Korean J Physiol Pharmacol 2024;28(3):219-227
https://doi.org/10.4196/kjpp.2024.28.3.219

KJPP

Original Article

Novel artesunate-metformin conjugate inhibits bladder cancer cell growth associated with Clusterin/SREBP1/FASN signaling pathway

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ARTICLE INFO

Received April 28, 2023
Revised November 22, 2023
Accepted November 24, 2023

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Key Words
Artesunate
Lipogenesis
Metformin
Urinary bladder neoplasms

ABSTRACT Bladder cancer remains the 10th most common cancer worldwide. In recent years, metformin has been found to have potential anti-bladder cancer activity while high concentration of IC_{50} at millimolar level is needed, which could not be reached by regular oral administration route. Thus, higher efficient agent is urgently demanded for clinically treating bladder cancer. Here, by conjugating artesunate to metformin, a novel artesunate-metformin dimer triazine derivative AM2 was designed and synthesized. The inhibitory effect of AM2 on bladder cancer cell line T24 and the mechanism underlying was determined. Anti-tumor activity of AM2 was assessed by MTT, cloning formation and wound healing assays. Decreasing effect of AM2 on lipogenesis was determined by oil red O staining. The protein expressions of Clusterin, SREBP1 and FASN in T24 cells were evaluated by Western blotting. The results show that AM2 significantly inhibited cell proliferation and migration at micromolar level, much higher than parental metformin. AM2 reduced lipogenesis and down-regulated the expressions of Clusterin, SREBP1 and FASN. These results suggest that AM2 inhibits the growth of bladder cancer cells T24 by inhibiting cellular lipogenesis associated with the Clusterin/SREBP1/FASN signaling pathway.

INTRODUCTION

Bladder cancer remains the 10th most common cancer worldwide. According to the GLOBOCAN database, there were 573,278 new cases of bladder cancer and 212,536 deaths worldwide in 2020 [1]. This disease can present as non-muscle-invasive bladder cancer and muscle-invasive bladder cancer (MIBC) [2]. Studies have shown that cisplatin-based combination neoadjuvant chemotherapy can improve survival outcomes in MIBC patients [3]. However, cisplatin is limited due to its predisposition to nephrotoxicity [4]. In recent years, the Food and Drug Administration has approved several targeted drugs for the treatment of bladder cancer, such as atezolizumab, pembrolizumab, and avelumab [5-7]. However, these drugs are often associated with a plethora of adverse events during treatment, many manifesting in the skin [8]. Therefore, there is an urgent need to develop a new anti-bladder cancer chemotherapy drug with high efficiency and low toxicity.

Metformin is a biguanide compound, which has been used clinically for the treatment of diabetes in 1950 [9]. In recent years, metformin has been found to have a certain role in anti-cancer, and its various anti-cancer mechanisms have also been proposed [10]. However, IC_{50} in bladder cancer cells is at millimolar level, too high to be reached orally, and is prone to cause gastrointestinal adverse reactions at high concentration [11,12]. Therefore, in order to enhance the anti-bladder cancer activity of metformin, novel metformin derivatives with higher anti-bladder cancer ef-

Author contributions: P.L., X.Y., L.W., and X.Z. designed research and conducted experiments. L.M., C.X., and X.Y. analyzed data and reviewed the manuscript. P.L. wrote the manuscript. All authors read and approved the manuscript.

ficiency and lower toxicity should be developed. Artesunate is a water solubility derivative of artemisinin and the most effective drug for the treatment of severe malaria [13]. In recent years, its anti-tumor effects were explored as well [14,15]. Thus, introducing the artesunate group to metformin moiety may improve its anti-bladder cancer activity. Along with this thought, we [15] designed and obtained a new artesunate dimer triazine derivative AM2 by esterification of artesunate and metformin.

Lipids are a critical component of cell membranes with important energy fuel functions in cells [16]. Studies have shown that lipogenesis was up-regulated in human cancer cells [17]. Besides, it has been shown that increased lipid synthesis promotes tumor proliferation and migration [18,19]. Regulating lipogenesis has become a new strategy for the treatment of cancer. Furthermore, increased level of fatty acid synthase (FASN) has emerged as a typical phenotype of most human carcinomas [20]. SREBPs, transcription factors of the helix–loop–helix leucine zipper family, are strongly involved in the control of lipogenesis. Its SREBP1 subtype mainly regulates the synthesis of FASN, phospholipids and triacylglycerols [21]. Clusterin is a chaperone protein that is widely involved in the initiation, progression, metastasis, and treatment resistance of many types of cancer [22]. In the present study, the detailed synthesis procedure and anti-bladder cancer activity evaluation of novel artesunate-metformin conjugate AM2 were presented.

METHODS

Chemicals

Artesunate was purchased from Energy Chemical (Anhui Zesheng Technology Co., Ltd). Metformin hydrochloride and 4-Dimethylaminopyridine (DMAP) were from Aladdin Biochemical Technology (Shanghai Aladdin Biochemical Technology Co., Ltd). 1-Ethyl-(3-dimethylaminopropyl) carbonyldiimide hydrochloride (EDCI) was bought from Macklin Biochemical (Shanghai Macklin Biochemical Technology Co., Ltd). All other chemicals were from Chron Chemicals (Chengdu Kelong Chemical Co., Ltd). Dulbecco's modified Eagle's medium (DMEM), Eagle's Minimum Essential Medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin solution, 0.25% trypsin and phosphate buffer were bought from Hyclone company (Hyclone). McCoy's 5A (Procell Life Science and Technology Co., Ltd) was obtained from Procell company. Clusterin and SREBP1 protein anti-bodies were from Santa Cruz Biotechnology. FASN protein anti-body was purchased from CTS.

Synthesis and purification

In this study, novel artesunate-metformin conjugate AM2 was synthesized by two steps. In brief, metformin hydrochloride reacted with sodium hydroxide in dichloromethane to obtain free metformin. Free metformin reacted with artesunate in dichloromethane under the catalysis of DMAP and EDCI. Reactions were monitored by thin-layer chromatography (Yantai Jiangyou Silica gel Development Co., LTD) using glass sheets coated with silica gel HSGF254, and the spots were visualized using UV lamp and vanillin sulfuric acid solution color development. After the reaction at room temperature for 12 h, the crude product is separated by a silica gel column to obtain the pure product.

Structural identification

The structure of the target product was identified by ^{1}H NMR, ^{13}C NMR and HR-MS, and the structure of AM2 was confirmed to be artesunate dimer triazine compound.

Cell lines and culture conditions

Human bladder cancer cells T24 and UMUC3 originated from Dr. Guo Peng (Xi'an Jiaotong University, China) and T24 cells were cultured in 5A supplemented with 10% FBS and 1% penicillin-streptomycin, UMUC3 cells were cultured in MEM supplemented with 10% FBS and 1% penicillin-streptomycin. RT4 cells were obtained from ATCC (HTB-2) and cultured in 5A supplemented with 10% FBS and 1% penicillin-streptomycin. Human umbilical vein endothelial cells (HUVEC) cells obtained from Prof. F. Y. Chen (Shanghai Jiaotong University, China) and were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were cultured in a 5% CO_2 wet incubator at 37°C. The cells were sub-cultured every 2 days.

MTT assay

In brief, RT4 cells, UMUC3 cells, T24 cells and HUVEC cells in log phase were prepared by seeding them in a 96-well plate at 7,000 cells/well and cultured 12 h. Then treated cells with different concentrations (0, 0.25, 0.5, 1 and 2 μM) of AM2, cisplatin and metformin for 72 h. The medium was removed after 4 h incubation with MTT (2 mg/ml, 50 μl) at 37°C and 150 μl dimethyl sulfoxide was added. Then, optical density (OD) values were measured at 490 nm with a microplate reader.

Clonogenic assay

In brief, T24 cells in log phase were seeded in a 24-well plate at 2,000 cells/well. After 12 h culture, T24 cells were treated with different concentrations (0, 0.125, 0.25 and 0.5 μM) of AM2 for about 7 days. Then we removed the medium and fixed T24 cells with 10% formaldehyde for 2 h. After that, 0.1% crystal violet was added to stain cells for 12 h. Finally, OD values were measured at 550 nm with a microplate reader.

Wound healing assay

Briefly, T24 cells in log phase were plated in a 12-well plate at 4×10^5 cells/well. When the cell density reached 90%, we used a 200 μl pipette tip to create a wound on the monolayer cell. The floating cells were rinsed by phosphate buffered saline (PBS) for three times. Then we added serum-free medium which contains different concentrations (0, 0.25, 0.5 and 1 μM) of AM2. Finally, the scratch width of 0, 12, 24 and 48 h were recorded and imaged by fluorescence microscope.

Oil red O staining

T24 cells in log phase were seeded in 12-well plate at 1×10^5 cells/well. After 12 h culture, we treated T24 cells with different concentrations (0, 0.25, 0.5 and 1 μM) of AM2 for 48 h. Then the medium was removed, T24 cells were fixed in 10% formalin for 1 h, washed with PBS, and then stained with 0.5% oil red O for 35 min at 37°C in the dark. Washed with 60% isopropanol to remove excess dye, and then rinsed three times with distilled water. Added hematoxylin staining solution to counterstain nuclei for 1–2 min, rinsed three times with distilled water again. Finally, the red lipid droplets were visualized by microscopy.

Western blotting analysis

Briefly, T24 cells in log phase were seeded in a 6-well plate at 4×10^5 cells/well and incubated for 12 h. Then T24 cells were treated with different concentrations (0, 0.25, 0.5 and 1 μM) of AM2. After 48 h, rinsed with cold PBS for twice and added lysate on the ice for 30 min. Then samples were boiled in boiling water for 10 min and stored at –20°C after cooling to room temperature. Western blotting experiment was carried out with regular procedure. In brief, the total proteins were separated by SDS-PAGE and then transferred onto a PVDF membrane. The PVDF membrane was blocked in 5% milk for 1 h and then incubated in first antibody at 4°C for 15–18 h. Washed the PVDF membrane three times with phosphate buffered saline with Tween 20 (PBST) every 10 min and then incubated in secondary antibody at room temperature. After 1 h, rinsed with PBST for three times. Finally,

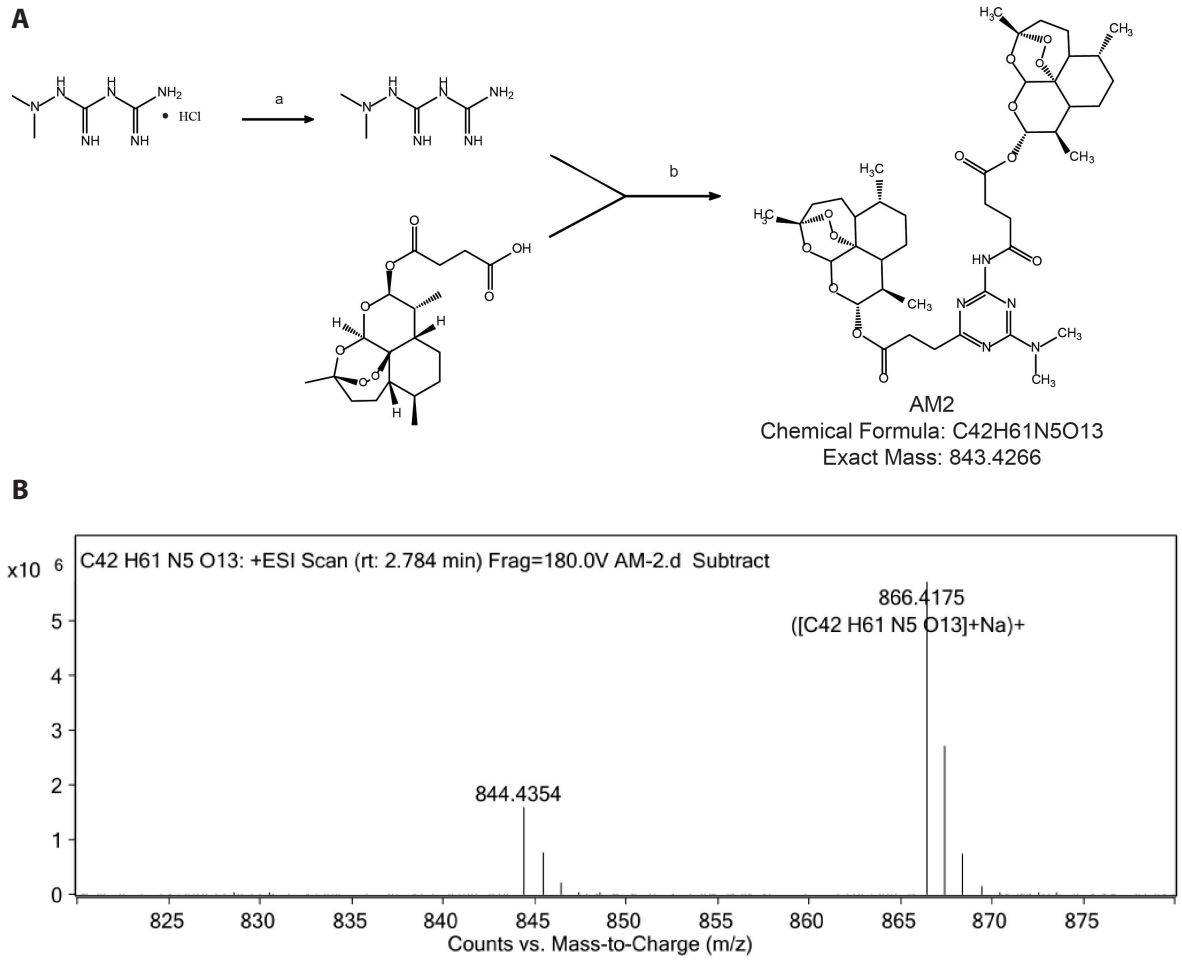

Fig. 1. The synthetic route and mass spectrometry results of AM2. (A) Free metformin obtained from metformin hydrochloride is esterified with artesunate. Reagents and conditions: (a) DCM, NaOH, rt, 2 h; (b) DCM, EDCI, DMAP, rt, 12 h; (B) Mass detection result of AM2.

bands on the membrane were revealed by developing solution and imaged on Chemi Doc (Bio-Rad).

RESULTS

Chemistry

The AM2 synthesis route was shown in Fig. 1A.

Preparation of free metformin: 1 g metformin hydrochloride was weighed and dissolved in 20 ml methylene chloride. After the solution was completely dissolved, added 10 ml 25% sodium hydroxide solution, stirred and reacted at room temperature for 2 h. After the reaction, filtered and dry the filtrate on the rotary evaporator to obtain free metformin.

Synthesis and separation of AM2: Artesunate (769 mg, 2 mmol) was dissolved in 20 ml dichloromethane, and free metformin (258 mg, 2 mmol), EDCI (575 mg, 3 mmol) and DMAP (366 mg, 3 mmol) were added after the solution was completely dissolved. The reaction was stirred at room temperature for 12 h and monitored by thin layer chromatography. After the reaction, water was washed once and saturated sodium chloride solution was washed 3 times. The lower layer was taken and anhydrous magnesium sulfate was added for drying and filtration. The filtrate was dry on the rotary evaporator to obtain crude products.

AM2 (50.15% yield). Orange powder, ^{1}H NMR (600 MHz, Chloroform-d) δ 7.76 (s, 1H), 5.79 (d, J = 9.9 Hz, 1H), 5.78 (d, J = 9.8 Hz, 1H), 5.42 (d, J = 4.8 Hz, 2H), 3.41–3.22 (m, 2H), 3.18 (s, 3H), 3.13 (s, 3H), 3.02–2.92 (m, 2H), 2.88–2.81 (m, 3H), 2.76 (dt, J = 17.5, 6.7 Hz, 1H), 2.59–2.51 (m, 2H), 2.37 (td, J = 14.0, 13.5, 4.0 Hz, 2H), 2.05–1.99 (m, 3H), 1.88 (dtd, J = 13.1, 6.8, 6.3, 3.2 Hz, 2H), 1.79–1.69 (m, 6H), 1.63–1.58 (m, 3H), 1.49–1.45 (m, 2H), 1.42 (s, 3H), 1.41 (s, 3H), 1.37 (dt, J = 13.6, 2.9 Hz, 2H), 1.28 (d, J = 2.0 Hz, 2H), 0.96 (s, 3H), 0.95 (s, 3H), 0.86 (d, J = 7.1 Hz, 3H), 0.80 (d, J = 7.1 Hz, 3H) (Supplementary Fig. 1). ^{13}C NMR (150 MHz, Chloroform-d) δ 177.21, 173.11, 171.95, 171.71, 165.04, 162.47, 104.58(×2), 92.20, 92.06, 91.64, 91.61, 80.28(×2), 51.71(×2), 45.39, 45.38, 37.41, 37.40, 36.63, 36.60, 36.36(×2), 34.23(×2), 32.82, 32.74, 31.96, 31.91, 30.73, 29.09, 26.09(×2), 24.72(×2), 22.13(×2), 20.35(×2), 12.19(×2) (Supplementary Fig. 2). HRMS (ESI) (m/z) $[M+1]^+$ calcd for $C_{42}H_{62}N_5O_{13}^+$, 844.4339; found, 844.4354. $[M+Na]^+$ calcd for $C_{42}H_{61}N_5NaO_{13}$, 866.4158; found, 866.4175 (Fig. 1B).

AM2 inhibited the proliferation of T24 cells

The ability to inhibit cell proliferation of AM2 in different bladder cancer cells (RT4, UMUC3 and T24) was assessed by MTT assay. As shown in Fig. 2A and B, compared with the control group, AM2 showed obvious inhibition of cell proliferation in different bladder cancer cells in a dose-dependent manner. In

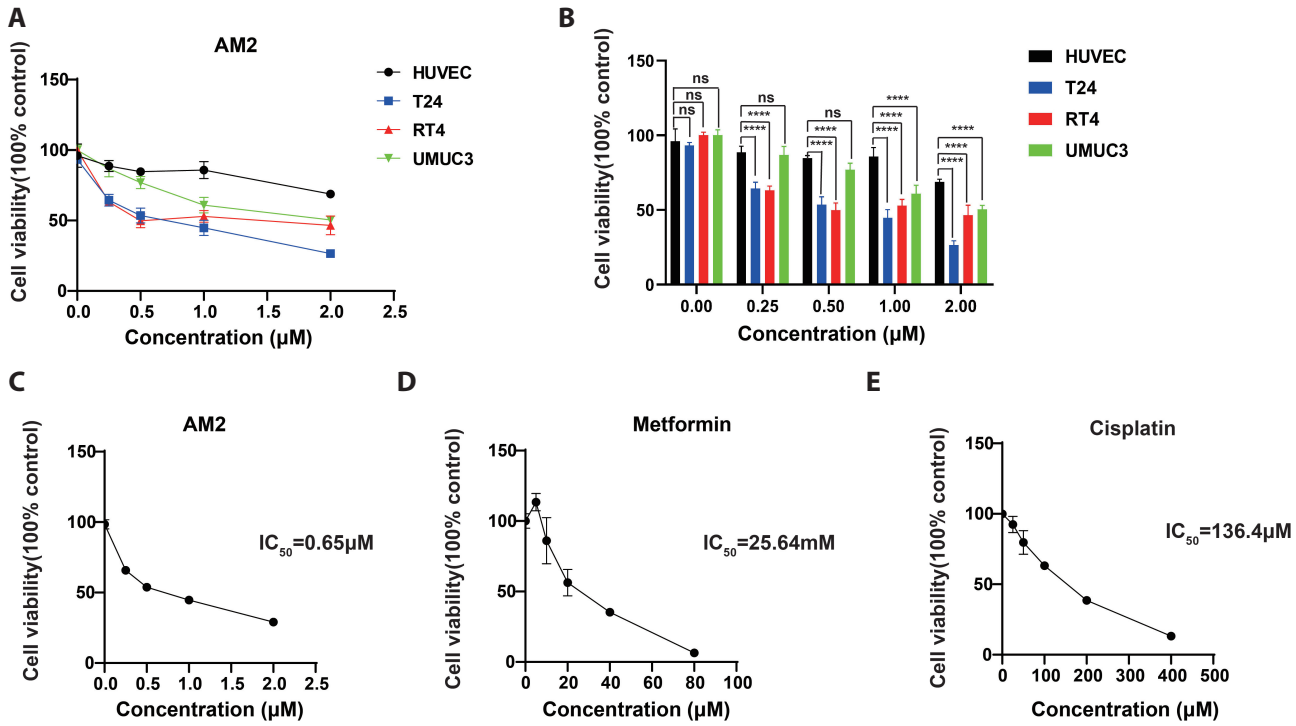

Fig. 2. The anti-proliferation activities of AM2 on a variety of bladder cancer cells. (A) RT4, UMUC3, T24, and HUVEC cells were treated with 0, 0.25, 0.5, 1, and 2 μM of AM2 for 72 h, then observed the cell viability. (B) Comparison of the inhibitory effects of AM2 on cell proliferation in various of bladder cancer cells and HUVEC cells. (C–E) T24 cells were treated with different concentration of AM2, metformin and cisplatin for 72 h, then observed the cell viability. Data are presented as the mean ± SD of three independent experiments. ns, no significance. ****$p < 0.0001$.

addition, by comparing the proliferation inhibition effect of AM2 in T24 and HUVEC with the same concentration, it can be seen that AM2 has low toxicity to normal cells. The calculated half inhibitory concentration (IC_{50}) showed that AM2 had the best activity in T24 cells (Table 1). Besides, AM2 displayed more than 200 times higher inhibitory effect than cisplatin in the T24 cancer cell line and more than 39,000 times than metformin (Fig. 2C–E).

AM2 inhibited colony formation of T24 cells

The ability of AM2 in inhibiting the clonogenicity of T24 cells was evaluated. At 1 μM concentration, AM2 inhibits T24 clone formation by up to 80%, at 0.5 μM concentration, AM2 inhibited T24 clone formation by up to 70%, even at lower concentrations exceeding 60%. Overall AM2 exerted potent inhibition on colony formation of T24 cells and the effect was in a dose-dependent manner (Fig. 3A, B).

AM2 inhibited migration of T24 cells

The inhibitory ability of AM2 on T24 cell migration was observed. As shown in Fig. 3C and D, at 24 h, the control cells migrated at 107 μm, while the 0.5 μM and 1 μM AM2-treated cells migrated at 75 μm and 57 μm. At 48 h, the migration distance of control cells reached 140 μm, while the migration distances of 0.5 μM and 1 μM AM2-treated cells were 84 μM and 77 μM, respectively. There is no significant change in the migration distance of T24 cells under 0.125 μM AM2 treatment for 48 h. It can be determined that AM2 significantly inhibits the migration of T24 cells in a dose-dependent manner at 24 and 48 h.

AM2 inhibited lipogenesis in T24 cells

Previous studies in our laboratory showed that metformin inhibited the growth of bladder cancer cells by inhibiting fatty acid synthesis [23]. In order to verify whether AM2 inhibits lipogenesis in T24 cells, oil red O staining was used. As shown in Fig. 4A, with the effect of different concentrations of AM2, the content of lipid droplets in T24 cells was significantly reduced in a dose-dependent manner.

Table 1. Inhibitory concentration 50% (IC_{50}) for AM2

AM2 (μM)	Bladder cancer cell lines		
	RT4	UMUC3	T24
	1.26 ± 0.13	1.95 ± 0.19	0.65 ± 0.05

IC_{50} (mean ± standard deviation).

Expressions of lipogenesis-related proteins in T24 cells treated with AM2

In order to gain insights into the mechanism by which AM2 inhibits T24 cells proliferation, the expressions of lipogenesis-related proteins were determined by Western blotting. As shown in Fig. 4, AM2 treatment could significantly reduce the expressions of SREBP1 precursor (pSREBP1), mature forms of SREBP1 (nSREBP1) and FASN in a dose-dependent manner, consistent with the result of oil red O staining, indicating that AM2 affects cell growth by inhibiting lipogenesis. At the same time, Clusterin, as the target of metformin in bladder cancer cells [23], also showed significant down-regulation under the effect of different concentrations of AM2 in a dose-dependent manner. In addition, we measured the basic expression level of Clusterin in T24 cells and HUVEC cells, and the results showed that the expression level of Clusterin in T24 was much higher than that in HUVEC (Supplementary Fig. 3). Based on the MTT results, the inhibitory effect of AM2 on T24 cells is much higher than that of HUVEC cells, suggesting that Clusterin may be a potential target of AM2. These results imply that AM2 inhibits T24 cells associated with Clusterin/SREBP1/FASN signaling pathway.

DISCUSSION

Artesunate and metformin are both drugs that have been used clinically for many years. Previous studies have shown that artesunate in combination with metformin has a synergistic effect on high-fat high-sugar diet/superior salivatory nucleus-induced dry mouth in diabetic rats [24]. It has been discovered that both the metformin and artesunate have antitumor activity in recent years. Latest research has shown that artesunate in combination with metformin can be used to treat glioblastoma multiforme, which requires 75 μM of artesunate and 20 mM of metformin [25]. However, metformin has significant cytotoxicity at high doses (20 mM to 50 mM), which means that the combination regimen is still not suitable for clinical treatment [26].

In this study, we linked artesunate, a derivative of artemisinin, to metformin to synthesize novel compound AM2. Through MTT experiments, we found that AM2 has an excellent inhibitory effect on the growth of a variety of bladder cancer cells, and IC_{50} reaches the micromoles level, among which the activity is the best in T24 cells. In contrast, in HUVEC cells, the effect of the same concentration of AM2 is much smaller, which indicates that AM2 has a high safety profile.

In addition, in T24 cells, the IC_{50} of AM2 was reduced by 40,000 times compared to its parent metformin, and even 213 times lower than the first-line treatment drug cisplatin which made it possible for AM2 to become a highly effective and low-toxicity new drug. In addition, we also experimentally demonstrated that AM2 significantly inhibits the colony formation and

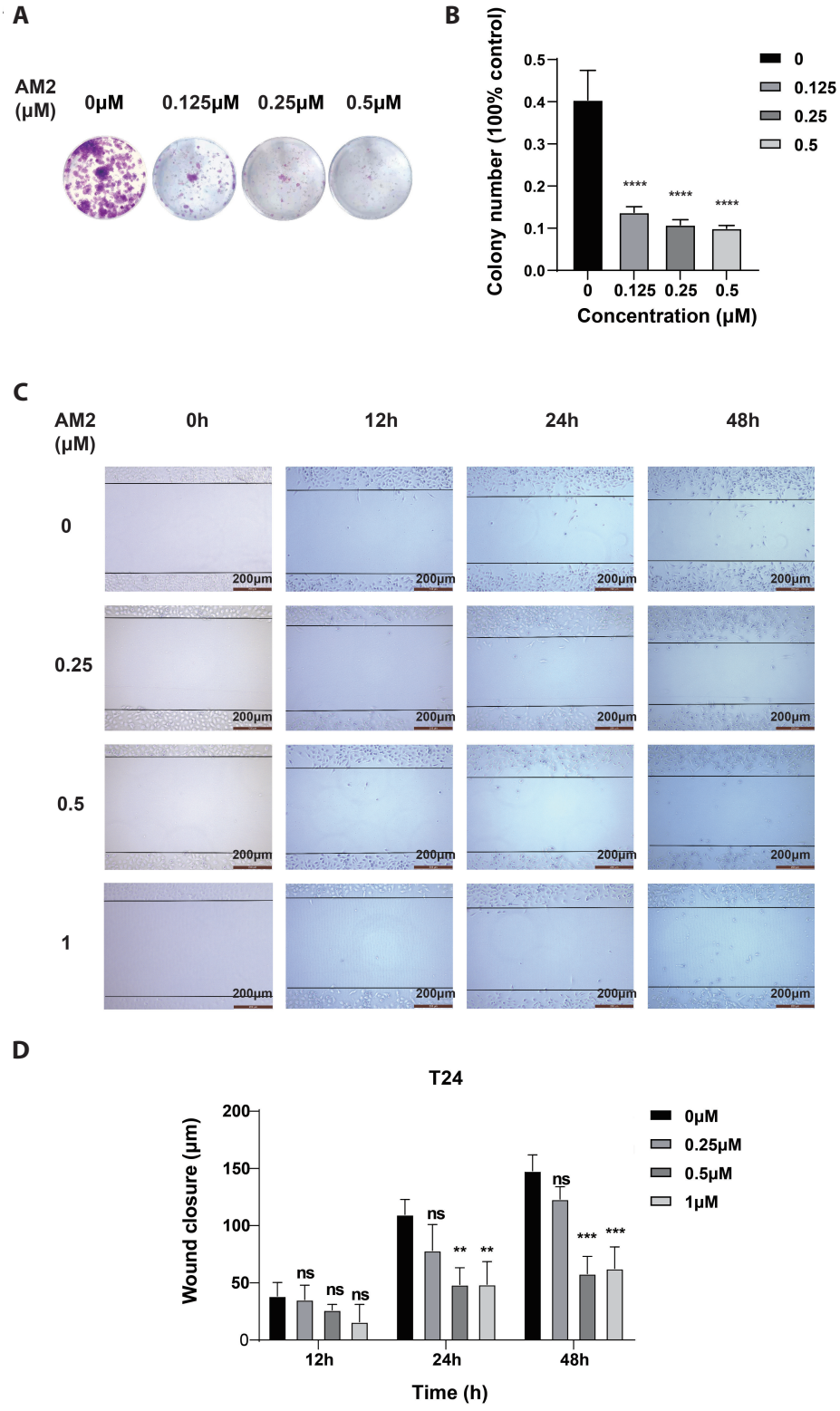

Fig. 3. The inhibition of colony formation and migration of AM2 on T24 cells. (A) Evaluation of colony suppression by AM2. (B) Quantification of the colony formation. OD values were scanned at a wavelength of 550 nm. (C, D) Inhibitory migration effect of AM2 on T24 cells for 12, 24, 48 h. Scale bar was 200 μm. Data are presented as the mean ± SD of three independent experiments. ns, no significance. **p < 0.01, ***p < 0.001, ****p < 0.0001.

migration ability of T24 cells.

We further explored the mechanism of action by which AM2 inhibits bladder cancer cells. Previous studies in our laboratory found that the expression of Clusterin was positively correlated with the progression of bladder cancer and was the target of metformin [23]. Silencing Clusterin causes a decrease in SREBP-1 and its downstream protein and mRNA expression. Metformin targets Clusterin, inactivates SREBP-1c and its downstream target

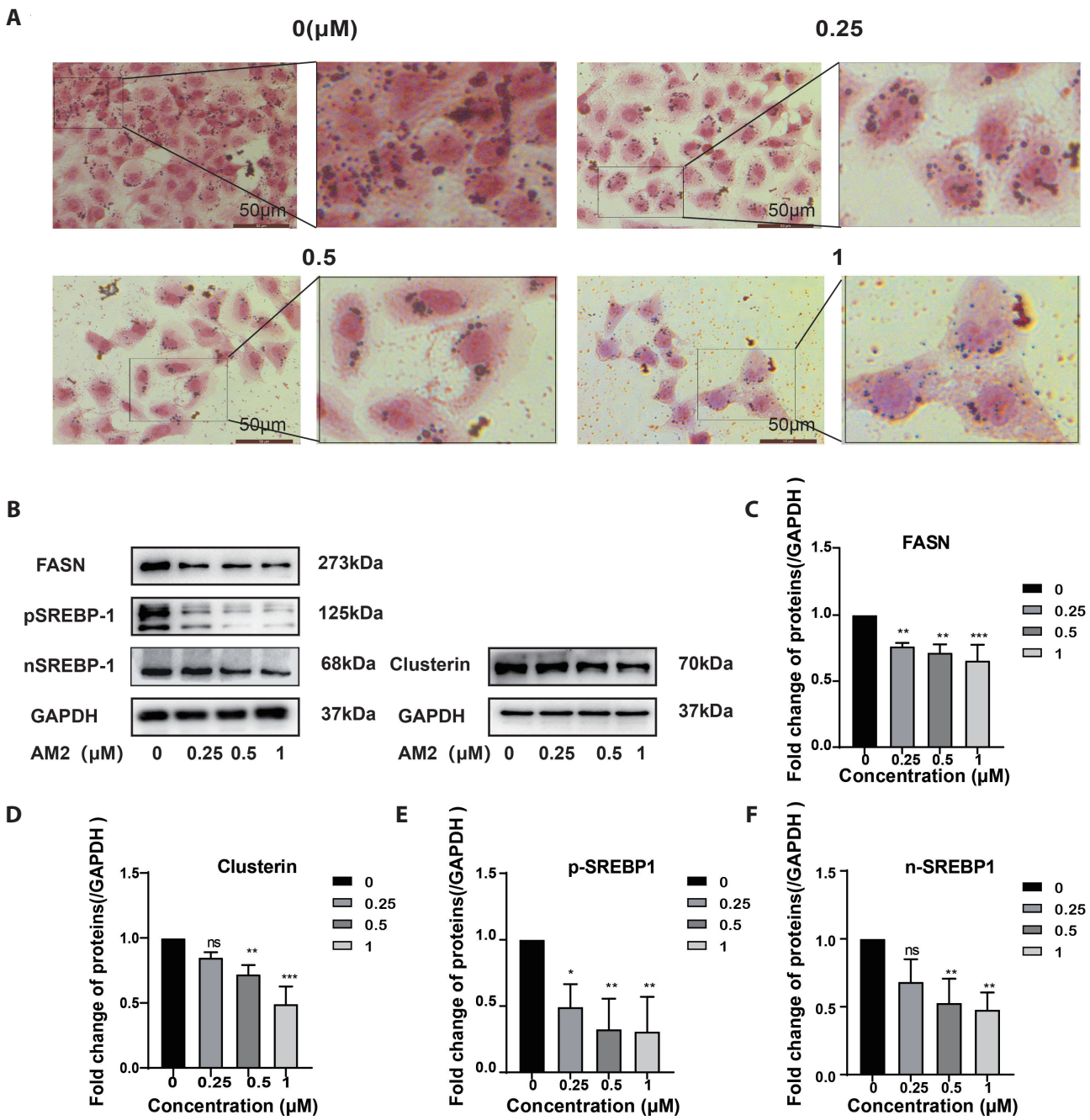

Fig. 4. AM2 regulated lipogenesis in T24 cells. (A) Oil red O staining images of T24 treated with different concentrations of AM2. (B) Effect of AM2 on the expression of FASN, SREBP1 and Clusterin in T24 cells. (C) The expression level of FASN protein. (D) The expression level of Clusterin protein. (E) The expression level of p-SREBP1. (F) The expression level of mature forms of n-SREBP1. Data are presented as the mean ± SD of three independent experiments. ns, no significance. *p < 0.5, **p < 0.01, ***p < 0.001.

FASN, blocks the synthesis of new fatty acids, and thus inhibits the growth of T24 cells. We think that AM2, as a derivative of metformin, may have a similar mechanism of action. Through oil red O staining experiments, we found that AM2 significantly inhibited lipid synthesis in T24 cells, which matched the guess. In addition, through Western blotting experiments, we found that AM2 downregulated Clusterin, SREBP 1, and FASN proteins in a dose-dependent manner, which implied that AM2 inhibited lipogenesis associated with the Clusterin/SREBP1/FASN signaling pathway, thereby inhibiting the growth of bladder cancer cells. However, it is not clear whether inhibition of lipid synthesis is the only mechanism by which AM2 works. The target of AM2 and other possible mechanisms of action are to be studied later.

In summary, we designed and synthesized a novel artesunate

dimer triazine compound AM2 through linking artesunate with metformin moiety. AM2 is a highly effective anti-bladder cancer agent that inhibits bladder cancer cells by blocking lipogenesis associated with the Clusterin/SREBP1/FASN signaling pathway. Overall, AM2 exhibits the potential of becoming a new chemotherapeutic drug for bladder cancer treatment.

FUNDING

This work was supported by the Natural Science Foundation of Hunan Province (No.2023JJ40460); General guidance subject of Hunan Provincial Health Commission (D202313059420); National College Student Innovation and Entrepreneurship Training Program (S202212652002) and Hunan Normal University School of Medicine Open Project (KF2022049).

ACKNOWLEDGEMENTS

None.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary data including three figures can be found with this article online at https://doi.org/10.4196/kjpp.2024.28.3.219

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