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A synthesized butyrolactone derivative in combination with chloroquine can inhibit cancer cell growth and lysosome vacuolation induced by chloroquine in A549 lung cancer cells

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Xin-Peng Chen,[a] Chuan-Dong Fan,[a] Le Su,[a] Bao-Xiang Zhao*[b] and Jun-Ying Miao*[a]

We found that a synthesized butyrolactone derivative(3BDO) in combination with chloroquine could elevate the Na^+,K^+-ATPase activity and decrease the expression of competing endogenous non-coding RNA *TGFB2-OT1*, resulting in inhibitory effect on autophagy. Therefore, the combination inhibited the cells growth and suppressed lysosomal vacuolation induced by CQ in A549 lung cancer cells.

Autophagy is an evolutionarily conserved mechanism of adaptation to adverse microenvironmental conditions, including limited nutrient supplies[1]. Autophagy is considered to have two contrasting roles. It is assumed to provide energy for cancer cells under such unfavourable conditions, and thereby, have a cancer-promoting role. On the other hand, autophagy also has a cancer-suppressing role[2]. Some pharmaceuticals are likely to act through autophagy and used to treat cancer.

Chloroquine (CQ), a drug for antimalarial, was a novel promising drug[3] . In the cancer treatment, CQ could enhance PH of lysosome and inhibit several lysosomal enzymes, and resulted in blocking the degradation of the autolysosome inducing tumour cell death including apoptosis[4]. Therefore, CQ was used as an inhibitor of autophagy. CQ was reported to induce tumour apoptosis and inhibit autophagy *in vitro*[5-7] and *in vivo*[8] at high dose. Many more toxic effects occurred at higher doses, such as visual disturbances, gastrointestinal upset, electrocardiographic changes, headache, and pruritus[2] . At a low dose, the side effects have been firmly detected in vitro in some tumour cell type. The lysosome vacuolation after CQ-treated tumour cell line was also significantly increased in absence of CQ toxicity[5]. The lysosome dysfunction could be harmful to autophagy flux. Considering that the use of chloroquine in combination with other chemo-therapeutic reagents may enhance cancer treatment efficiency, we hope to find a novel small molecule in combination with CQ[9, 10]. The combination could be used to inhibiting growth of cancer cell instead of lysosomal vacuolation.

3-benzyl-5- ((2-nitrophenoxy) methyl)-dihydrofuran-2(3H)-one (3BDO), which is one of the butyrolactone derivatives, could inhibit the growth of A549 lung cancer cells and smooth muscle cell migration and proliferation[11, 12]. In previous report, 3BDO could activate mTOR by targeting FKBP1A and decrease the level of a competing endogenous RNA *TGFB2-OT1* to inhibit autophagy in human umbilical vein endothelial cells (hUVECs) and neuronal cells[13]. 3BDO could inhibit CQ-induced accumulation of autophagic vesicles in VECs[14]. These findings reminded us to explore the inhibitory effect of 3BDO in combination with CQ on the A549 cell growth and lysosomal vacuolation.

In this study, we observed that 3BDO in combination with CQ suppressed the growth of A549 cell line and inhibited lysosomal vacuolation induced by CQ at clinical acceptable dose (32μM).The combination might decrease the expression level of competing endogenous RNA *TGFB2-OT1* to inhibit autophagy, exhibiting more efficient effect on the growth of A549 cells than CQ alone.

3BDO was reported to inhibit the growth of human lung cancer cell A549 and smooth muscle cell proliferation[12]. We wondered that 3BDO in combination with CQ had a more effective inhibitory effect on the growth of A549 cell line. As shown in Fig.1B and C, there was no obvious morphological change in the cells when treated with 3BDO and CQ separately. When added together, they inhibited vesicle formation induced by CQ in A549 cells and appeared to decrease the number of A549 cell line (Fig.1D). To ensure the results, the viability was examined. 3BDO in combination with CQ could markedly reduce the viability of A549 cells in a dose-dependent and time-dependent manner than CQ alone, exhibiting high efficiency of inhibiting growth of A549 cell line

[a] *Shandong Provincial Key laboratory of Animal Cells and Developmental Biology, School of Life Science, Shandong University, Jinan 250100, China. E-mail: miaojy@sdu.edu.cn; Fax: +86 531 88565610; Tel: +86 531 88364929*
[b] *Institute of Organic Chemistry, School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, China. E-mail: bxzhao@sdu.edu.cn*
† Footnotes relating to the title and/or authors should appear here.
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(Fig.1E and F).Considering that cell viability was caused by proliferation or cell necrosis, the effect on A549 cell proliferation was examined. 3BDO could increase the percentage of G0/G1 and G2/M cell cycle (Fig S1). In addition, LDH activity was assayed to detect the necrosis when CQ and 3BDO were added into the medium jointly. There was no significant difference between treated group and control group, indicating that 3BDO in combination with CQ did not cause necrosis (Fig.1G). These results suggest that 3BDO could obviously inhibit the growth of A549 cell line by inhibiting proliferation when coupled with CQ, thus hinting 3BDO in combination with CQ might be a promising scheme to cure tumour.

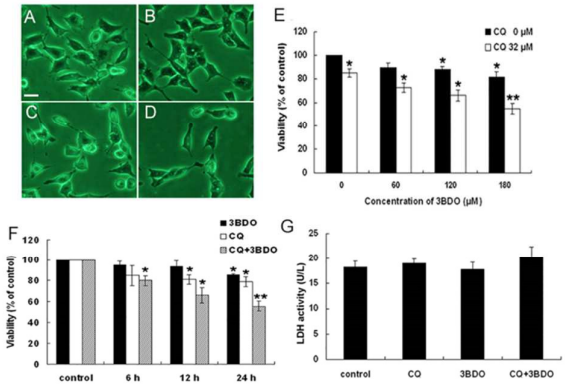

Fig.1 3BDO combination with CQ can significantly inhibit A549 cell line growth. Effects on A549 cell morphology with 3BDO and CQ for 24h (A-D). A: DMSO; B: CQ (32 μM); C: 3BDO (180 μM); D: CQ (32 μM) +3BDO (180 μM). E: the viability of A549 cells after treatment with different concentration of 3BDO for 24h in the presence of CQ (32μM). F: the viability of A549 cells with CQ (32μM), 3BDO (180μM) and CQ (32μM) combination with 3BDO (180μM) at 6h, 12h, and 24h. G: The effect of 3BDO, CQ and 3BDO combination with CQ on LDL activity in A549 cell line (Scale bars: 20μM;*p<0.05 and **p<0.01 vs control, n=3).

In previous report, the growth of human lung adenocarcinoma A549 could be inhibited by CQ at low dose. However, CQ could increase the lysosome volume and induce lysosomal vacuolation[5]. In fact, when any of components of the lysosome (enzymes, membrane proteins, cofactors, etc.) is lost or deficient, it gives rise to lysosomal dysfunction. The autophagosome fused with lysosome would increase the lysosome volume. 3BDO was an effective inhibitor of autophagic vesicle accumulation induced by CQ in VECs[14]. And then whether 3BDO could block the vacuolation induced by CQ remained unknown. AO staining was used to visualize acid vesicles formation induced by CQ. As shown Fig.S2B, CQ could obviously induce vacuolation in A549 cell line. Interestingly, 3BDO alone did not cause accumulation of acid vesicles and vacuolation. When coupled with CQ, 3BDO obviously decreased amounts of acid vesicles induced by CQ and protected A549 cell line from lysosomal vacuolation formation (Fig.S2D). To confirm the results, neutral red uptake assay was also performed and quantified. The results further suggested 3BDO at 180μM decreased almost 4 fold volumes of acid compartments (VAC) elevated by CQ and restrained the acid compartments with the increase of concentration (Fig.S2E). In addition, the effect of 3BDO on A549 cell line at different time was also observed and 3BDO could significantly cell lysosomal vacuolation at a time-dependent manner (Fig.S2F).

To understand the reason that 3BDO depressed the vacuolization induced by CQ, we speculated that the increase of VAC might be caused by the down-regulation of Na^+, K^+-ATPase activity. Accumulating evidences indicated endocytic pH was regulated by the Na^+,K^+-ATPase in living cells[15-18]. Removal of Na^+,K^+-ATPase (by recycling or degradation) would relieve the membrane potential and permit acidification to lysosomal levels, resulting in lysosome vacuolation[19-21]. Because CQ could bind to ion transporting ATPases and inhibit the activity of ion transporting ATPases, the effect of Na^+, K^+-ATPase activity was explored. In similarity, CQ (32μM) but not 3BDO decreased the Na^+, K^+-ATPase activity. When with additional 3BDO, Na^+, K^+-ATPase activity was also declined. However, 3BDO could partly elevate Na^+, K^+-ATPase activity down-regulated by CQ and effectively reduce the accumulation of acid compartments, preventing lysosome from vacuolation (FigS3).

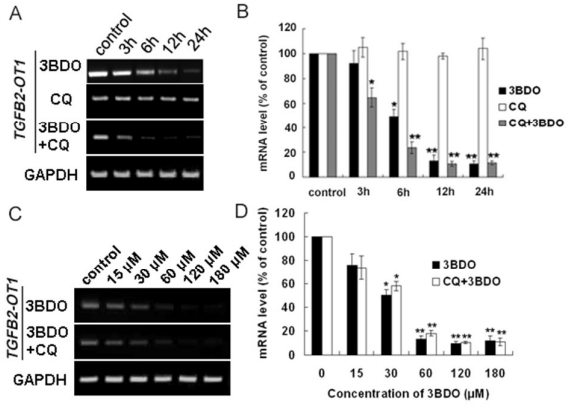

Fig.2 the expression analysis of *TGFB2-OT1* in A549 cells by semi-quantitative RT-PCR. (A) Treatment with 3BDO, CQ, CQ combination with 3BDO for 3h, 6h, 12h, 24h; （C） The level of *TGFB2-OT1* in the cells treated with 3BDO (0, 15, 30, 60, 120, and 180 μM) in the absence or presence of CQ (32 μM). The quantitative results of (A) and (C) were shown in (B) and (D) respectively. (* p<0.05 and **p<0.01vs control, n=3)

The competing endogenous RNAs (ceRNAs), as microRNA response elements, could compete for the binding of microMRA (miRNA) to regulate mRNA expression. To date, numbers lines of evidence in bioinformatics, cell biology and animal models from several famous laboratories have supported the ceRNA hypothesis[22] . CeRNAs were found to be involved in many tumours' development, such as prostate cancer and colorectal cancer[23, 24]. *TGFB2-OT1*, a competing endogenous RNA, was a key factor located at downstream of mTOR complex and could bind to *MIR4459* targeting proliferation-associated mRNA *CDC20B* and autophagy-associated mRNA *ATG13*. Previously, the down-regulation of *TGFB2-OT1* could prompt differentiation of human embryonic stem cells via cell cycle and autophagy[25]. Therefore, it implied that the inhibitory effect of growth might be associated with the expression of *TGFB2-OT1*. The mammalian target of rapamycin (mTOR) signalling pathway senses and integrates a variety of environmental cues to regulate organismal growth and homeostasis[26]. 3BDO was identified as an activator of mTOR[13]. When mTOR were activated and the level of *TGFB2-OT1* was declined. We speculated that 3BDO plus CQ could affect the gene expression of *TGFB2-OT1*. Therefore, the expression level of *TGFB2-OT1* was analysed by semi-quantitative RT-PCR. As shown in Fig.2A and B, CQ alone had no effect on the expression of *TGFB2-OT1*. 3BDO plus CQ could remarkably down-regulate the expression of *TGFB2-OT1* at 3h, 6h, 12h and 24h. After incubation with different concentration of 3BDO for 24h, the level of *TGFB2-OT1* was declined in the absence or presence of CQ (Fig.2C and D). Hence, 3BDO in the combination with CQ reduced the expression level of *TGFB2-OT1* and then bonded to less *MIR4459*. *TGFB2-OT1* might increase the more free level of *MIR4459*, resulting in the decline of mRNA *CDC20B* and then inhibiting cell cycle arrest. On the other hand; we inferred that 3BDO in combination with CQ acted on the inhibitory effect on autophagy. Autophagy provided energy for cancer cells and was beneficial to tumours' development in limited nutrients' supplies. When the energy was limited, it also had inhibitory effect on the proliferation of cancer cells.

Conclusions

In this study, we discovered that 3BDO combination with CQ upregulated the activity of Na^+, K^+-ATPase and decreased expression level of long non-coding RNA *TGFB2-OT1* to inhibit cell cycle and autophagy, thus inhibiting the growth of A549 cell line and protecting lysosome from vacuolation induced by CQ. Hence, 3BDO in combination with CQ might be a novel means to curing cancer.

Acknowledgements

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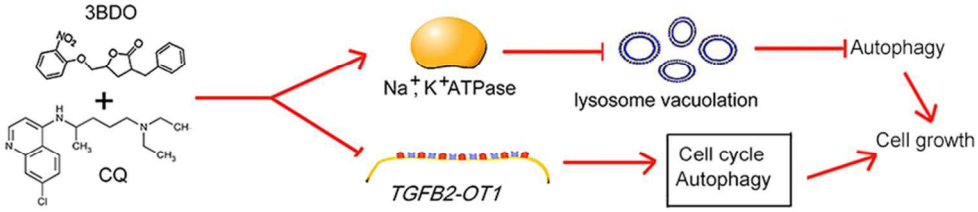

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