primary amines with cantharidin. These compounds were prepared according to similar procedures, and the reaction took place in high-pressure tubes. Cantharidin was added to a tube containing 3 mL of dry ethyl alcohol and triethylamime, and the solution was stirred and heated at 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.

Antineoplastic Bioassays  Preparation of Test Solution and Assay Procedure for Cytotoxicity  Media and sera for cell culture were purchased from Gibco/BRL (Grand Island, NY). Most chemicals were purchased from Sigma-Aldrich Co. and E. Merck. N-(3-Phenyl-1,2,4-thiazolyl)cantharidinimine (3f): mp 197–198°C; δ (ppm) 1.26 (d, 6H, J = 8.0 Hz, CH3); 8j (ppm) 1.84–1.88 (m, 2H, CH2), 1.31 (s, 6H, CH3); 1.85–1.87 (m, 2H, CH2), 1.25 (t, 2H, J = 2.6, 2.4 Hz, OCH2); 6.98 (s, 1H, H-4), 7.37 (t-like, 1H, H-4), 7.44 (dd, 2H, J = 7.7, 7.3 Hz, H-3, H-5), 7.73 (d, 2H, J = 7.5 Hz, H-2, H-6); IR (KBr): 1706 (carbonyl group) cm−1; HR-MS (EI, 80 eV) Calcd for C19H19N2O2S: 337.1425. Found: 337.1426.

MTT Assay for Cellular Viability  Cells were seeded into 96-well plates and allowed to adhere for 24 h before drug was introduced. Then drugs and media were removed, and each well was treated with 100 µL of 500 µg/mL MTT in culture medium. The plates were centrifuged at 450 g for 10 min, and supernatants were removed and replaced with 100 µL of dimethyl sulfoxide (DMSO). Absorbance, as a measure of viable cell number, was read the following day with 100 µL of dimethyl sulfoxide (DMSO). Absorbance, as a measure of viable cell number, was read the following day with 100 µL of dimethyl sulfoxide (DMSO). Absorbance, as a measure of viable cell number, was read the following day with 100 µL of dimethyl sulfoxide (DMSO). Absorbance, as a measure of viable cell number, was read the following day with 100 µL of dimethyl sulfoxide (DMSO). Absorbance, as a measure of viable cell number, was read the following day with 100 µL of dimethyl sulfoxide (DMSO). Absorbance, as a measure of viable cell number, was read the following day with 100 µL of dimethyl sulfoxide (DMSO). Absorbance, as a measure of viable cell number, was read the following day with 100 µL of dimethyl sulfoxide (DMSO).
N-(3-Amino-4-pyridyl)cantharidinimine (8m): mp 235–238 (MeOH); 1H-NMR (500 MHz, CDCl3): δ (ppm) 1.28 (s, 6H, CH3×2), 1.76–1.78 (m, 2H, CH2), 1.87–1.91 (m, 2H, CH2), 3.97 (bs, 2H, NH2-3), 4.69–4.70 (t, 2H, J=2.3 Hz, OCH×2), 6.95(s, 1H, H-5), 8.06–8.07 (d, 1H, J=4.7Hz, H-6), 8.24 (s, 1H, H-2); IR (KBr): 1717(C=O) cm−1; MS m/z (rel., int.): 287 [M]+ (13), 170 (100).

N-(4-Amino-3-pyridyl)cantharidinimine (8n): mp 273–276 (MeOH); 1H-NMR (500 MHz, CDCl3): δ (ppm) 1.29 (s, 6H, CH3×2), 1.76–1.78 (m, 2H, CH2), 1.89–1.92 (m, 2H, CH2), 4.51 (bs, 2H, NH2-4), 4.69 (s, 2H, OCH×2), 6.63–6.64 (d, 1H, J=5.6Hz, H-5), 8.04 (s, 1H, H-2), 8.21–8.30 (d, 1H, J=5.6Hz, H-6); IR (KBr): 1713 (C=O) cm−1; MS m/z (rel., int.): 287 [M]+ (100), 218 (75).

N-(5-Bromo-2-pyridyl)cantharidinimine (8o): mp 193–194 (MeOH); 1H-NMR (500 MHz, CDCl3): δ (ppm) 1.26 (s, 6H, CH3×2), 1.74–1.76 (m, 2H, CH2), 1.84–1.87 (m, 2H, CH2), 4.71–4.72 (t, 2H, J=2.41 Hz, OCH×2), 7.25–7.26 (t, 1H, H-3), 7.95–7.97 (q, 1H, J=2.3, 6.1, 2.4Hz, H-4), 8.69 (d, 1H, J=2.2Hz, H-6); IR (KBr): 1710 (C=O) cm−1; MS m/z (rel., int.): 350 [M]+ (2), 281 (100), 283 (97).

N-(4-Pyridyl)cantharidinimine (8p): mp 139–140 (MeOH); 1H-NMR (500 MHz, CDCl3): δ (ppm) 1.26 (s, 6H, CH3×2), 1.75–1.77 (m, 2H, CH2), 1.85–1.88 (m, 2H, CH2), 4.69–4.70 (t, 2H, J=2.7, 2.61 Hz, OCH×2), 7.46–7.47 (d, 2H, J=6.2Hz, H-3, H-5), 8.71–8.73(d, 2H, J=5.5Hz, H-2, H-6); IR (KBr): 1717 (C=O) cm−1; MS m/z (rel., int.): 272 [M]+ (38), 203 (100), 204 (95).

N-(4,6-Dimethyl-2-pyridyl)cantharidinimine (8q): mp 185–186 (MeOH); 1H-NMR (500 MHz, CDCl3): δ (ppm) 1.26 (s, 6H, CH3×2), 1.73–1.75 (m, 2H, CH2), 1.85–1.87 (m, 2H, CH2), 2.35 (s, 3H, CH3-6), 2.53 (s, 3H, CH3-4), 4.71–4.72 (t, 2H, J=2.9, 2.61Hz, OCH×2), 6.9 (s, 1H, H-5), 7.03 (s, 1H, H-3); IR (KBr): 1707 (carbonyl group) cm−1; MS m/z (rel., int.) 300 [M]+ (12), 231 (100).

N-(5-Hydroxy-6-pyridyl)cantharidinimine (8r): mp 289–291 (MeOH); 1H-NMR (500 MHz, CDCl3): δ (ppm) 1.25 (s, 6H, CH3×2), 1.71–1.73 (m, 2H, CH2), 1.83–1.85 (m, 2H, CH2), 4.69–4.70 (t, 2H, J=2.7, 2.7Hz, OCH×2), 7.21–7.23 (t, 1H, J=4.5, 4.5Hz, H-5), 7.35–7.37(d, 1H, J=6.9Hz, H-4), 8.10–8.12(d, 1H, J=3.2Hz, H-6); IR (KBr): 1712 (C=O) cm−1; MS m/z (rel., int.): 288 [M]+ (18), 219 (100).

Acknowledgments We thank Ms. Shwu-Huey Wang for help with mass and NMR spectra and data calculation.

References