Abstract. Aim: Brucea javanica was studied to identify nuclear factor kappaB (NF-κB) inhibitors exhibiting reactive oxygen species (ROS) intracellular amplification. Material and Methods: Eight compounds were evaluated for selective cytotoxicity using HT-29, HeLa, and HL-60 cells, and in a NF-κB assay. Active compounds were then tested using ROS and mitochrondria transmembrane potential (MTP) assays. NF-κB and nuclear factor activated T-cell (NFAT) translocation were also assessed using their respective whole cell assays. Results: Bruceajavanone B, bruceantin, bruceine A, (–)-hydnocarpin, and chrysoeriol exhibited cytotoxic potential and NF-κB p65 inhibition. Chrysoeriol exhibited selective cytotoxicity against leukemia cells with greater potency and also showed an ability to up-regulate NFAT transcriptional pathways through the amplification of intracellular ROS, in the presence of H₂O₂, to a greater degree than bruceantin and bruceine. Conclusion: Chrysoeriol selectively kills leukemic cells and potentiates the amplification of ROS levels. Therefore, chrysoeriol could serve as a potential chemotherapeutic modifier for leukemia chemotherapy since leukemia cells have a higher susceptibility to elevated ROS levels.

Brucea javanica (L) Merr. (Simaroubaceae) is a medicinal plant used by local populations in Southeast Asia to treat dysentery, malaria, and cancer (1). Phytochemical investigations on B. javanica have indicated this species to be a source of quassinoids that are known to possess antimalarial, cytotoxic, and antineoplastic activities (2, 3). As part of a drug discovery program on natural product anticancer agents, the combined leaves, twigs, and inflorescence of B. javanica were collected in Vietnam. In a prior phytochemical study, activity-guided isolation of a chloroform extract of these plant parts of B. javanica, using the MCF-7 breast cancer cell line to monitor fractionation, led to the isolation of several triterpenoids, two quassinoids, and a flavonolignan (2). Several of these compounds, bruceajavanone A 7-acetate (1), bruceajavaninone A (2), bruceajavanone C (3), bruceajavanone B (4), bruceantin (5), bruceine A (6), and (–)-hydnocarpin (7), as well as a flavonoid [chrysoeriol (8)] (Figure 1) isolated from the same plant source, were selected for further biological evaluation. These were tested for selective cytotoxicity using HT-29 colon cancer, HeLa cervical cancer, and HL-60 leukemia cells, and then also evaluated in a nuclear factor kappaB (NF-κB) inhibition assay (4, 5). Compounds found active in both assays were then tested for their antioxidant potential and for their ability to activate nuclear factor of activated T-cells (NFAT) transcriptional pathways while also repressing NF-κB, via the rapid intracellular amplification of reactive oxygen species (ROS) possibly secondary to mitochondrial damage (6-10). Thus, in the present study these compounds were further studied for their multi-target biological potential.

Materials and Methods

Materials. Compounds 1 to 7 were isolated and purified from a sample of the combined leaves, twigs, and inflorescence of B. javanica (L) Merr. (Simaroubaceae), collected in Vietnam, as described previously (2). A further constituent of this plant (8) was also isolated from this same plant source, as described below. Vitamin C, ellipticine, and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Staurosporine was obtained from Cayman Chemical Company (Ann Arbor, MI, USA), and thapsigargin from Pierce Biotechnology (Rockford, IL, USA). Rocaglamide was isolated in pure form in a previous investigation in our laboratories (10).

Isolation. The flavone, chrysoeriol (8), was isolated from the same partially detannified chloroform-soluble extract of B. javanica as compounds 1-7 (2). A precipitate deposited from fraction F2 from
this chloroform extract was further purified by preparative TLC 20x20 cm, 500 µm thickness, Si gel 60 F254 glass plates (Whatman, Clifton, NJ, USA), using CH2Cl2-acetone (5:1) as developing solvent, to yield compound 8 (3 mg). This pure isolate (8) was identified as chrysoeriol by comparing its physical and spectroscopic data with literature values (11).

Sample preparation. Serial dilutions were performed on test compounds at 50 or 20 µg/ml. A 10-fold dilution was made from the stock solution (10 mg/ml in 100% dimethylsulfoxide (DMSO)) with water or corresponding buffer, while subsequent dilutions were made with 10% DMSO in water or buffer. The concentration of the solvent remained constant at 0.5% v/v or lower.

Cell culture. The HT-29, HL-60, and HeLa cells were purchased from the American Type Culture Collection. HT-29 colon cancer (ATCC #HTB-38) and HL-60 leukemia (ATCC #CCL-240) cells were cultured at 37°C in 5% CO2 with RPMI-1640 medium (ATCC #HTB-38) and HL-60 leukemia (ATCC #CCL-240) cells from the American Type Culture Collection. HT-29 colon cancer (ATCC #HTB-38) and HL-60 leukemia (ATCC #CCL-240) cells were cultured at 37°C in 5% CO2 with RPMI-1640 medium supplemented with 5% (v/v) heat-inactivated fetal bovine serum, 2.5 mg/l amphotericin B, 50 mg/l gentamicin and 100,000 UI/l penicillin. HeLa cells (ATCC #CCL-2) were cultured with D-MEM containing L-glutamine and sodium pyruvate, and also supplemented in the same manner as RPMI-1640 medium. The culture medium was changed twice per week and passes were conducted when cells reached 70% confluence.

Cytotoxicity. Cells were grown to the desired level of confluence and appropriate test cell concentrations were made with fresh medium to incubate cell solutions in the absence or presence of the test sample and ellipticine as a positive control for 3-4 days in a CO2 atmosphere at 37°C. A zero-day control test was performed by incubating cells in at least 16 wells for 30 min at 37°C in a CO2 incubator. Cells were fixed by adding trichloroacetic acid (TCA), incubating for 30 min at 4°C, and washing three times with tap water. Cells were stained with sulforhodamine B (SRB) for 30 min, unbound dye was rinsed, and the cells were then dried under air, and bound dye dissolved in Tris base for 5 min on a gyratory shaker. Optical densities were measured on a 96-well plate reader (5).

Reactive oxygen species (ROS) assay. Intracellular ROS were estimated using a fluorescent probe, DCFH-DA. DCFH-DA readily diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent dichlorofluoroscein (DCF) in the presence of ROS. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly. In brief, cells were incubated for 5 h with either the test compound with and without FeSO4 and H2O2 for 30 min to induce hydroxyl radical damage to the cells. Vitamin C was used as a negative control to protect against hydroxyl radical damage and FeSO4 and H2O2 were used as positive controls. An aliquot of the cell suspension (160 µl) was loaded into a 96-well plate along with the test compounds (10 µl). FeSO4 and H2O2 (20 µl) were used to induce hydroxyl radical damage in the HT-29 cells, and then DCF-DA (10 µl, final concentration 5 mM) was added separately to wells after incubation. The DCF fluorescence intensity was detected at different time intervals using a FLUOstar Optima fluorescence plate reader (BMG Labtechnologies GmbH, Inc, Durham, NC, USA) with an excitation wavelength of 485 nm and emission wavelength of 530 nm (12). All treatments were performed in triplicate and are representative of at least two independent experiments.

NF-κB assay. A commercially available kit (EZ-Detect™ transcription factor assay system; Pierce Biotechnology, Rockford, IL, USA) was used to assess the ability of test compounds to interfere with the specific binding between the biotinylated- consensus sequence for the respective factor and the active form of NF-κB transcription factor. A nuclear extract of HeLa cells, treated with each test compound and tumor necrosis factor alpha (TNF-α), was used for evaluation of specific binding. The detection of NF-κB activity was based on the measurement of a chemiluminescent signal in a plate reader (FLUOstar Optima, BMG Labtechnologies GmbH, Inc). TNF-α-stimulated nuclear extract was used as a negative control and rocaglamide as a positive control in the NF-κB assay (9, 10).

NF-κB evaluation in whole cells. A cell-based NF-κB assay was carried out according to the protocol previously reported (13). In brief, HeLa cells were pretreated with a test compound or rocaglamide for 2 h and then stimulated with interleukin-1 alpha (IL-1α) for 40 min in black 96-well clear bottom plates. Cells were fixed with 10% formaldehyde (pH 7.2) at room temperature for 30 min and washed with wash buffer (WB). Cells were then incubated for 1 h with NF-κB primary and secondary antibody (Cellomics, Inc, Pittsburgh, PA, USA). Cells were then incubated with detergent buffer for 15 min and washed twice with WB. NF-κB staining reagent was added; plates were then covered with aluminum foil and incubated for 1 h at room temperature in the dark. Samples were observed under an inverted phase-contrast Axiosvert 40 CFL fluorescence microscope equipped with a color camera ProRes™ C10 Plus (Zeiss) to assess the inhibition of NF-κB translocation from the cytoplasm to the nucleus by active compounds. All treatments were performed in duplicate.

NFAT-1 assay. Translocation of NFAT-1 from the cytoplasm to the nucleus was detected by a similar assay as above. In brief, cells in a 96-well plate were incubated with thapsigargin (60 nM) or test compound for 30 min. Cells were fixed and incubated with NFAT-1 primary and secondary antibody and then stained with Hoechst

Table I. Cytotoxic potential and NF-κB p65 inhibition of compounds from Brucea javanica.

<table>
<thead>
<tr>
<th>Compound</th>
<th>HT-29 ED50 µg/ml</th>
<th>HeLa ED50 µg/ml</th>
<th>HL-60 ED50 µg/ml</th>
<th>ELISA NF-κB IC50 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>2</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>3</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>4</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>5</td>
<td>5.3</td>
<td>5.3</td>
<td>5.3</td>
<td>5.3</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>8</td>
<td>11.5</td>
<td>11.5</td>
<td>11.5</td>
<td>11.5</td>
</tr>
<tr>
<td>Ellipticinea</td>
<td>3.5</td>
<td>3.6</td>
<td>1.3</td>
<td>-</td>
</tr>
<tr>
<td>Rocaglamideb</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.038</td>
</tr>
</tbody>
</table>

aEllipticine was used as a positive control in the cytotoxicity assays.
bRocaglamide was used as a positive control in the NF-κB p65 assay.
dye (Cellomics, Inc, Pittsburgh, PA, USA). The plates were then scanned using fluorescence microscopy (Carl Zeiss Microimaging, Inc, Thornwood, NY, USA) to assess the ability of the active compounds to translocate NFAT from the cytoplasm to the nucleus. All observations were performed in duplicate and were representative of at least two independent experiments.

**MTP assay.** Changes on the mitochondria transmembrane potential were detected and quantified by a fluorescence cell-based assay. In brief, cells cultured in black 96-well plates or black clear bottom 96-well plates at a density of 6x10^4 were incubated overnight at 37°C in a CO₂ incubator. Cells were then treated with the test compounds or staurosporine (positive control) for two hours. Immediately afterwards, cells were incubated with the lipophilic cationic dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanide (JC-1) (Cayman Chemical Company, Ann Arbor, MI, USA) for 30 min. After incubation, cells were washed with a wash buffer to remove unbound staining reagent. The clear bottom plates were then scanned with fluorescence imaging microscope (Axiovert 40 CFL, Carl Zeiss Microimaging, Inc). Black 96-well plates were analyzed by a FLUOstar Optima fluorescence plate reader (BMG Labtechnologies GmbH, Inc.) with an excitation wavelength of 485 nm and emission wavelength of 530 nm for JC-1 monomers and an excitation wavelength of 560 nm and emission wavelength of 595 nm for J-aggregates. Measurements were performed in triplicate and are representative of at least two independent experiments (14).

**Results**

**Cytotoxicity.** The eight pure isolates (1-8) from *Brueca javanica* were assessed for selective cytotoxicity against HT-29, HeLa, and HL-60 cells (Table I). In the cytotoxicity assays, compounds 5 and 6 were shown to be highly cytotoxic against all three cancer cell lines, while compounds 4 and 8 exhibited selective cytotoxicity against the HL-60 cell line. Compound 7 showed moderate cytotoxicity against both the HT-29 colon cancer and HL-60 human leukemia cell lines.

**NF-κB assay.** Eight compounds (1-8) were also tested using an ELISA NF-κB p65 inhibition assay (Table I). A nuclear extract of HeLa cells treated with each test compound and TNF-α was used for evaluation of specific binding. TNF-α-stimulated nuclear extract was used as a control and rocaglamide as a positive control in this assay. Compounds 4, 6, and 8 showed NF-κB inhibitory activities with IC₅₀ values of 1.0, 2.2, and 0.012 μg/ml, respectively, while the positive control rocaglamide had an IC₅₀ value of 0.038 μg/ml. The NF-κB inhibitory activity of 8 (IC₅₀ 0.012 μg/ml) was comparable to that of the positive control.
Compounds 4-8, which exhibited cytotoxicity against at least one of three different cancer cell lines used, were evaluated using an ROS assay in the absence and presence of H2O2. Firstly, compounds were tested alone in the ROS assay to assess their capability to increase ROS levels in cancer cells (Figure 2A). Secondly, the compounds were tested in combination with H2O2 to assess their potentiating effect in increasing ROS levels (Figure 2B). Only compounds 4 and 8 exerted a potentiating effect in the presence of H2O2. Compound 8 induced the most significant elevation of intracellular ROS in the absence and in the presence of H2O2.

MTP Assay. ROS generation has also been associated with a rapid increase in intracellular calcium and rapid dissipation of the mitochondrial membrane potential. Compounds 4-8 were tested in the present investigation using an MTP assay. Compound 8 was found to be able to compromise the integrity of the mitochondrial membrane to a larger extent than the other test compounds (Figure 3).

NF-κB evaluation in whole cells. Compound 8, exhibiting most significant inhibition in the NF-κB assay (Table I), was evaluated for its ability to translocate NF-κB from the cytoplasm to the nucleus using a whole cell NF-κB assay. It showed inhibition of translocation of NF-κB into the nucleus at 5 μg/ml (Figure 4A). Immunofluorescence staining of NF-κB in HeLa cells showed that NF-κB is mainly located in the cytoplasm with a minimal amount in the nucleus (PBS control A, Figure 4B). Upon stimulation with IL-1α (control B) for 30 min, a significant amount of the NF-κB is translocated to the nucleus (Figure 4C) and treatment with the positive control (Rocaglamide control C) inhibited the translocation (Figure 4D).

NFAT-1 Assay. Compound 8 was also evaluated for its ability to translocate NFAT from the cytoplasm to the nucleus while up-regulating the NFAT transcriptional pathway through the amplification of intracellular ROS (Figure 5). Induced activation of NFAT was confirmed by its translocation to the nucleus (Figure 5A).

Discussion

Oxidative stress plays a well-known role in cellular change, and the dynamic state of cellular ROS depends on the equilibrium between the internal generation of ROS and the cell’s antioxidant system (15). The basal level of ROS in malignant cells is usually higher due to higher metabolic rates, and at the same time, malignant cells are more vulnerable to mitochondrial dysfunction (16). These two properties can be used by redox-targeting therapeutic strategies.

In the present study, we evaluated eight compounds isolated from Brueca javanica in terms of their multitargeting effects using several bioassays. Although the quassinoids, bruceantin (5) and bruceine A (6), are well
known for their cytotoxic potential, (2,17, 18), there is no previous report on their selective cytotoxicity toward leukemia cells. Compounds 5 and 6 were also found to exhibit moderate NF-κB inhibitory activity. Despite their cytotoxic potential and their effect on NF-κB, NFAT, ROS levels, and MTP, compounds 5 and 6 did not potentiate the elevation of intracellular ROS when treated in the presence of H₂O₂. Bruceajavanone B (4) and chrysoeriol (8) were able to potentiate the elevation of intracellular ROS when treated in the presence of H₂O₂. A dose-dependent dual targeting of NF-κB and ROS by these compounds was observed. Chrysoeriol (8), in particular, was also found to exhibit selective cytotoxicity against leukemia cells and also to up-regulate NFAT transcriptional pathways through the amplification of intracellular ROS to a greater degree. It was also able to exponentially potentiate the elevation of intracellular ROS when treated in the presence of H₂O₂.

Our findings are consistent with data that show leukemia cells have a higher susceptibility to elevated ROS and are more sensitive to cytotoxic agents that elevate ROS levels (19). ROS susceptibility is often enhanced following chemotherapy, possibly secondary to accrued mitochondrial DNA damage (7). It might be possible to exploit these unique properties in the treatment of chronic lymphocytic leukemia refractory to chemotherapy. The therapeutic activity of antileukemia agents and the potentiating effect of compound 8 could be combined suggesting that this compound may be useful in combination therapy with other chemotherapeutic agents that use ROS in cytotoxic pathways for the treatment of either refractory chronic lymphocytic leukemia or other leukemias. Chrysoeriol was found to be moderately active when using the P-388 cell line and has also shown some activity when screened using a different ROS assay (20, 21, 22). However, there are no prior reports on its selective cytotoxicity using HL-60 cells or on similar effects in the ROS assay used in the present research.

**Conclusion**

In summary, the flavone, chrysoeriol, which has been found to selectively kill HL-60 leukemia cells and potentiate the amplification of ROS levels, represents a potential chemotherapeutic modifier in CLL chemotherapy.

**Acknowledgements**

This research was supported by a new faculty start-up package from the Ohio State University to E.J.C. de B. and the Program Project Grant P01-CA125066 funded by the National Cancer Institute, NIH.
We are grateful to Dr. A. Douglas Kinghorn for providing the natural product samples used in the present research, and for helpful comments. We thank Mr. Cuong M. Nguyen and Mr. Jonathan Gladden for figure formatting. We also wish to acknowledge the plant taxonomists who collected the plant material used in this investigation.

References