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Novel anthraquinone derivatives: Synthesis via click chemistry approach and their induction of apoptosis in BGC gastric cancer cells via reactive oxygen species(ROS)-dependent mitochondrial pathway

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ABSTRACT

Three water soluble anthraquinone derivatives were designed and synthesized employing click chemistry to prepare novel and potent antitumor drugs. An MTT assay indicated that all compounds had significant inhibitory activity against BGC gastric cancer cells in vitro. Apoptosis induced by these compounds was observed by flow cytometry and laser confocal microscopy. Mechanistic analysis showed that these compounds induced the generation of several reactive oxygen species, the loss of mitochondrial membrane potential ($\Delta\psi m$), the transition of mitochondrial permeability, and the release of cytochrome C from the mitochondrion to cytoplasm. These results suggest that the anthraquinones might be potential lead compounds for the cancer chemotherapy.

Induction of apoptosis for malignancy of tumors has become an area of intense research in oncology.[1,2] This is because this process allows for the selective apoptotic destruction of tumor cells without causing vicinal inflammation in normal body tissue. Confocal microscopy and flow cytometry are efficient techniques to identify the processes involved in apoptosis,[3,4] and recently, many antitumor compounds have been found to induce the apoptotic process in tumor cells.[5,6] Anthracycline antibiotics are an important class of drugs for cancer chemotherapy, and several have been successfully administered in the clinic, including daunomycin and adriamycin.[7] However, long-term exposure to these drugs could lead multidrug resistance (MDR). As such, modified anthracycline derivatives are being studied as alternate, second line therapeutics in order to improve the overall effectiveness of this drug class.[8–10]

In the 1990s, 'click chemistry' was invented by K. Barry Sharpless, based on an efficient 1,3-dipolar cycloaddition reaction between alkynes and azides.[11] It has found widespread use in fields of drug discovery and synthetic organic chemistry.[12,13] In an attempt to identify more effective anthracycline derivatives as anticancer treatments, we have prepared three cationic anthraquinone derivatives **1–3**, which are disubstituted using click chemistry (see Supporting Information). We believe that planar, heteroaromatic triazole-derived compounds might lead to a more facile interaction with DNA, proteins, or cells. To assess their abilities for apoptotic induction, we employed a BGC target cell line, which is a common model for testing general antitumor compound activities and for clarifying molecular mechanisms.[14] In this letter, we report the synthesis of novel anthraquinone derivatives, the cytotoxicity of these compounds against BGC cells lines in vitro, the observation of apoptosis induced by these compounds, and the mechanic study of the apoptotic induction, and we discuss the structure–activity relationships (SARs) of these compounds. Overall, our results indicate that the synthesized anthraquinones might be potential lead compounds for the cancer chemotherapy (Scheme 1, Detailed synthesis procedures see supporting information).

Selective toxicity toward tumor cells is a crucial characteristic of compounds in the development of anticancer drugs.[15] A concentration-dependent decrease in cell proliferation was observed in an MTT assay. All synthesized compounds displayed good inhibitory activities against BGC cells with IC_{50} values of **1–3** of 9.31, 6.52 and 4.02 μM, respectively.

In order to determine whether the anthraquinone derivatives successfully killed tumor cells involved in the apoptotic pathway, cell cycle analysis was performed using a flow cytometry assay, staining with propidium iodide. Our results suggested that all anthraquinones successfully recruited the cells in the apoptotic sub-G1 peak (Fig. S1), (AP) representing cells undergoing apoptosis.[16] The apoptotic percentage in the DNA histogram was 39.4% when BGC cells were exposed to 25.0 μM compound **3** for 24 h.

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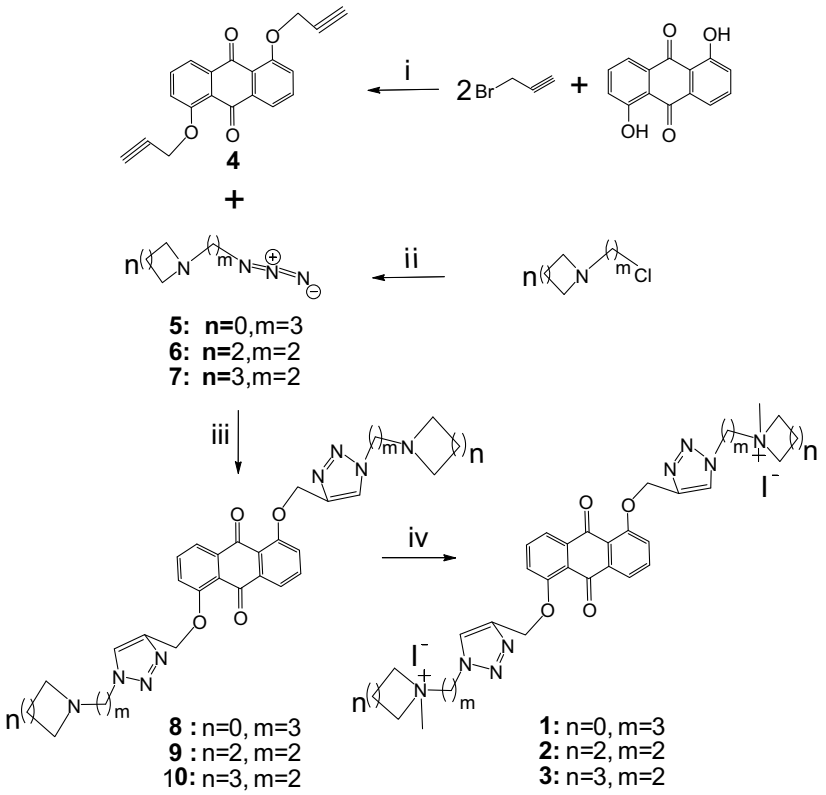

Scheme 1. Synthetic route for preparation of compounds **1**–**3**. Reagents and conditions: (i) acetone, potassium carbonate, potassium iodide, reflux; (ii) DMSO, sodium azide, sodium iodide, 50 °C; (iii) *t*-BuOH, H_2O, $CuSO_4$, sodium ascorbate, 60 °C; (iv) CH_3I, $CHCl_3$, reflux.

Flow cytometry analysis indicated that the anthraquinones induced apoptosis of the BGC cells in a concentration-dependent manner (Fig. S1). Gratifyingly, compounds **2** and **3** had more efficiency than compound **1** in the induction of apoptosis, possibly due to their improved lipophilicity.

The percentage of apoptosis can be reflected by the phosphatidylserine content (PS) on the outer leaflet of the plasma cell membrane (PS externalization) with FITC-conjugated Annexin V.[17] Although negative control cells induced little apoptosis (ca. 5.4%), the percentages of apoptotic cells treated with anthraquinones were significantly greater (generally between 18% and 23%) than those of control cells. This further demonstrated the superior apoptosis-inducing abilities of compounds **2** and **3**, consistent with the former cell cycle assays (Fig. S2).

Apoptosis can be characterized by morphological and biochemical changes in the cell nucleus, including chromatin condensation and nuclear shrinking.[18] To further confirm the existence of apoptosis in the BGC cells treated with our anthraquinones, the morphology of apoptosis was investigated. BGC cells treated with 12.5 μM compound **3** produced distinct morphological changes in apoptosis, including membrane budding, chromatin condensation, and the formation of apoptotic bodies after being stained with Hoechst 33258 (Fig. S3). Additionally, the anthraquinones also induced apoptosis in BGC cells in a concentration-dependent manner (Fig. S3), which is in accord with the results obtained from the previously described flow-cytometric analysis (see Fig. 1).

The mitochondrion is an important organelle for the production of cellular energy through metabolism and has been reported to be involved in programmed cell death.[19] The bursting of the ROS in the mitochondrion may result in functional disorder of the mitochondrion. To determine whether this event occurs in anthraquinone-induced apoptosis, we examined the intracellular generation of ROS using confocal scanning microscopy and flow cytometric analysis with DCFH-DA. DCFH-DA is cleaved intracellularly by esterases and subsequently oxidized to highly fluorescent 2,7-dichlorofluorescein (DCF) by ROS. As shown in Figure 2, an ROS burst was observed when cells were exposed to 5 μM anthraquinone **3**, and flow cytometry analysis revealed that this ROS burst can be generated in a concentration-dependent manner. *N*-Acetylcysteine (NAC), a synthetic precursor to intracellular glutathione (GSH) and cysteine, is known to be a general antioxidant.[20] It can scavenge ROS and subsequently increase the intracellular levels of GSH.[21] To demonstrate whether anthraquinone-induced apoptosis is dependent on the intracellular levels of ROS, we investigated the effects of NAC on anthraquinone-treated cells. Pretreatment of the cells with 1.5 mM NAC for 24 h successfully attenuated the elevation of ROS and also the apoptosis induced by anthraquinone treatment. These data indicate that the cytotoxicity of the anthraquinones was dependent on ROS.

The maintenance of mitochondrial membrane potential ($\Delta\psi m$) is significant for mitochondrial integrity and bioenergetic function.[22] Mitochondrial changes, including loss of $\Delta\psi m$, are key events that take place during drug-induced apoptosis. To determine the changes in $\Delta\psi m$, we examined the lipophilic dye JC-1, specific for mitochondria. Mitochondria that maintain normal $\Delta\psi m$ concentrate JC-1 into aggregates (red fluorescence). However, JC-1 forms monomers (green fluorescence) in depolarized mitochondria. The loss of $\Delta\psi m$ can thus be monitored by a shift in fluorescence from red to green (Fig. S7). As shown in Figures 3 and S7, red fluorescence decreased with a corresponding increase in green fluorescence when cells were treated with our synthesized anthraquinones, suggesting a distribution from polarized subpopulations to depolarized ones. Flow cytometry analysis (Fig. S8) demonstrated that the anthraquinones induced a loss of $\Delta\psi m$ in a concentration-dependent manner, which was consistent with our previous apoptosis results. These results indicate that a decrease in $\Delta\psi m$ was involved in anthraquinone-induced apoptosis.

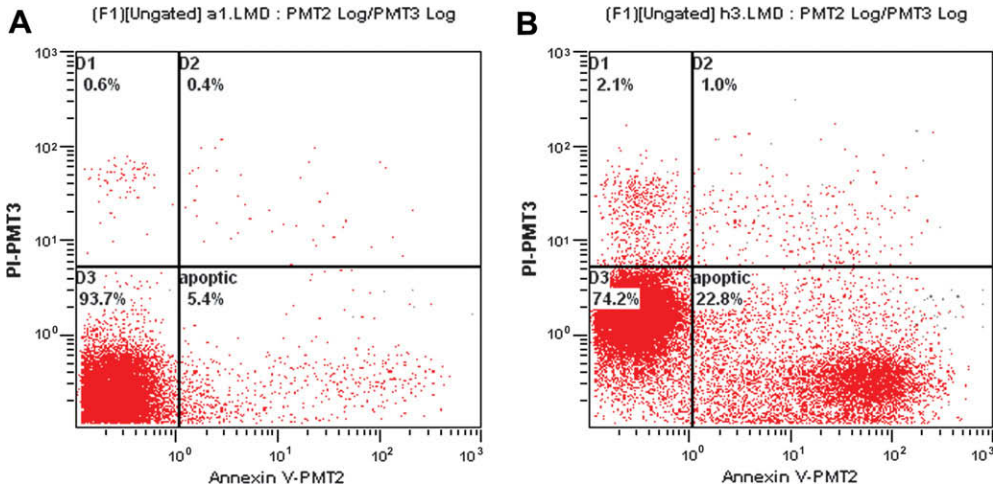

Figure 1. Apoptosis detection in BGC cells using the Annexin V assay after 24 h. The percentage of apoptosis was represented by the region named 'apoptic'. (A) and (B) represent the cells treated with 0 and 25 μM compound **3**, respectively.

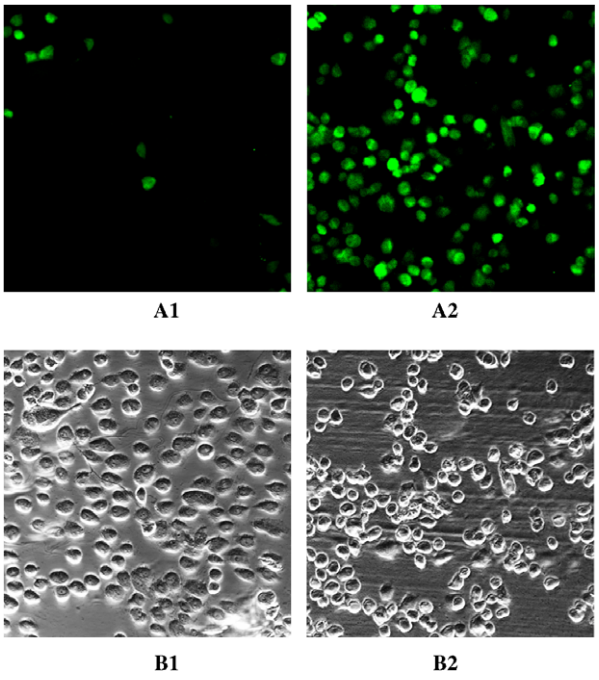

Figure 2. Burst of ROS imaged by fluorescence scanning confocal microscopy. BGC cells were treated 5 μM compound **3** then followed staining with 10 μM DCFH-DA at 37 °C for 20 min. (A) The green passage (excitation 488 nm, emission 530 nm) and (B) the phase-contrast passage. (1) Control; (2) 5 μM compound **3**.

When cytochrome C is released from the mitochondria into cytoplasm, it is removed from permeabilized cells into blocking buffers, and it can be subsequently washed.[23] During this process, a decrease in immunofluorescence intensity can be observed, which is directly correlated to the release of cytochrome C from the mitochondria. Anthraquinone-treated cells produced significant reductions in cytochrome C immunofluorescence relative to a control, demonstrating that the release of cytochrome C was involved in anthraquinone-induced apoptosis.

It has been shown that the occurrences and onset of stomach cancer are directly related to the reduction of cell apoptosis and the disturbance of cell differentiation.[24] To date, chemotherapy remains the most effective and conventional method of treatment. In

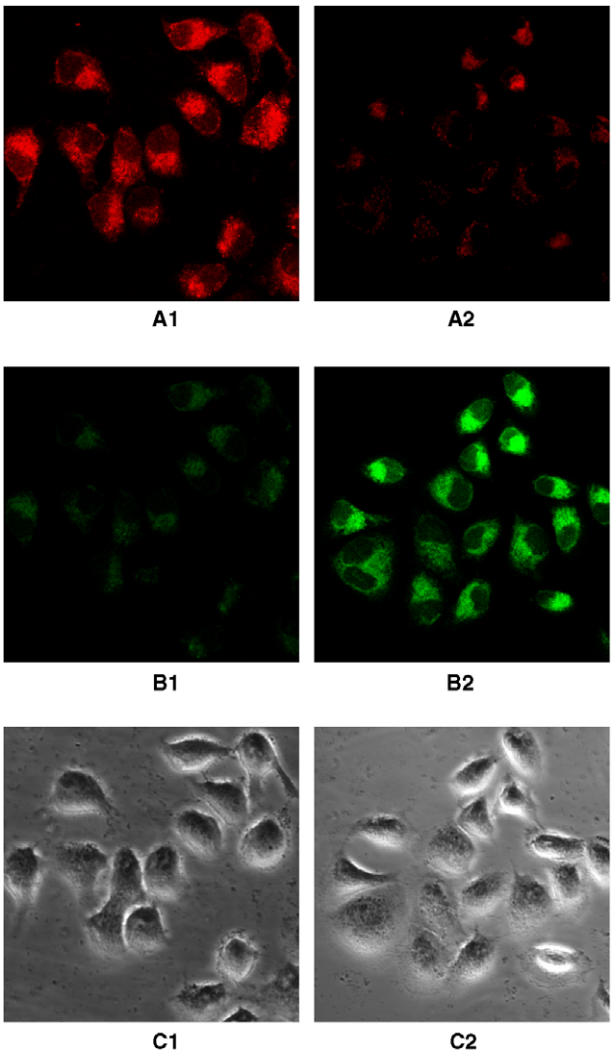

Figure 3. Changes of mitochondrial membrane potential ($\Delta\psi m$) of BGC cells when incubated with compound **2** for 24 h. The LSCM images of cell samples. (A) The red passage (excitation 525 nm, emission 590 nm), (B) the green passage (excitation 488 nm, emission 530 nm) and (C) the phase-contrast passage. (1) Control; (2) 10 μM compound **2**.

recent years, several natural anthraquinones found in the roots of the rhubarb plant, such as rhein, emodin, and aloe-emodin, have been reported to inhibit the proliferation of various tumor cells in vivo, which may be associated with apoptotic induction.[25] However, a daily average dosage of such rhubarb is so high that the applied concentration of the overall anthraquinones reaches the millimole level.[25] Results of this study indicate that synthetically modified anthraquinones can significantly induce apoptosis in BGC cells at micromolar concentrations in vitro. Mitochondria are known to play an important role in the regulation of apoptosis.[26] Bursting of DNA-damaging ROS can disrupt redox homeostasis in cells and activate mitochondrial permeability transition, resulting in a loss of mitochondrial membrane potential ($\Delta\psi m$).[27] Our studies suggest that the anthraquinone-generated ROS may be vital for apoptotic induction because pretreatment of cells with antioxidant NAC showed a distinct protective effect against apoptosis. Many previous results have demonstrated that caspases (cysteine aspartic-acid-specific protease) play important roles in the regulation of apoptosis.[28] As such, the activity of caspases is an important biochemical indicator of the apoptotic processes. Studies of the enzymatic activity of caspases in cells incubated with the synthesized compounds are in progress.

In summary, we have demonstrated for the first time the synthesis of novel anthraquinone derivatives though click chemistry. Anthraquinone-induced apoptosis of BGC cells might involve an ROS-dependent and mitochondrial dysfunction mechanism. The induction of apoptosis by these compounds in other cancer cell lines is the subject of on-going investigations.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.10.047.

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