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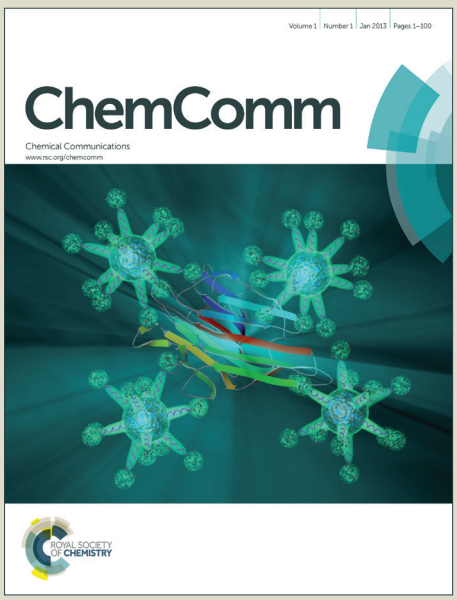

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COMMUNICATION

Potential sonodynamic anticancer activities of artemether and the liposome–encapsulated artemether

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The potential application of artemether as a novel sonosensitizer for sonodynamic therapy (SDT) was explored and illustrated for the first time. In addition, liposome–encapsulated artemether exhibited significant enhanced sonodynamic anticancer activity. Our findings indicated that artemisinin derivatives may serve as a new kind of sonosensitizers for SDT.

It is more and more difficult to explore new chemical entities (NCE) in clinical settings recently due to the fact that the development of a brand-new drug is time consuming, costly and risky.[1] However, with the increasing knowledge on the pathological mechanisms of diseases and the rapidly development of biological techniques, accumulating evidence revealed that numerous approved drugs might have additional therapeutic functions which shed light on clinical management.[2] This promising strategy has been recognized as drug repurposing or drug repositioning during the past decade, providing unprecedented opportunities to find an existing old drug or investigational drug for an additional therapeutic area.[3] This approach can significantly reduce the time, decrease costs and improve success rates, resulting in development of several repositioned drugs such as sildebafil,[4] thalidomide,[5] and methotrexate.[6]

Artemisinin containing a biologically relevant 1,2,4-tiroxane ring system originated from the traditional Chinese medicinal plant *Artemisia annua* and its derivatives including itself are commonly used as antimalarial drugs for more than 30 years.[7] Recently, growing amount of research has demonstrated that artemisinin and many of its bioactive derivatives especially artemether exhibit anticancer effects in a range of human cancer cell models.[8] Increasing clinical evidence has suggested that the treatment of artemether may improve the survival rates of cancer patients with good tolerability and significantly reduced/or minimal side effects.[9] However, the clinical development of artemisinins for cancer therapy has been hampered by its relatively moderate potency.[7] Despite many efforts on the development of novel artemisinin analogues by rational modification in order to improve the anticancer potency and drug-like properties, these new artemisinin derivatives are still far from the clinical application.[10]

Sonodynamic therapy (SDT) as a promising noninvasive approach for human cancer was developed on the basis of photodynamic therapy (PDT).[11] Different from PDT, SDT utilizes low-intensity ultrasound to trigger a certain sonosensitizer, eventually resulting in significant synergistic anticancer effects. As ultrasound is capable of penetrating deep-seated tissues, SDT can treat deep lesions effectively, indicating that SDT has more potential for cancer therapy.[12] In particular, the preliminary clinical trial of sonoflora 1 as a new sonosensitizing agent (SF1, one of chlorophyll analogues, also known as a PDT photosensitizer) in the treatment 3 advanced refractory breast cancer patients exhibited the positive therapeutic effects.[13] However, the recent studied sonosensitizers are mainly photosensitizers, most of which exhibit deficiencies of skin photosensitivity and unsatisfactory sonocytotoxicity.[14] Therefore, more efforts should be put in the development of new kinds of sonosensitizers with low or no photocytotoxicity and desirable sonochemical properties. In our continuing effort to develop new efficient sonosensitizers for SDT,[15] we reported the potential applications of artemether (Fig. 1A) as a novel sonosensitizer for the first time. In addition, we also reported the liposome-encapsulated artemether which exhibited significant enhanced sonodynamic anticancer activity.

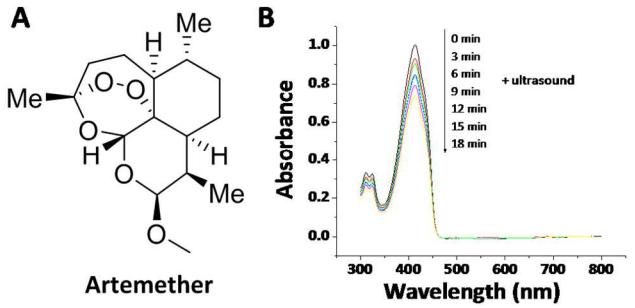

Fig. 1 (A) Chemical structure of artemether; (B) Electronic absorption spectra of DPBF and artemether at 413 nm under ultrasound treatment (1.0 MHz, 2 W/cm^2) for 0 min and t min.

As artemisinins contain the biologically relevant 1,2,4-tiroxane ring system, we predicted that this kind of scaffold would benefit for the potential production of ROS (singlet oxygen) under the condition of ultrasound irradiation. Herein we choose artemether as a model compound because it is one of the essential medicines on the WHO model List (18th list) and very safe (especially in pregnancy).[7] To determine the potential production of singlet oxygen by artemether under ultrasound irradiation, we firstly utilized a steady-state method using 1,3-diphenylisobenzofuran (DPBF) as the scavenger according to the process for

photosensitizers (The experimental set-up for ultrasound exposure is showed in Fig. S1).[16] After comparison of numerous solvents including DMSO, DMF and EtOH, we found that 2-methoxyethanol can be used as a prefect solvent for artemether. 2-Methoxyethanol is very stable under ultrasound irradiation without any change in the DPBF absorbance at 413 nm for several hours. Specifically, 2 mL of 2-methoxyethanol solution of artemether (5 μM) containing DPBF (50 μM) was prepared and irradiated with ultrasound (1.0 MHz, 2 W/cm^2, 3 min), then DPBF degradation at 413 nm was monitored along with different irradiated time. Notably, Nomikou et al. also used DPBF to determinate singlet oxygen generation of water-soluble microbubble-sonosensitizer conjugate in the mixed solvent (EtOH/H_2O = 1/1 by volume).[17] As expected, artemether showed remarkable enhancement of the singlet oxygen with quenching rate of 10.33 (Fig. 1B and Table S1), indicating the potent synergistic relationship between artemether and ultrasound.

To determine whether the singlet oxygen generated under ultrasound irradiation would have the desired cytotoxic effect on cancer cells, we next examined the cytotoxic effects of sonication with artemether. HepG2 cells were incubated with artemether at the concentration of 100 μM. These cells were then treated without or with ultrasound (1.0 MHz, 2 W/cm^2, 3 min). We included control without artemether for comparison. Following irradiation, the cells were incubated for 24 h and the cell viability was determined using the MTT assay. The results showed that artemether displayed about 50% inhibition in cell viability upon ultrasound irradiation, while artemether without ultrasound treatment were significantly less effective with about 15% reduction in cell viability (Fig. 2A). Consistent with the previous report, 100 μM of artemisinins do not show significant anticancer effects on HepG2 cell.[18] It is worth mentioning that artemether did not show any photocytotoxicity at 100 μM (Fig. S2). In order to confirm the sonocytotoxicity of artemether, we also evaluated the sonodynamic and photodynamic anticancer effect on the proliferation of breast cancer cell lines MCF-7 (Fig. S3 and Fig. S4). These results further demonstrated that artemether only exhibited efficient sonocytotoxicities without obvious photodynamic effect.

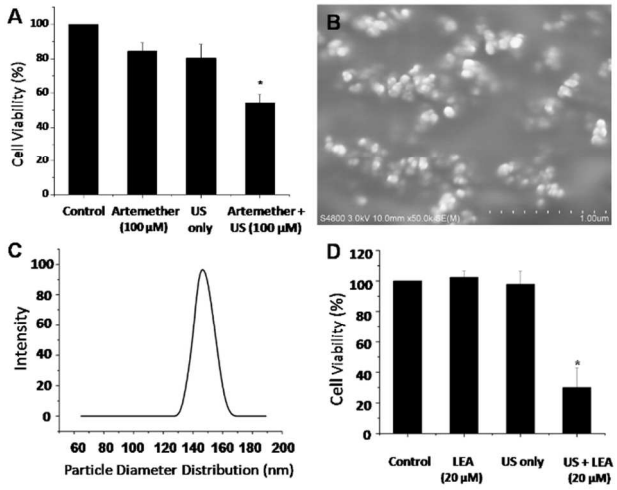

Fig. 2 (A) Cytotoxicity of artemether (100 μM) against HepG2 cells; (B) SEM images and (C) particle diameter distribution of liposome–encapsulated artemether (**LEA**); (D) Cytotoxicity of **LEA** (20 μM) against HepG2 cells (*, $p < 0.01$).

Dai et al. proposed that the application of sonosensitizers in combination with liposomes might enhance anticancer efficiency.[19] Very recently, Lu et al. reported new functional paclitaxel plus artemether liposomes.[20] This kind of liposome can significant enhance efficacy of drugs for cancer treatment. In order to increase the cytotoxic effect of artemether, we prepared the liposome–encapsulated artemether to verify the hypothesis of Dai.[19] The liposome–encapsulated artemether (**LEA**) was prepared by a conventional thin-film hydration method.[21] Generally, the chloroform solution of artemether, soybean lecithin, cholesterol and vitamin E (mass ratio = 10:80:20:0.5) was evaporated to form lipid film, and then the film was hydrated with PBS at 30 ^oC for 2 h. The resultant suspension was sonicated for 10 min to form liposomes. The final liposomes were filtrated through 0.22 μm microporous membrane and sealed in vial. The particle size distribution of liposomes was measured by dynamic laser-light scattering (DLS) and scanning electron microscopy (SEM) and the results were shown in Fig. 2B and Fig. 2C. The particle diameter of liposomes is about 150 nm. The size distribution of liposomes comprised only one peak, which indicated that particle distribution of **LEA** is uniform. SEM showed that the morphology of liposomes is spherical or near-spherical. The amount of artemether in **LEA** was also determined by HPLC. The results revealed that the drug encapsulation efficiency of liposome–encapsulated artemether is about 76.7%.

To determine whether the application of artemether in combination with liposomes would enhance anticancer efficiency, we next carried out the similar experiment using HepG2 cells. The cell viability of liposome–encapsulated artemether mediated SDT is shown in Fig. 2D. The results showed that **LEA**-mediated SDT at 20 μM induced a 70.0 ± 13.0% reduction in cell viability. This finding also suggested that **LEA**-mediated SDT displayed more potent antiproliferative effect against HepG2 cells than artemether-mediated SDT (cytotoxicity, 45.7 ± 4.8% at 100 μM, Fig. 2A) and ultrasound sonication alone (*, $p < 0.01$, Fig. 2D). This result confirmed that the application of artemether in combination with liposomes significantly enhanced anticancer efficiency. The enhancement may be due to the uptake increase of artemether after encapsulated in liposome.[22] Ultrasound can induce liposomal drug release by transient formation of pore like defects in the liposome membrane through which the drug is rapidly released.[23] Moreover, ultrasound-induced cavitation can make the cell membranes and capillaries more permeable to drugs.[24] These favourable terms eventually resulted in the enhancement of anticancer efficiency.

To investigate whether intracellular ROS have been involved in the induction of cell death after ultrasound exposure. We next monitored the intracellular ROS formation after SDT treatment by measuring the conversion of non-fluorescent DCFH–DA to fluorescent DCF using flow cytometry. The HepG2 cells were treated with DCFH-DA staining 30 min after sonodynamic therapy at the ultrasonic intensity of 2 W/cm^2 for 3 min. As shown in Fig. 3, there were no any cells in control group displayed DCF fluorescence (Fig. 3A), while about 0.5% of cells in US alone group showed slight higher DCF fluorescence (Fig.

3B). The DCF fluorescence intensity increased significantly when the HepG2 cells were treated with 20 μM of **LEA** after ultrasound irradiation (13.3% cells displayed higher DCF fluorescence, $p < 0.01$). This result suggests that **LEA**-mediated SDT has a strong effect on ROS production. When the cells were pre-treated with **LEA**, the level of ROS was slightly increased compared to that of only US-treated cells. These results demonstrated that artemether-loaded liposomes might enhance intracellular uptake, resulting in significant increasing the generation of ROS in HepG2 cells. Our in vitro studies confirmed that liposomal-encapsulated sonosensitizers could enhance the killing efficiency of tumor cells by ultrasound radiation. However, the cellular mechanisms of the application of artemether and the combination with liposomes need to be further clarified in future experiments.

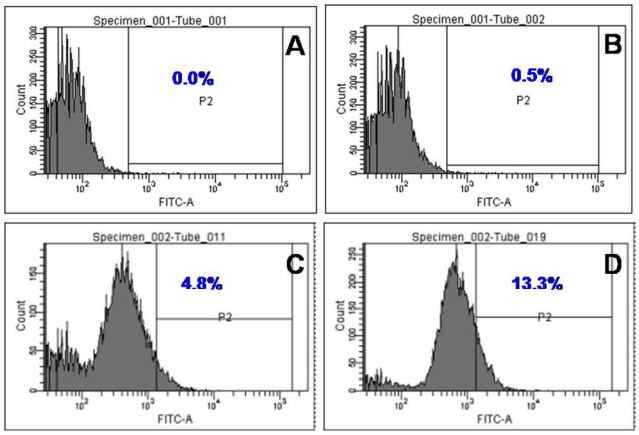

Fig. 3 Measurement of reactive oxygen species (ROS) in HepG2 cells was accomplished using flow cytometry with DCFH-DA staining 30 min after sonodynamic therapy at an ultrasonic intensity of 2 W/cm^2 for 3 min. (A) Control; (B) US alone; (C) **LEA** alone (20 μM); (D) **LEA** + US (20 μM).

Conclusions

The major challenge in recent cancer chemotherapy is to minimize toxicity and side effects of therapeutic drugs for patients. In this paper we have explored and illustrated the potential applications of artemether, one of the safe drugs, as a novel sonosensitizer for the treatment of human cancer for the first time. In addition, we also verified the liposome–encapsulated artemether exhibited enhanced sonodynamic anticancer activity. Such liposome formulation can serve as a model platform to further investigate the potential applications of other sonosensitizers or old drugs. Our recent work might open up new avenues that ultrasonic exposure has been shown to trigger efficient anticancer effects of current safe drugs on the malignant cells. Research is currently underway to elucidate the potential mechanism and to apply this strategy to other artemisinins.

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