Apoptosis-Inducing Activity of New Pyrazole Emodin Derivatives in Human Hepatocellular Carcinoma HepG2 Cells

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A series of new pyrazole derivatives from emodin synthesized in our lab have been shown to have much stronger cytotoxicity than emodin against various tumor cell lines. This study was to examine the apoptosis-inducing activity of these new emodin derivatives in human hepatocellular carcinoma HepG2 cell culture for a better understanding of their cytotoxic effects on the cancer cells. Several major events in the induction of cell apoptosis, nuclear chromatin condensation, DNA fragmentation, caspase-3 activation and poly ADP-ribose polymerase (PARP) cleavage were detected in the cells after treatment with the compounds at various concentrations. Of the seven emodin derivatives tested at a dose of 10 μM and within a treatment period of 24 h, only compounds 1 and 3 effectively induced all these apoptotic events in the cancer cells. The apoptosis-inducing activity of the compounds showed a positive correlation to their cytotoxic activity, suggesting a close connection between the growth inhibition and apoptosis induction of the cancer cells by these pyrazole emodin derivatives.

Key words emodin derivative; apoptosis; HepG2 cell; caspase-3

MATERIALS AND METHODS

Cell Culture and Drug Treatment Human hepatocellular carcinoma HepG2 cell (ATCC, Rockville, MD, U.S.A.) was cultured on RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in 25-cm2 culture flasks at 37 °C in a humidified atmosphere with 5% CO2. All cells to be tested in the following assays had a passage number of 3—6. For the drug treatment experiments, the HepG2 cells were harvested from the culture during the exponential growth phase, and seeded into multi-well culture plates at 5×103—1×104 cells/ml in fresh medium. After overnight growth, the cells were treated with the compounds (predissolved in DMSO) at selected concentrations for a period of 1—2 d.

MTT Assay of Cell Viability At the end of the drug treatment period, 50 μl of 1 mg/ml MTT solution in PBS (without MTT as the blank) was fed to each well of the culture plate (containing 100 μl medium). After 4 h incubation, the formazan crystal formed in the well was solubilized with 100 μl DMSO for optical density reading at 570 nm.

Detection of Nuclear Morphology Change and DNA Fragmentation At the end of the drug treatment period, the cells in each well were washed once with PBS and fixed with 4% formaldehyde in PBS at 4 °C for 30 min. The cells were then washed with PBS and stained with 1 μg/ml Hoechst 33258 in PBS at 37 °C for 15 min, and then viewed under a fluorescent microscope for nuclear morphology analysis.

Emodin is a major anthraquinone in numerous herb species such as Rheum and Polygonum (Polygonaceae), Rhamnus (Rhamnaceae) and Senna (Cassieae). It has shown a wide range of pharmacological activities including antitumor and antibacterial, anti-inflammatory, and vasorelaxant activities. In particular, emodin has been shown to inhibit the growth and to induce the apoptosis of various cancer cells such as human lung adenocarcinoma, human promyeloleukemia, human cervical cancer and human hepatoma. Apoptosis or programmed cell death has been recognized as an important physiological event in the development and pharmacology of anticancer agents and cancer therapies. Previous studies have demonstrated that a variety of chemotherapeutic agents induce apoptosis in malignant cells.

Since the clinical use of two anthraquinones, mitoxantrone and daunorubicin, for cancer treatment 30 years ago, emodin and numerous other anthraquinones abundant in herbs have been widely explored as the lead compounds for developing new anticancer drugs through chemical modification. These anthraquinones are usually potent inhibitors of topoisomerase II in DNA, and some also induce apoptosis in cancer cells. However, the anti-proliferative activity of emodin is usually of low potency, detectable only at relative large concentrations. In a recent study in our lab, several new anthrapyrazole derivatives of emodin were synthesized by attaching various cationic alkyl amino side chains onto a pyrazole ring fused under a fluorescent microscope for nuclear morphology analysis.

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changes. For the DNA fragmentation analysis, the cells were extracted using a mini-scale apoptosis DNA isolation kit (Watson Biotechnologies, Inc., China) according to the supplier’s manual. The extract (20 μl) was then loaded onto a 2% agarose gel, and viewed with a gel documentation system.

**Caspase-3 Activity Assay** The caspase-3 activity of cells was determined using a caspase-3 colorimetric assay kit (Keygen Biotech Co., Ltd., Nanjing, China) according to the supplier’s manual. The blank was treated with the same reagent but without the caspase-3 substrate. The optical density of the assay solutions was measured at 405 nm with a spectrophotometer.

**Poly ADP-Ribose Polymerase (PARP) Cleavage Analysis** Cells harvested from each well of the culture plates were lysed in 150 μl extraction buffer consisting of 100 μl solution A (50 mM glucose, 25 mM Tris–HCl, pH 8, 10 mM EDTA, 1 mM PMSF) and 50 μl solution B (50 mM Tris–HCl, pH 6.8, 6 M urea, 6% 2-mercaptoethanol, 3% SDS, 0.003% bromphenolblue). The suspension was centrifuged at 10000 rpm and 4 °C for 5 min, and the supernatant (10 μl for each sample) was loaded onto 10% polyacrylamide gel and then transferred to a microporous polyvinylidene difluoride (PVDF) membrane. Western blotting was performed using rabbit polyclonal anti-PARP antibody (Roche, Mannheim, Germany) and horseradish peroxidase-conjugated anti-rabbit secondary antibody (Zhongshan Golden Bridge Biotechnology Co., Ltd., Guangzhou, China). Protein was visualized using super-signal west pico-chemiluminescence substrate (Pierce, Rockford, IL, U.S.A.).

**RESULTS**

**Cells Growth Inhibition** After 48 h treatment of the HepG2 cells with emodin and the derivatives, the cell viability (relative to the untreated control) showed a dose-dependent decrease with compounds 1—4 and 6, 7 in the range of 0.1—10 μM, and with emodin and compound 5 in the range of 1—100 μM (Fig. 2). Regardless of the large variability in the data, the relative cytotoxicity potency of all compounds can be distinguished and ranked from 1 and 3 as the most potent, to compound 5 and emodin as the least potent. This ranking order is consistent with the cytotoxicity levels (IC₅₀ values) of these compounds in the HepG2 and two other tumor cell lines reported previously.¹)

**Nuclear Morphology Change and DNA Fragmentation** With a drug dose of 10 μM and a treatment period of 24 h, chromatin condensation could be observed in the majority of cells in the groups treated by compounds 1 and 3, and in much fewer of the cells treated by other emodin derivatives such as compounds 2 and 6, but in none of the cells treated by emodin (Fig. 3). At a larger drug dose (20 μM) or longer treatment time (48 h), the chromatin condensation was more widespread in the cultures treated by most of the emodin derivatives (data not shown). Similarly, from the DNA fragmentation analysis, ladders, as the indication of DNA degradation, were clearly observable only in the cells treated by compounds 1 and 3 (10 μM for 24 h treatment), but were barely visible in the cells treated by other compounds or emodin (Fig. 4A). At a larger dose of 20 μM, the DNA ladders became clearly visible in the cells treated by another two derivatives compounds 2 and 6, but not in the cells treated by other derivatives (4, 5, 7) or emodin (Fig. 4B). As chromatin condensation and DNA degradation are two hallmark symptoms of apoptosis,¹⁶) the results here proved that the emodin derivatives compounds 1—3 and 6 had significant apoptosis-
inducing activity in the HepG2 cells, and they were much stronger than emodin and the other derivatives.

Caspase-3 Activation and PARP Cleavage The caspase-3 activity in the HepG2 cells treated by emodin and the derivatives at a fixed dose of 10 μM for 24 h was increased most significantly by compounds 1 and 3, and slightly by compounds 2 and 6, but not significantly affected by emodin and compound 5 (Fig. 5A). The caspase-3 activation caused by the effective compounds was dependent on both the drug dose and treatment time. With compound 1 fixed at 10 μM (Fig. 5B), for example, the caspase-3 activity showed a slight increase between 0 and 12 h and a notable increase between 12 and 24 h of treatment, and leveled off thereafter. The caspase-3 activity after 24 h treatment by compound 1 showed a gradual increase with the drug concentration from 2.5—10 μM, but no further increase from 10—20 μM. Caspase-3 is the major executor caspase at the downstream of the apoptosis cascade, which is activated by other initiators and upstream caspasas.16)

The treatment of HepG2 cells with some of the emodin derivatives also induced a dose- and time-dependent cleavage of the 113 kD PARP protein into 89 kD fragments. The cleavage was induced by compound 1 at 10 μM after 24 h treatment (Figs. 6A, B), and by other compounds at a larger dose or longer treatment time. PARP is a nuclear enzyme involved in the DNA repair process but is specifically cleaved into 89 and 24 kD fragments by caspase-3 in the process of apoptosis.17) Therefore, the cleavage of PARP shown here is another evidence for the caspase-dependent apoptosis induction by these compounds.

DISCUSSION

The above experimental results have shown that some of the pyrazole emodin derivatives were much more active than emodin itself to induce the apoptosis of the HepG2 cells through the activation of caspase-3. The degrees of nuclear morphology change and DNA fragmentation, the two hallmark symptoms of cell apoptosis, are in good correlation with the level of caspase-3 activation, which may prove the involvement of caspase-3 as an execution enzyme in the apoptosis induction. A good correlation has also been found between the cell cytotoxicity and the significance of these apoptosis events induced by these compounds, suggesting that the induction of apoptosis in the cells be a cause of cytotoxicity. Therefore, the cytotoxicity and apoptosis-inducing activity of the new pyrazole emodin derivatives share a similar structure–activity relationship (SAR). As reported previously,13) compounds 1—3 and 6 bearing the monocationic side chains had much stronger cytotoxicity than compounds 4, 5 and 7 bearing the bicaticonic side chains. Likewise, compounds 1—3 and 6 also showed a higher apoptosis-inducing activity than compounds 4, 5 and 7 according to the results of the nuclear morphology change and DNA fragmentation from this study (Figs. 3, 4). In terms of the caspase-3 activation (Fig. 5), compounds 1, 2 and 3 with the monocationic alkyl amino group were the most active, followed by compounds 6 and 7 with the heterocycle group, and compounds 4 and 5 with the bicaticonic alkyl amino were the least active.

Similar to the two anticancer drugs mitoxantrone and daunorubicin, most of the anthrapyrazole derivatives act as DNA intercalators and topoisomerase II inhibitors to inhibit the growth or cause the death of tumor cells.12,13) On the other hand, a recent study has shown that emodin induces the apoptosis of human lung adenocarcinoma cells through the generation of reactive oxygen species (ROS).6) The ROS-generating ability of emodin was mainly attributed to its quinone structure as several quinoes are putative radical-generating agents. However, the anthrapyrazole derivatives of emodin have an altered chromophore with the addition of a fourth ring, which can prevent the quinone portion of the molecule from semi-quinone free radical formation. Therefore, the cytotoxic and apoptosis-inducing properties of our new pyrazole emodin derivatives may be mostly through their interactions with the DNA molecule, interfering with DNA functions such as the inhibition of topoisomerase II, instead of through the ROS-generation.
Although a variety of anthrapyrazole derivatives from emodin or natural compounds with a similar structure have been synthesized with improved cytotoxic activity and/or DNA binding ability in cancer cells,\textsuperscript{13,18} the structure–activity relationship is still not well understood. It has been suggested that the space between the two nitrogen atoms in the side chain is an important factor for the activity, and the optimal is about two methylenes in between. All pyrazole emodin derivatives tested in our study have this structural feature. In addition, a dimethyl group has been identified as a favorable substituent on the terminal nitrogen of the side chain,\textsuperscript{18} which is a distinct structural feature of the most active compound 1 from the other less active derivatives in our study.

In conclusion, this study has demonstrated for the first time the apoptosis-inducing activity of several new pyrazole emodin derivatives, which is in close correlation with their cytotoxicity in the HepG2 cancer cells. The most active compounds were those with monocationic alkyl amino side chains, which may be promising lead compounds for further structural modification with improved antitumor activity. More efforts should also be made to understand the structure–activity relationship for more rational design and modification of the active compounds.

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**REFERENCES**