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The “dextran-magnetic layered double hydroxide-fluorouracil” drug delivery system exerts its anti-tumor effect by inducing lysosomal membrane permeability in the process of cell death

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Summary

The “dextran-magnetic layered double hydroxide-fluorouracil” (DMF) drug delivery system is a new type of pharmaceutical preparation that can cause cancer cell oncosis. In the present study, we used different experimental methods such as MTT, cycle assay, reactive oxygen species (ROS) assay, Annexin V-FITC/PI, Giemsa stainings, transmission electron microscopy, immunofluorescence staining and Western blotting to study the mechanism of expansion death by using Hydroxychloroquine (HCQ) as a positive control and 5-Fluorouracil (5-Fu) as reference. The results showed that DMF exhibited a better anti-tumor effect than 5-Fu in the process of cell death, and the pharmacological mechanism of 5-Fu was changed by its preparation DMF. The mechanism of cancer cell death induced by DMF was similar to that of HCQ. But DMF intervention did not cause a large amount of accumulation of mitochondrial reactive oxygen species, and the location of lysosomotropic LysoTracker Red (LTR) staining induced by DMF was closer to the nucleus or nuclear membrane. Lysosomal membrane permeability (LMP) and its subsequent the explosive death of cancer cells may be mainly related to the direct action of DMF with different organelles.

Keywords Dextran-magnetic layered double hydroxide-fluorouracil, Oncosis, Lysosomal membrane permeability, Cathepsin

Introduction

5-Fu is a commonly used broad-spectrum anti-cancer drug. However, its clinical application and anti-efficacy are largely limited by the dose-limiting toxicity and multi-drug resistance ¹⁾. Dextran-magnetic layered double hydroxide-fluorouracil (DMF) is a new type of drug delivery form that combines the broad-spectrum anti-cancer drug 5-Fluorouracil (5-Fu) and nano-carrier Magnetic Layered Double Hydroxides (MLDH) with magnetically targeted sustained release function. In the present study, we used MLDH as the carrier and developed a new pharmaceutical dosage form of DMF to achieve both magnetic targeting and release control. The structural model of DMF, the characteristics of cell transport and the phenomenon leading to oncosis of cancer cells are shown in the following figure 1²⁾:

Extensive studies have focused on the role of 5-Fu in apoptosis signaling pathway. However, some recent studies suggested that DMF might be involved in another important pathway ³⁾. Electron microscopic observations showed that the way of cell death induced by DMF was different from that of 5-Fu, and the intracellular lysosome was increased, so we speculated whether DMF caused cell death by inducing lysosomal membrane permeability (LMP) ³⁾.

Dove and his colleagues discovered the lysosome in the late 1950s. They speculated that the instability of the lysosome might lead to cell death and therefore called the lysosome a "suicide bag" ⁴⁾. Lysosomes contain a large number of cathepsin and other acidic proteins. There are mainly two lysosomal cathepsin families: one is the cysteine (cysteine) family containing 11 members known as cathepsins B, C, F, H, K, L, O, S, V, W and X, and most current studies have focused on Cathepsin B (CB). The other is the aspartic acid (aspartic) family, and the most common one is Cathepsin D (CD). When lysosomes are damaged or destroyed, these enzymes will be released into the cytoplasm, causing cell change or death through some specific signals.

Lysosomal alterations are closely related to lysosomal dysfunction and have been shown to be crucial in the plethora of cell death scenarios and pathological contexts ^{5, 6)}.

Lysosome instability can be caused by many factors, such as oxidative stress, lysosomal photooxidation, withdrawal of growth factors or activation of Fas ^{7, 8, 9, 10, 11)}. Lysosomal instability will increase LMP. Reactive oxygen species (ROS) is a well-known inducer of LMP ^{12, 13)}. Studies on lysosomal stability under oxidative stress have showed that lysosome is not a kind of organelle and not easy to be disturbed by other factors; on the contrary, lysosome was very sensitive to oxidative stress ¹⁴⁾. Exposure of the lysosomal membrane to a large amount of ROS may trigger lipid peroxidation in the intimal membrane, leading to the release of lysosome contents, these acid hydrolases will cause damage to other organelles in the cell and thus accelerating cell death ⁷⁾. In addition to mediating mitochondrial release of cytochrome C and activating the caspase family, lysosome and lysosomal protease cathepsin D and B are also mediators of cell death ⁷⁾. Therefore, the stability of lysosomes is very important for cell homeostasis and survival.

Using gastric adenocarcinoma with lymph node metastasis SGC-7901 in combination with fluorescence imaging, flow cytometry and Western blotting assays, we demonstrate that DMF could induce cell death through the lysosomal pathway.

Materials and Methods

Cell Viability Analysis By MTT Assay SGC-7901 were spread on 96-well plates at a density of 9000 cells/well for 24 h, interfered with different concentrations (62.5, 125, 250, 500, 1000 and 2000 μ g/mL) of 5-Fu and DMF at 5% CO₂ 37°C for 24 h. Thereafter, cells were added with complete medium containing 200 μ L MTT and

incubated in the cell incubator for 4 h. After sucking out the MTT medium, 100µL dimethyl sulfoxide (DMSO) was added to each well and shaken well on a plate shaker. Finally, the light absorption value was measured at 490nm using an enzyme labeling instrument (MULTISKAN GO, Thermo Fisher, U.S.A) , used statistical analysis software SPSS 26 to calculate the cell viability rate.

Giemsa Cells were seeded in 12-well plates through overnight incubation at 5% CO₂ 37°C for 24 h and given drug treatment (30µg/mL HCQ, 350µg/mL 5-Fu and 1 mg/mL DMF (contain approximately 35% 5-Fu)) for 6 h and 12 h. Giemsa staining was performed using a kit from Solarbio (G1020). Cells were stained for 5 min, washed once with PBS, and observed under a microscope (OLYMPUS, Japan).

Transmission electron microscopy (TEM) After exposed to 30µg/mL HCQ, 350 µg/mL 5-Fu and 1mg/mL DMF (contain approximately 35% 5-Fu) for 3h, then cells were fixed with 2% glutaraldehyde, post fixed in 1% osmium acid, dehydrated with different concentrations ethanol (30-50-70-80-90-100%), embedded in epoxy resin, sliced into ultrathin sections, and finally observed under an electron microscope (Hitachi, Japan).

Apoptosis Analysis By Annexin V-FITC/PI Cells were seeded in 6-well plates at 1×10^6 per well overnight and subjected to the indicated treatment 30µg/mL HCQ, 350µg/mL 5-Fu and 1mg/mL DMF (contain approximately 35% 5-Fu) for 24 h. In the inhibitor group, Bafilomycin A1 (Baf A1, 0.1µM, Apexbio) was added one hour before administration. After intervention, the cells were treated with trypsin digestion and washed twice with phosphate buffered saline (PBS). 100µL $1 \times$ Binding Buffer, 5µL PI solution and 5µL Annexin FITC (BD Biosciences FITC Annexin V Apoptosis Detection Kit, 556547) solution was added to each tube, gently blown until they were well mixed, and then placed in the dark at room temperature (25°C) for 15-20 min.

Cells were then resuspended in 1× Binding Buffer, and per sample was determined by flow cytometry (BD Accuri C6).

Cell Cycle Assay Cultured cells (1×10^6) were incubated under drug stimulation for 24 h. The inhibitor N-benzyloxycarbonyl-ValAla-Asp-fluoromethylketone (z-VAD-fmk, 100 μ M, Apexbio) was added one hour in advance. After digestion and cleaning, 75% alcohol was added for fixation overnight. Cells were washed twice, resuspended with 500 μ L RNase, and stained with propidium iodide (PI, KeyGEN BioTBCH, final concentration 25 mg/ml) at room temperature for 0.5 h. The stained cells were subjected to a flow cytometry (BD Accuri C6) and analyzed with a modfit LT-BD software.

Western Blotting After drug treatment, cells were collected, washed and added with whole protein lysate at 4°C cells following the manufacturer's instructions (KeyGEN BioTBCH, KGP902). The protein concentration was determined by using the BCA protein quantification kit (KeyGEN BioTBCH, KGP250), SDS-PAGE electrophoresed and transferred onto the polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked for 1 h in TBS-Tween-20 containing 5% non-fat milk, probed with the primary antibodies against cathepsin B (CB, Rb mAb ab125067 from Abcam), cathepsin D (CD, Rb mAb ab75852 from Abcam), anti-alpha Tubulin antibody (α -Tubulin, Rb mAb ab52866 from Abcam), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz, Sc-47724) and Bax (Rb pAb ab53154 from Abcam) at 4°C overnight. Then incubated with the secondary antibodies (ZSGB-BIO, ZB-2301) at room temperature for 1 h and washed with PBS. According to the manufacturer's instructions, the protein bands were visualized using an enhanced chemiluminescence reagent (ECL) kit (NCM Biotech, P10100).

Immunofluorescence For immunofluorescence staining, cells were fixed with

paraformaldehyde (4% w:v) after cell culture and drug administration (30 µg/mL HCQ, 350 µg/mL 5-Fu and 1mg/mL DMF (contain approximately 35% 5-Fu) for 6 h), Baf A1 was added 1 h before the addition of drug. Subsequently, cells blocked with 1% BSA for 1h and washed in PBS for three times. Then stained for the detection of cathepsin D (CD, Rb mAb ab75852 from Abcam) and Lamp-1 (Mouse mAb ab25630 from ABcam), all detected by a goat anti-mouse or goat anti-rabbit IgG conjugated with fluorochromes (Abbkine). All cover glass was placed on a slide with 4',6-diamidino-2-phenylindole (DAPI) to seal and looked at it under a microscope. LysoTracker Red (LTR) stainings were performed using a kit from KeyGEN BioTBCH (KGMP006). After drug stimulation for 15 min, the dose is the same as before. The 1µM LysoTracker Red was incubated at 37°C for 20 min and photographed using the DP20-5 inverted microscope (OLYMPUS, Japan).

ROS Assay Cells of logarithm growth were harvested and seeded in the 6-well plate. After 24 h incubation, 30 µg/mL HCQ, 350 µg/mL 5-Fu and 1 mg/mL DMF (contain approximately 35% 5-Fu) were added to the 6-well plates at 37°C with 5% CO₂ for 6, 12 and 24 h, washed once with PBS. Incubated with 0.2 µmol/L DCFH-DA at 37°C for 10 min in the dark, washed again with PBS, and detected for ROS production by flow cytometry (BD Accuri C6). The experiment was performed for at least three times.

Statistical Analysis The results are presented as the mean ± standard deviation (SD) from three individual experiments. Data were statistically analyzed by one-way analysis of variance, followed by Dunnet t's multiple comparison tests. A level of $p < 0.05$ was considered to be statistically significant.

Results

DMF has a better anti-tumor effect than 5-Fu The MTT experiment results are shown in Table 1. The IC_{50} of DMF and 5-Fu in SGC-7901 cells was 440.93 ± 0.78 and 2261.18 ± 0.61 $\mu\text{g/mL}$, respectively. To ensure the clinical equivalence of DMF and 5-Fu (the equivalence is equal according to the content of 5-Fu), the 5-Fu dose was 350 $\mu\text{g/mL}$ and the DMF dose was 1 mg/mL . Compared with the original drug 5-Fu, the DMF targeting preparation had a significantly enhanced inhibitory effect on tumor cell proliferation.

Morphologic observation of cell death by Giemsa and TEM The state of cell organelles was shown by Giemsa staining (Fig. 2a) and TEM (Fig. 2b). Giemsa staining showed obvious marginal changes in individual cells after intervention with HCQ or DMF, and the cell volume and nuclear volume were larger than those of normal cells. In different intervention periods, the organelle swelled greatly in SGC-7901 treated with HCQ, but the retention of cytoplasmic contents was high. Although there was obvious vacuolization but no nuclear apoptosis occurred after 12 h treatment, while in cells treated with DMF, the cytoplasmic loss was serious, the intercellular fluid was increased, the nuclear swelling was higher and the nuclear retention became lower. In 5-Fu group, no obvious cell swelling was observed but with the increase of the intervention time, cell chromatin agglutination and loss of membrane structural integrity were observed.

The degree of damage to the cell organelles was further observed by TEM. It was found that the organelles in the normal control group were intact, the scattered mitochondrial crest was clearly visible, and the dictyosome and nuclear membrane was intact. After administration of the positive drug HCQ, cells showed obvious autophagy, meanwhile the organelles wrapped in the autophagy were digested, in

which the double-layer membrane structure of one of the digested organelles remained, and the other organelles were digested severely (➡). After administration of DMF, the number of intracellular lysosomes (▲) and ruptured mitochondrial crests (●) were increased as compared with normal cells. In 5-Fu administration group of 5-Fu, myeloid metaplasia of organelles(Δ) was observed in SGC-7901 cells. The electron microscope experiment showed that the activity of lysosome in the cells after administration of DMF was higher than that in the normal control group.

Annexin FITC-PI determination of cell death We cited a positive drug — HCQ, which is an antimalarial drug for the treatment of autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, inflammation and skin diseases, as well as autophagy inhibitors. It is reported that HCQ can also induce LMP¹⁵⁾. To further verify the expression of the lysosomal pathway we also introduced the specific inhibitor of the lysosomal pump (vacuole H⁺ ATPase), Bafilomycin A1 (Baf A1), knowing that they can help determine whether lysosome-induced cell death was caused by DMF¹⁶⁾.

The apoptosis rate of each drug on cell lines was detected by flow cytometry and Annexin V FITC-PI method. The apoptosis rate of SGC-7901 cells induced by HCQ, DMF and 5-Fu was 18.01 ± 4.50 %, 19.68 ± 2.67 % and 14.52 ± 4.74 %, respectively (Fig. 3a and the statistical chart is shown by 3c). It was found that cells were most sensitive to the drug DMF. After administration of Baf A1, the apoptosis rate of SGC-7901 cells induced by HCQ, DMF and 5-Fu was 14.37 ± 2.29 %, 17.78 ± 6.06 % and 15.13 ± 7.78 %, respectively. Compared with the direct intervention effect of the drugs, Baf A1 inhibitors had more obvious protective effects on apoptosis induced by HCQ and DMF, and the decrease of the apoptosis rate induced by polyhydroxyl compound HCQ was greater, indicating that the mechanism of cancer

cell death induced by DMF was similar to that of HCQ. That is to say, both of them may be closely related to the lysosome mechanism. After the use of Baf A1, the apoptosis rate of SGC-7901 cells induced by 5-Fu was increased, indicating that the mechanism of cancer cell death induced by 5-Fu was quite different from that of DMF.

Detection of cell cycle arrest The cells were collected and the distribution of cell cycle was detected by flow cytometry. The experimental results showed that the distribution ratio of G0/G1 phase in SGC-7901 cells treated with HCQ, DMF and 5-Fu was $66.32 \pm 1.41 \%$, $72.77 \pm 0.53 \%$ and $66.75 \pm 2.13 \%$ respectively (Fig. 3b and the statistical chart is shown by 3d). It was found that each drug could increase the distribution proportion of cells in G0/G1 phase, indicating that the growth of these cells was blocked in G0/G1 phase. The pan caspase inhibitor N-benzyloxycarbonyl-ValAla-Asp-fluoromethylketone (z-VAD-fmk) was added to the drug intervention group of SGC-7901 1 h in advance, and no significant difference in the cycle change of SGC-7901 cells was observed as compared with the non-inhibitor group. The results showed that HCQ, DMF and 5-Fu could block the growth of tumor cells in G0/G1 phase, but in terms of the mechanism of cell death, the pharmacological mechanism of 5-Fu was highly correlated with caspase signal pathway, while the pharmacological mechanism of HCQ and DMF was not very sensitive to Caspase signal pathway. Caspase signal pathway is closely related to apoptosis. The phenomenon observed by electron microscopy further confirms our conjecture that the signal pathway of DMF induced cancer cell death may not be exactly the same as the original drug. In the experiment of methylglyoxal combined with 5-fluorouracil to improve the chemosensitivity of breast cancer, Sonali Ghosh¹⁷⁾ observed nuclear condensation and breakage by laser confocal, which is also the

morphological change of early apoptosis.

Release of Cathepsin B and D accelerates cell death In addition to caspases, the growing list of proteases that act as positive mediators of apoptosis can be extended to include the lysosomal aspartic cathepsin D and a few lysosomal cysteine proteases, for instance cathepsins B and L¹⁸⁾. It is also reported that an increase in the cytosolic concentration of cathepsin D concentration may have a specific effect on apoptosis.¹⁸⁾ Cathepsin B is an important downstream medium of a cell apoptotic cascading response initiated by tumor necrostatic factor (TNF) triggering and caspase¹⁹⁾. It can be concluded that cathepsin plays an important role in the process of cell death. The release of enzymes in the lysosomes are associated with lysosomes membrane permeability.

As shown in Fig. 4a (CB) and 4b (CD). We detected the protein content of CB and CD in the whole protein of the cell. The above experimental results showed that the CB protein level of SGC-7901 cells increased after the intervention of 5-Fu and DMF (compared with the blank control group). The short-term effect (2 h experimental group) caused a significant increase, meanwhile, the time-dependent growth trend was obvious between 2 and 24 hours. However, in the inhibitor group, DMF had a significant response, while 5-Fu had no effect. The CB protein level of HCQ intervention decreased (compared with the blank control group), but the effect of 24 hour intervention was inhibited by inhibitors. HCQ and DMF drug group were increased the level of CD protein in cells and the effect of intervention was inhibited (compared with the control group). DMF had a more significant inhibitory response, while 5-Fu intervention group decreased (compared with the control group), it was indicated that the inhibition was ineffective.

From the comparison of different drugs and preparations, cells were most

sensitive to DMF, as represented by the increase level of CB and CD within 2 h. The effect of DMF intervention was relatively more sensitive to the inhibitors and the trend of protein level increase was generally inhibited. The protein level of CB and CD in SGC-7901 cells had a certain time-dependent response to HCQ, reaching the peak at 24 h, which could be reversed by the inhibitor. Noticeably, a certain time-dependent response to the intervention of 5-Fu was observed. Through the above comparison, at least two conclusions could be drawn. On the one hand, the polyhydroxyl structure of the carrier and the original pharmacological properties indicated that the mechanism of cancer cell death of DMF had phenotypes similar to HCQ and characteristics related to 5-Fu. On the other hand, the characteristic of cancer cell death caused by DMF through LMP pathway was more prominent than that of HCQ, which was characterized by the sensitivity of histone CB and CD levels and their response to vacuole H^+ ATPase inhibitor Baf A1. Through the detection of CB and CD, it is proved that they can promote the occurrence and development of LMP.

LysoTracker Red LysoTracker Red probe can be enriched in acidic organelles and protonated, resulting in a weak base of the dye molecule to reduce the fluorescence quenching phenomenon, so that the fluorescence intensity produced by the dye is enhanced. As shown in Fig. 4c, SGC-7901 cells were stained with the LTR and selectively accumulated in the cellular acidic compartments (mainly lysosomes)⁵⁾. The phenotype of the lysosomal membrane leakage mechanism of DMF preparation was similar to that of HCQ. The LTR staining results showed that DMF could cause stronger and more localized lysosomal membrane leakage than HCQ; Baf A1 could significantly improve the lysosomal membrane leakage induced by HCQ, but could not significantly reduce the lysosomal staining degree of DMF at the nuclear location.

This may be related to the nuclear targeted transport characteristics of DMF nanoparticles, and the lysosomal membrane will be protected by Baf A1. However, after the DMF enters the nucleus, the effect of inhibitors may be disappeared, which reflects that the DMF is different from positive control drug HCQ.

CD translocation induced by DMF In 1998, Ann-Charlotte Johansson and colleagues found that aspartate protease cathepsin D could redistribute from lysosome to cytoplasm during apoptosis induced by oxidative stress²⁰⁾. Lysosomal membrane proteins has play an indispensable role in maintaining normal cell physiological activities and lysosomal function, therefore, lysosomal membrane proteins may also become targets for cancer therapy. At present, it has been identified that more than 25 kinds of lysosomal membrane proteins, among which the most abundant lysosomal membrane proteins are lysosome associated membrane protein 1 (Lamp-1) and lysosomal associated membrane protein 2 (Lamp-2), which account for 50% of the total lysosomal membrane proteins²¹⁾. These proteins play an important role in lysosome function, lysosomal acidification, metabolite transport and molecular chaperone-mediated autophagy (CMA)²¹⁾.

SGC-7901 cells were stained for immunofluorescence detection of the lysosome-associated membrane proteins 1 (Lamp-1) and CD. It was found that CD colocalized with Lamp-1 in control cells (Fig. 5) as shown by the blend of the red and green fluorescence (yellow). HCQ induced the translocation of CD to the cytosol as well as to the nucleus. Pretreatment with Baf A1 greatly reduced the frequency of cells manifesting the lysosomal release of CD. HCQ triggered the release of CD from lysosomes to the cytosol and the nucleus. This translocation of CD was inhibited by Baf A1. DMF showed the same fluorescence as HCQ. However, there were two obvious differences between DMF experimental group and HCQ experimental group.

Without the inhibitor, the coincidence degree of the lysosomal membrane and CD staining site in HCQ experimental group was high, and it was mainly distributed in the cytoplasm. The activity of lysosome in DMF experimental group was much higher than that in HCQ experimental group, and the high intensity staining of Lamp was significantly closer to the nucleus, while the staining site of CD (or the coincidence of Lamp and CD) mainly appeared in the cytoplasm. The proton pump inhibitor could significantly reduce the staining intensity of Lamp, but had little effect on the staining intensity of Lamp-CD codomain. The location of Lamp-CD could be limited to the cytoplasm, and the coincidence between the lysosomal membrane and cathepsin D staining site was low in 5-Fu experimental group. Proton pump inhibitors had little effect on Lamp staining intensity and Lamp-CD co-localization staining intensity. Altogether, these data suggest that HCQ and DMF caused selective, Baf A1 can inhibit the phenomenon of LMP, and DMF intervention could induce a more intense and lasting lysosomal organoplasmic reaction, which is the tendency of targeted distribution of the nucleus.

ROS production promotes LMP Exposure of the lysosomal membrane to a large amount of ROS may trigger lipid peroxidation in the inner membrane of cells, leading to the release of lysosome contents, which further leads to penetration of the lysosomal membrane. Lysosomal membrane leakage can cause the release of lysosomal proteases (Cathepsin B and D), erode the mitochondrial membrane and cause mitochondrial membrane leakage. Mitochondrial damage may lead to the release of ROS, and the increase of ROS level will trigger or amplify the signal of cell death ²²⁾.

As shown in Fig. 6a, the ROS level of SGC-7901 cells increased with the increase of the 5-Fu intervention time, and there were significant differences between

the different time groups. 5-Fu treatment can cause time and dose dependent increase of ROS. According to the detection results of LMP related indexes, ROS accumulation may be mainly related to mitochondrial dysfunction, which is the embodiment of mitochondrial apoptosis signal pathway. HCQ intervention within a certain period of time (within 12 h) could cause ROS accumulation, which was relatively low in DMF intervention group, but ROS was decreased significantly in 24 h as compared with the blank group and other experimental groups. The results showed that DMF intervention usually did not cause a large amount of accumulation of mitochondrial ROS. The level of ROS in the group treated with HCQ for 24 h was similar to 12 h, but DMF showed a more obvious and special consumption trend. We speculated that the depletion of ROS induced by DMF may be related to the dissolution of dextran (DET) and the increased exposure of the polyhydroxyl carrier in the surface layer of DMF preparation accompanied by lysosome acid etching, resulting in the oxidation of the carrier layer of the preparation. Therefore, the later cell death may be more related to the direct reaction of DMF particles with the organelle or cytoplasm and nucleus. In addition to the potential reasons for the possible oxidation consumption of the MLDH carriers resulting in a constant (or very low) level of ROS, this experimental result also reflected the particularity of the LMP mechanism of DMF different from HCQ.

Bax plays a promoting role in LMP Recent studies have demonstrated that Bax is a member of the pro-apoptotic Bcl-2 family, having a direct effect on lysosomes²³⁾. Therefore, Bax can be transported from the cytoplasm to the lysosomal membrane and induce LMP. It has been reported that down-regulation of Bax expression mediated by Si RNA can protect cells from death induced by LMP and hydrogen peroxide²⁴⁾. In SGC-7901 cells, the expression of Bax protein in 5-Fu experimental

group showed an obvious time-dependent increasing trend (Fig. 6b), and the rising trend in 24 h experimental group could be restrained by the Baf A1, which was consistent with the law reported in the literature ²⁴⁾. After the intervention of the positive drug HCQ, the expression of Bax protein in the cell lines fluctuated slightly, but the inhibitory effect of Baf A1 was clear. That is to say the accumulation of pro-apoptotic proteins could be reduced by inhibiting LMP. However, DMF showed particularity different from both 5-Fu and HCQ. After 2 hours of intervention with DMF, the expression of Bax protein increased markedly, but the inhibitor Baf A1 could not down-regulation Bax expression in 24 h experimental group. These findings suggest that the lethal effect of DMF on cancer cells might similar to that of HCQ, indicating that it had an obvious phenotype of LMP mechanism, and the increased expression of pro-apoptotic proteins was higher and the effective time was shorter. At the same time, the production of a large number of pro-apoptotic protein Bax was consistent with the process of oncosis induced by DMF, and so the effect of Bax protein may also be one of the factors promoting oncosis. In other words, the permeation of mitochondrial membrane can accelerate the oncosis process induced by DMF. This may be that the pharmacodynamic characteristics of DMF are related to the great difference between HCQ and 5-Fu, DMF has well cell transport ability and nuclear targeting, at the same time, the speed of transport and efficacy is fast.

Discussion

As one of the important organelles of cells, lysosomes not only play an important role in regulating the balance of the intracellular environment but participate in the mode of cell death. Although not all cell death models induce cell death by involving lysosomal pathways, the role of lysosomes could not be ignored. The above

experimental results showed that (1) DMF had a better anti-tumor effect than 5-Fu in the process of cell death, while DMF mainly induced cell death by increasing LMP. (2) The increase of lysosome activity caused by DMF can make lysosome more sensitive to LMP. (3) Lysosomes are highly sensitive to ROS which it may promote the occurrence and development of LMP. (4) The release of lysosomal proteases (Cathepsin B and D) may damage the mitochondria, then lead to mitochondrial damage, ROS accumulation and release of cytochrome C, resulting in cell oncosis and death.

Some studies demonstrated that the increase in cathepsin D activity and expansion of the lysosome volume could lead to prostate cell death, which is consistent with our finding that the increase in lysosome death and the increase in intracellular lysosomal enzyme release accelerated cell death, which may further lead to caspase-dependent or independent cell death with or without mitochondrial involvement^{25, 26}. In the Lamp-CD co-localization staining experiment (Fig. 4), HCQ positive control drugs showed high coincidence of staining sites and synchronization of events. The experimental results of DMF inhibitor group showed that proton pump activation had a clear upstream and downstream relationship with LMP, but the release of cathepsin (CD) was not necessarily synchronized with LMP. Proton pump inhibitors could inhibit the LMP phenomenon induced by DMF, but could not block the nuclear targeted delivery trend of DMF-LMP transport vesicles. It can also be inferred that DMF and the positive control drug HCQ have similar upstream signal transduction pathways in the pharmacological mechanism, but there may be specific differences in the downstream pathways that induce oncosis. In other words, the occurrence of cell death induced by HCQ may mainly follow the signal pathway of LMP to cathepsin protease release, which activates Bax, and then MMP continue to

activate Caspase-3^{13, 27)}.

We found that lysosomal membrane leakage led to the release of lysosomal proteases (Cathepsin B and D) when intracellular ROS was accumulated, which eroded the mitochondrial membrane and led to mitochondrial membrane leakage. Mitochondrial damage may lead to the release of ROS, and the increase of ROS level would trigger or amplify the signal of cell death. The timeliness of ROS induced by 5-Fu intervention with cells showed an increasing trend, while the increase of ROS in the 24 h DMF and HCQ intervention group was slower than that in the 12 h experimental group, and even showed obvious reactive oxygen species depletion in DMF. After 24 h intervention with DMF, the contribution of ROS accumulation to cell death decreased, while the depletion of ROS induced by DMF may be related to the dissolution of DET and the increased exposure of polyhydroxyl carriers in the surface layer of DMF preparations accompanied by lysosome acid etching, resulting in the oxidation of the carrier layer of the preparation. Therefore, we speculate that the death mechanism of the polyhydroxyl drugs or agents in the treatment of cancer cells is different from that of 5-Fu. Specifically, DMF can induce LMP that is also controlled by proton pump inhibitors, but the upstream stimulation signal of LMP may not necessarily be the accumulation of ROS in the cytoplasm, this may be related to the direct action of DMF particles with lysosome.

As the downstream of its signal pathway, the LMP phenomenon induced by HCQ may cause the increase of mitochondrial membrane permeability and the production of more ROS, which in turn further strengthens the degree of LMP and activates the cell death pathway downwards (such as activating Caspase-3). DMF induces LMP and ROS increased at first and then decreased, while the explosive death of cancer cells may be more related to the direct action of DMF particles with

different organelles. In particular, it is related to the damage of cell membrane and nuclear membrane and the release of small molecular substances and energy in the nucleus. At the same time, the family of Bcl-2 gene such as Bax and Bak can promote apoptosis, induce the formation of MMP and promote cell death after activation and translocation to mitochondria ²⁸⁾. The process of oncosis induced by DMF is accompanied by the descending of LMP signal pathway, but here may also be other more complex pharmacological or biochemical reactions related to the direct action of DMF nanoparticles-organelles (such as mitochondria and nucleoli). Further studies, which take these variables into account, will need to be undertaken.

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Conflict of Interest

The authors declare no conflict of interest.

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Table 1 MTT test results of SGC-7901 cells interfered with DMF and 5-Fu

Dosage μg/ml	DMF		5-Fu	
	Survival rate (%)	SD	Survival rate (%)	SD
62.5	87.75	0.89	78.74	7.18
125	70.05	0.90	75.07	3.44
250	52.87	1.04	73.23	1.52
500	46.57	0.58	66.28	5.02
1000	36.32	2.31	63.78	2.58
2000	25.29	3.48	53.36	5.32

Fig. 1 Research background. 5-Fu was inserted into MLDH to form MLDH-FU, and then the surface of MLDH-FU was compounded with dextran to form DMF, which has the function of magnetic targeting-sustained release. The mode of cell death caused by DMF nanoparticles is different from that of 5-Fu, showing the typical characteristics of oncosis and death. (Color figure can be accessed in the online version.)

Fig. 2 Morphological observation. (a) Cell morphological observation by Giemsa staining. (b) Transmission electron microscopy. (C) The number of lysosomes in the electron microscope picture. (Color figure can be accessed in the online version.)

Fig. 3 Detection of apoptosis and cell cycle arrest. (a) Detection of apoptosis.

Addition of Baf A1 before drug administration 1 h, SGC-7901 cells were incubated with 30 μ g/mL HCQ, 1mg/mL DMF (contains 35% of 5-Fu) and 350 μ g/mL 5-Fu for 24 h. The percentage of apoptotic cells was measured by cytofluorometry. (b) Detection of cell cycle arrest. Doses are as in Fig (a), the cell arrest period was detected by PI staining and cytofluorometry. (c) Statistical graphs of apoptosis (n=3), Data were statistically analyzed by one-way analysis of variance. (d) Statistical graphs of cell cycle arrest (n=3), the statistical method is the same as before. (Color figure can be accessed in the online version.)

Fig. 4 Lysosomal dysregulation induce by different drugs. (a) The CB protein level of

lysosome enzymes at different time points by western blotting. (b) The CD protein level of lysosome enzymes at different time points by western blotting. The results are from three independent experiments (n=3). Data were statistically analyzed by one-way analysis of variance. [#]* $P < 0.05$ and ** $P < 0.01$. (c) After drug modification, SGC-7901 cells were stained with acid probe staining solution LTR, observed and photographed. (Color figure can be accessed in the online version.)

Fig. 5 CD translocation induced by different drugs. SGC-7901 cells were stained for immunofluorescence detection of the Lamp-1 (red fluorescence) and CD (green fluorescence). Pretreatment with Baf A1 reduced the frequency of cells manifesting the lysosomal release of CD. (Color figure can be accessed in the online version.)

Fig. 6 ROS production promotes LMP. (a) ROS was detected by fluorescence probe DCFH-DA. (n=3). Data were statistically analyzed by one-way analysis of variance. (b) Detection of Bax protein expression by western blotting. The results are representative of at least three independent experiments results. the statistical method is the same as before. * $P < 0.05$ and ** $P < 0.01$. (Color figure can be accessed in the online version.)