Matrine Can Inhibit the Growth of Colorectal Cancer Cells by Inducing Ferroptosis

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Abstract
Colorectal cancer (CRC) is a common malignant tumor of the digestive system that can seriously threaten human health. Chinese matrine is known to have a wide range of antiviral and immunomodulatory effects. In this study, we evaluated the effect of matrine on ferroptosis using the HCT116 human colon cancer cell line. We evaluated cell viability and proliferation using the cell counting kit-8 assay and carried out cell clone formation experiment by measuring reactive oxygen species (ROS) production, as well as levels of glutathione (GSH), lipid peroxide (MDA), Fe2+, glutathione peroxidase 4 (GPX4), activating transcription factor 4 (ATF4), solute carrier family 7 member 11 (SLC7A11), transferrin receptor protein 2 (TFR2), and Sigma-1 receptor (Sigma-1R) in order to evaluate the redox status of cells. These results indicate that matrine can significantly reduce the cell viability of HCT116 and decrease cell proliferation. After treatment with matrine, ROS, Fe2+, and MDA levels increased significantly, while the GSH content decreased. In addition, the expression of GPX4, SLC7A11, and Sigma-1R decreased significantly, while the expression of ATF4 and TFR2 increased significantly. These results indicate that matrine can induce ferroptosis in CRC, which can provide new clues for further pharmacological research of matrine and provide experimental evidence for understanding its mechanism of inhibiting CRC.

Keywords
matrine, ferroptosis, colorectal cancer, mechanism

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Colorectal cancer (CRC) is the third most malignant tumor of the digestive system, as well as the fourth deadliest cancer in the world. CRC is most frequently diagnosed among people aged 40-50, with a male-to-female ratio of 2-3: 1.2 The incidence of CRC has risen significantly over recent years, with nearly 900,000 deaths per year. However, it lacks effective prevention and control measures. Today, multidisciplinary comprehensive treatment based on surgery is an important treatment principle for CRC, as this includes surgery, radiation therapy, chemotherapy, immunotherapy, traditional Chinese medicine, and other treatment methods. However, more than 50% of CRC patients have already progressed to stage III-IV when cancer is diagnosed. If the best time for diagnosis and treatment is missed, then the 5-year survival rate is less than 40%.3 Therefore, in addition to managing adverse risk factors, including obesity, lack of physical activity, poor diet, drinking, and smoking, we need to implement an organized screening plan to detect and treat early in order to reduce the incidence and mortality of CRC.5,6 The pathogenesis of CRC is complex, and treatment is very limited. In chemotherapy, capecitabine, oxaliplatin, and fluorouracil are often used as first-line drugs. However, these drugs have limited efficacy, as well as varying degrees of side effects.7 Therefore, it is very important to discover and develop new effective drugs.

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Ferroptosis is a newly discovered form of iron-dependent, nonapoptotic cell death. Ferroptosis is mainly regulated via cystine-glutamate reverse transporter (System xc-) and glutathione peroxidase 4 (GPX4). The consumption of intracellular glutathione (GSH), decrease in GPX4 activity, and an increase in lipid peroxide and reactive oxygen species (ROS) production are characteristics of ferroptosis. The system xc-light chain subunit solute carrier family 7 member 11 (SLC7A11) is generally highly expressed in tumor cells. Hence, blocking SLC7A11 can induce tumor cells to undergo ferroptosis, which leads to inhibition of tumor growth. By restoring intracellular GSH levels, activating GPX4 expression, and inhibiting lipid peroxidation and ROS accumulation, the occurrence of ferroptosis in cancer cells will remain limited. As a novel form of cell death regulation, ferroptosis is closely related to regulating the growth of various tumor cells, including ovarian cancer, head and neck tumors, liver cancer, cervical cancer, pancreatic cancer, and rectum cancer. Drug-induced ferroptosis can be an effective method to fight tumor.

Ferrostatin-1 is a synthetic antioxidant that is able to effectively inhibit lipid peroxidation and eliminate alkoxy groups. Ferrostatin-1 is a more effective ferroptosis inhibitor compared with phenolic antioxidants. Matrine is an active ingredient in the traditional Chinese medicine Sophora flavesens, which is known to have low toxicity to humans and animals and has antibacterial, antiviral, anti-inflammatory, immune regulation, antihepatic fibrosis, sedative, hypnotic, analgesic, and additional pharmacological effects. Recent evidence indicates that matrine has an inhibitory effect on various tumor cells, which includes cervical cancer, leukemia, pancreatic cancer, gastric cancer, lung cancer, and breast cancer. However, the exact mechanism of the antitumor activity of matrine has not yet been fully understood. In this study, we found that matrine induces the occurrence of ferroptosis in CRC cells. Specifically, when matrine induces a decrease in cell viability and an increase in intracellular ROS levels, cells tend to exhibit a decrease in GSH levels, downregulation of GPX4, SLC7A11, and Sigma-1 receptor (Sigma-1R) protein expression, as well as an upregulation of activating transcription factor 4 (ATF4) and transferrin receptor protein 2 (TFR2) proteins, all of which significantly reduce growth and proliferation of colon cancer cells.

Materials and Methods

Reagents and Materials

Matrine (#110805-200508) was purchased from the National Institutes for Food and Drug Control of China. Antibodies targeting NRF2 antibody (#16396-1-AP), HSPA5 (#11587-1-AP), and ATF4 (#10835-1-AP) were purchased from Proteintech group, Inc. (Wuhan, China). GPX4 antibody (#bs-3884R), SLC7A11 antibody (#bs-5111), and TFR2 antibody (#bs-9894R) were acquired from Beijing Boosen Biotechnology Co., Ltd (Beijing, China), SLC7A11 antibody (#ab175186), and anti-rabbit immunoglobulin G (#ab150077), and iron assay kit (#ab83366) were bought from Abcam Technology (Abcam, Cambridge, UK), glyceraldehyde 3-phosphate dehydrogenase antibody (#21118) was purchased from Cell Signaling Technology (CST, USA), while ferrostatin-1 (#HY-100579) was bought from MedChemExpress (New Jersey, USA). ROS detection kit (#CA1410), reduced GSH content detection kit (#BC1175), malondialdehyde (MDA) content detection kit (#BC0025), and bicinchoninic acid assay (BCA) protein concentration determination kit (#PC0020) were bought from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). The cell counting kit-8 (CCK-8) kit (#CK04) was purchased from Dojindo (Japan). Trypsin-ethylenediaminetetraacetic acid (EDTA) (#GNM25200), phosphate-buffered saline (PBS) (#GNM20012), and D-Hanks (#GNM14170) were bought from Gino Biomedical Technology Co., Ltd. (Hangzhou, China), and Crystal Violet Stain (#AS1086) was acquired from Wuhan Aspen Biotechnology Co., Ltd. (Wuhan, China).

Cell Culture

The cell line HTC116 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HCT116 was cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS) (Grand Island, NY) with 1% antibiotics, and maintained at 5% CO₂ and 37 °C in a humidified incubator.

Cell Viability

Cell viability was detected using the CCK-8 kit, as per the manufacturer’s guidelines.

Intracellular ROS Level Determination

The ROS assay kit utilizes the fluorescent probe 2’,7-dichlorofluorescein diacetate (DCFH-DA) for active oxygen detection. DCFH-DA itself has no fluorescence and can therefore freely pass through the cellular membrane. After entering the cell, DCFH-DA can be hydrolyzed into DCFH within the cell. ROS in cells can oxidize nonfluorescent DCFH to produce fluorescent DCF. By detecting DCF fluorescence, levels of ROS in the cells can become known. We performed ROS detection according to the manufacturer’s instructions. In brief, after we carried out cell treatment, the culture solution was removed, the cells were collected after trypsin digestion, and washed 3 times with PBS. First, 1 mL of 10 μmol/L DCFH-DA was added and then the mixture was incubated at 37 °C in the dark for 20 minutes. Next, the cells were washed 3 times with PBS. After resuspending cells in PBS, they were analyzed using flow cytometry.

Clone Formation

Cells were trypsinized with 0.25% Trypsin-EDTA and resuspended in 1 mL of medium. After the cells were counted, they were seeded onto a 6-well plate at an inoculation amount of
approximately 1000 cells per well. Additionally, it was incubated at 37 °C in 5% carbon dioxide. After cells were allowed to grow, they were treated with drugs for 24 hours and cultured for 2 weeks. The cells were then fixed with 4% paraformaldehyde, stained with crystal violet at room temperature, washed 3 times with PBS, and visualized after drying. The cloning rate was calculated using the equation:

\[
\text{Cloning rate} = \frac{\text{Number of clones}}{\text{number of inoculated cells}} \times 100\%.
\]

**Determination of Intracellular GSH**

The reagents were prepared as per the GSH assay instructions. We collected no less than 10^6 cells, which were washed twice with PBS. The cells were then resuspended in PBS, centrifuged at 600g for 10 minutes, and 3 times the volume of the cell pellet was added to resuspend the cells. The freezing and thawing were repeated 2-3 times (freeze in liquid nitrogen and dissolve in a 37 °C water bath), centrifuged at 8000g for 10 minutes, the supernatant was collected, and the absorbance was measured at 412 nm on a microplate reader.

**Determination of Fe^{2+} Level**

As per the manufacturer’s instructions, an iron detection kit was utilized to determine the Fe^{2+} levels.

**Lipid Peroxide Detection**

The MDA content in the cells was identified as per the operating instructions of the MDA content detection kit.

**Western Blot**

The cells were then collected and washed twice with PBS. After cells were lysed to obtain the protein, the concentration of the protein was determined using the BCA kit. The protein concentration of each group was adjusted to be 2 µg/μL with 5× loading buffer and then heated at 95 °C for 5 minutes. Next, 20 µg of protein was separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation gel. After electrophoresis and membrane transfer, the reaction was blocked using 5% skimmed milk powder for 1.5 hours. The primary antibody was incubated overnight at 4 °C. The membrane was washed 3 times with Tris-buffered saline and Tween 20 (TBST) buffer, and it was incubated with secondary antibody at room temperature for 1 hour. After washing the membrane 3 times with TBST buffer, the membrane was developed using enhanced chemiluminescence. The membrane was then exposed and analyzed using a Bio-Rad gel imager.

**Statistical Methods**

Graphpad Prism 7.0 was utilized for data analysis, while data were expressed as mean ± SD. Additionally, analysis of variance was used for comparisons between multiple samples. A statistical value of \( P < 0.05 \) indicated that the differences were statistically significant.

**Results**

**The CCK-8 Bioassay**

Different concentrations of matrine (5, 10, or 20 µmol/L) significantly reduced cell viability in a dose-dependent manner (Figure 1). Since the use of 20 µmol/L matrine led to a good inhibitory effect, this concentration was used for subsequent experiments.

**ROS Detection and Clone Formation**

ROS accumulates after unfavorable external stimuli and may exceed the peroxidase system’s ability to clear, leading to oxidative stress, destruction of cell membranes, and eventually induction of ferroptosis. We initially tested changes in intracellular ROS after matrine intervention and found that ROS led to a significant increase after matrine intervention (Figure 2(A)). In order to further observe whether matrine affects cellular ferroptosis during the inhibition of cell growth, we introduced the ferroptosis inhibitor ferrostatin-1 for comparison (introduction dose was 5 µmol/L). We found that matrine inhibited the clonal formation of HTC116 cells (Figure 2(B)). Furthermore, the addition of the ferroptosis inhibitor ferrostatin-1 while treating cells with matrine led to a reduction in the inhibitory effect of matrine on clonal formation. These results indicate that the mechanism of matrine inhibiting cell growth may be partly related to the induction of ferroptosis. Based on these results, we measured several indicators related to ferroptosis in the subsequent experiments.
Detection of GSH, Fe\(^{2+}\), MDA

GSH is an important nonenzymatic antioxidant and plays an important role in restoring physiological function by removing lipid peroxides. Therefore, levels of GSH are an important factor in determining antioxidant capacity. GSH and Fe\(^{2+}\) are key regulators of ROS in the process of ferroptosis. MDA is a product of lipid peroxidation. It has strong biological toxicity, and its production can aggravate damage to biofilm. Therefore, MDA production serves as an index for membrane lipid peroxidation, which indirectly reflects the antioxidant capacity of cells, as well as the degree of damage to cells.\(^9\) In order to confirm the effect of matrine on the antioxidant capacity of HTC116 cells, we estimated the levels of GSH and MDA. In comparison to the control group, GSH levels significantly decreased in the drug intervention group, while MDA levels significantly increased (\(P < 0.01\)). Treatment with ferrostatin-1 reversed changes present in GSH and MDA levels. When compared with the control group, the Fe\(^{2+}\) level in the matrine group was significantly increased (\(P < 0.01\)) (Figure 3(C)). However, after treatment with ferrostatin-1, the levels of Fe\(^{2+}\) significantly decreased (\(P < 0.01\)). Therefore, we can conclude that matrine can promote Fe\(^{2+}\) and inhibit GSH to increase the levels of ROS, thereby promoting hypertrophy.

Expression of Ferroptosis-Related Proteins

As shown in Figure 4(A) and (B), when compared with the control group, the expression of GPX4, SLC7A11, and Sigma 1R in the matrine group significantly decreased (\(P < 0.01\)), while expression of ATF4 and TFR2 in the matrine group significantly
increased \((P < 0.01)\). After treatment with ferrostatin-1, levels of GPX4, SLC7A11, and Sigma 1R increased \((P < 0.01)\), while levels of ATF4 and TFR2 were reduced.

**Discussion**

Ferroptosis is a form of cell death that is caused by iron-dependent lipid peroxidation, as well as ROS accumulation. Excess iron levels due to abnormal iron metabolism or imbalance of 2 major redox systems (lipid peroxidation and thiol) is the main cause behind ROS production. The synthesis of GSH, a thiol-containing tripeptide, depends on the constant introduction of cysteine (Cys2) onto the cell surface Cys2/glutamate anti-transporter xCT (also known as SLC7A11). As a specific light chain presenter of Cys2/glutamate reverse transport protein, SLC7A11 has a key function in the negative regulation of ferroptosis. Additionally, iron overload can cause ferroptosis of cancer cells. Genes related to iron metabolism, such as transferrin (TF) and transferrin receptor (TFR), are key mediators in the process of promoting iron. \(^{20}\) ATF4 is a key medium for metabolism, oxidative homeostasis, and cell survival. Activation of ATF4 induces HSPA5 expression, which, in turn, binds GPX4 and protects degradation of GPX4 protein, as well as subsequent lipid peroxidation. Among them, the genetic or pharmacological inhibition of GPX4 increases lipid peroxidation, which results in cancer death mediated by ferroptosis. \(^{21}\) Sigma-1R can regulate the accumulation of ROS through nuclear factor erythrocyte-like factor 2 (NRF2), and high expression of Sigma-1R inhibits the occurrence of ferroptosis. \(^{22}\)

Ferroptosis has been recently discovered to involve the production of iron-dependent ROS. GPX4 can catalyze the reduction of lipid peroxides in a complex cellular membrane environment. Under normal circumstances, GPX4 prevents cells from ferroptosis by inhibiting the accumulation of intracellular lipid peroxides. While GPX4 has been inhibited, it can cause intracellular ROS accumulation and induce ferroptosis. \(^{23}\) At the molecular level, cysteine availability, GSH biosynthesis, and proper functioning of GPX4 are the main source of keeping ferroptosis in check, whereas conditions that culminate in
GPX4 inhibition/destabilization sensitize or can even trigger ferroptotic cell death. The mainstay of ferroptosis is the generation of specific phospholipid hydroperoxides in the presence of catalytically active iron, which can be endogenously counteracted by the systemic xc-/GSH/GPX4 axis. The xCT (SLC7A11) light chain, which constitutes the substrate-specific subunit of system xc-, is subject to complex transcriptional regulation. Once taken up by the system xc-, cysteine is reduced to cysteine by GSH and/or thioredoxin reductase 1, which can then be used for GSH biosynthesis. Since cysteine is a rate-limiting substrate in GSH biosynthesis, and GSH is the prevailing antioxidant across mammalian cells, conditions that impede on intracellular cysteine, and consequently GSH levels impact directly on GPX4 activity, thus predisposing cells to ferroptosis. A large number of studies have indeed confirmed that GPX4 plays a key regulatory role in ferroptosis across various tumors. The identification of an ever-growing list of GPX4 inducers and inhibitors, as well as the elucidation of their mode of action, have greatly helped clarify the key role of GPX and potential for treatment in diseases, not just limited to cancer.

SLC7A11 is a cystine/glutamic acid xCT transporter that controls the production of phenylalanine pigment. Studies have identified that high expression of SLC7A11 can enhance the dependence of cancer cells on glucose by transporting glutamate, which suggests that SLC7A11 also plays a role in the Warburg effect. Extensive evidence shows that SLC7A11 promotes cysteine uptake and glutathione biosynthesis, thereby preventing oxidative stress and ferroptosis cell death. In addition, SLC7A11 can also mediate metabolic reorganization that is able to promote cancer cell progression. System xc- is a sodium-dependent retrotransporter of cysteine and glutamate and is known to be a pivotal protein in the transport of extracellular cystine. It consists of 2 subunits, including the light chain subunit SLC7A11 and the heavy chain subunit SLC3A2. The light chain subunit SLC7A11 is responsible for primary transport activity and is highly specific for cysteine and glutamate, while the heavy chain subunit SLC3A2 primarily functions as a chaperone protein and regulates trafficking of SLC7A11 to the plasma membrane. By introducing cystine and promoting GSH biosynthesis, SLC7A11 is able to prevent the accumulation of lipid hydrogen peroxide and protect cells from ferroptosis. GPX4 is a phospholipid peroxidase that can catalyze the reduction of lipid peroxides in the presence of GSH as an essential cofactor. Downregulation of SLC7A11 can cause loss of intracellular cysteine levels, as well as subsequent loss of GSH biosynthesis, which indirectly leads to the inhibition of GPX4 activity and leads to accumulation of lipid peroxides and subsequent activation of ferroptosis. The established role of SLC7A11 in inhibiting ferroptosis and protecting cells from oxidative stress indicates that SLC7A11 has the function of promoting tumors. Targeting SLC7A11 may therefore provide a novel and effective treatment strategy to treat cancer patients that overexpress SLC7A11.

Sigma-1R is a unique multitask lipid microdomain and plasma membrane localization chaperone protein. It can serve as an important therapeutic target for the treatment of various forms of neurodegenerative diseases across humans. The direct and indirect effects of Sigma-1R involve changes in enzyme activity and are able to regulate signaling pathways. NRF2 is a signaling pathway that is regulated via Sigma-1R. NRF2 is an important antioxidative transcriptional factor that regulates the expression of a number of cytoprotective genes that are involved in detoxification and antioxidant and drug metabolism by binding to its response element, the antioxidant response element. Studies have shown that activation of NRF2 has an inhibitory effect on the ferroptosis of cancer cells. NRF2 and Sigma-1R have a similar pattern in maintaining redox balance. Sigma-1R regulates ROS through NRF2, and NRF2 in turn negatively regulates Sigma-1R gene expression. All these proteins are closely related to ferroptosis.

ATF4 is commonly expressed in human organs and can be activated in response to various stress signals including hypoxia, endoplasmic reticulum stress, amino acid deprivation, and oxidative stress. High expression of ATF4 is known to be related to the growth of malignant tumors. Tumor growth induced by ATF4 can be restored by inhibiting SLC7A11, which has been identified as the major driving force of ATF4 function. ATF4 expression leads to an increase in tumor angiogenesis and vascular growth, and ATF4 is resistant to ferroptosis-mediated cell death. In the process of inducing ferroptosis, ATF4 may be activated in order to exert its resistance.

In vertebrates, transferrin transfers iron safely to cells through circulation. Transferrin-bound iron is incorporated into cells via transferrin receptor-mediated endocytosis. TRR2 promotes the transport of iron from lysosomes to the mitochondria, where its activation causes a large increase in iron levels in cells. As an iron-dependent form of cell death, the increase in intracellular iron is undoubtedly favored by ferroptosis. Edna Ooko et al have confirmed that induction of ferroptosis is accompanied by TRR2 activation.

Iron-dependent cell death is mainly mediated by ROS, while GPX4 and SLC7A11 are key regulatory factors. Sigma 1R, ATF4, and TFR2 are also important regulators. Their expression not only characterizes the phenotype of ferroptosis but can directly or indirectly control the development of ferroptosis by targeting these proteins. In our study, we found that matrine significantly increased levels of ROS, MDA, Fe 2+, ATF4, and TFR2 protein, while significantly downregulating GSH and GPX4, Sigma 1R, and SLC7A11 protein expression.

Conclusion

Matrine increases ferroptosis of cancer cells, and its mechanism likely depends on the regulation of the oxidative stress pathway. However, the pharmacological effects of matrine are diverse. In this study, we found that matrine plays a role across many aspects of ferroptosis, including regulating levels of Fe 2+, the GSH/GPX4 pathway, and regulation of Sigma 1R, ATF4, and TFR2 proteins (Figure 5). These functions of
matrine aggravate the accumulation of ROS, induce ferroptosis, and inhibit the growth of cancer cells. In the future, the specific mechanism of matrine in ferroptosis and CRC needs to be explored. This study provides a scientific basis for the treatment of CRC and induction of ferroptosis and also provides some experimental evidence for clarifying the pharmacological effect of matrine.

Declaration of Conflicting Interests

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References

1. Benson AB 3rd, Bekaii-Saab T, Chan E, et al. Metastatic colon cancer, version 3.2013: featured updates to the NCCN guidelines. J Natl Compr Canc Netw. 2013;11(2):141-152. doi:10.6004/jnccn.2013.0022
2. Zhao Y, Hu X, Zuo X, Wang M. Chemopreventive effects of some popular phytochemicals on human colon cancer: a review. Food Funct. 2018;9(9):4548-4568. doi:10.1039/C8FO00850G
3. Simon K. Colorectal cancer development and advances in screening. Clin Interv Aging. 2016;11:967-976. doi:10.2147/CIA.S109285
4. Keller DS, Berho M, Perez RO, Wexner SD, Chand M. The multidisciplinary management of rectal cancer. Nat Rev Gastroenterol Hepatol. 2020;17(7):414-429. doi:10.1038/s41575-020-0275-y
5. Hugen N, Brown G, Glynne-Jones R, de Witt JHW, Nagtegaal ID. Advances in the care of patients with mucinous colorectal cancer. Nat Rev Clin Oncol. 2016;13(6):361-369. doi:10.1038/nrclinonc.2015.140
6. Brody H. Colorectal cancer. Nature. 2015;521(7551):S1. doi:10.1038/521S1a

Figure 5. Possible mechanism of matrine induced ferroptosis. ATF4, activating transcription factor 4; GPX4, glutathione peroxidase 4; GSH, glutathione; NRF2, nuclear factor erythocyte-like factor 2; ROS, reactive oxygen species; SLC7A11, solute carrier family 7 member 11; Sigma 1R, Sigma-1 receptor; TFR2, transferrin receptor protein 2.
7. Hong YS, Nam B-H, Kim K-P, et al. Oxaliplatin, fluorouracil, and leucovorin versus fluoruracil and leucovorin as adjuvant chemotherapy for locally advanced rectal cancer after preoperative chemoradiotherapy (ADORE): an open-label, multicentre, phase 2, randomised controlled trial. *Lancet Oncol.* 2014;15(11):1245-1253. doi:10.1016/S1470-2045(14)70377-8

8. Xie Y, Hou W, Song X, et al. Ferroptosis: process and function. *Cell Death Differ.* 2016;23(3):369-379. doi:10.1038/cdd.2015.158

9. Yagoda N, von Rechenberg M, Zaganjor E, et al. RAS-RAF-MEK-dependent oxidative cell death involving voltage-dependent anion channels. *Nature.* 2007;447(7146):865-869. doi:10.1038/nature05859

10. Dixon SJ, Lemberg KM, Lamprecht MR, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell.* 2012;149(5):1060-1072. doi:10.1016/j.cell.2012.03.042

11. Latunde-Dada GO. Ferroptosis: role of lipid peroxidation, iron and ferritinophagy. *Biochim Biophys Acta Gen Subj.* 2017;1861(8):1893-1900. doi:10.1016/j.bbagen.2017.05.019

12. Stockwell BR. A powerful cell-protection system prevents cell death by ferroptosis. *Nature.* 2019;575(7784):597-598. doi:10.1038/d41586-019-03145-8

13. Miootto G, Rossetto M, Di Paolo ML, et al. Insight into the mechanism of ferroptosis inhibition by ferrostatin-1. *Redox Biol.* 2020;28:101328. doi:10.1016/j.redox.2019.101328

14. Wang W, You RL, Qin WJ, et al. Anti-tumor activities of active ingredients in compound Kushen injection. *Acta Pharmaceut Sin.* 2015;36(6):676-679. doi:10.1038/aps.2015.24

15. Huang J, Xu H, Matrine: bioactivities and structural modifications. *Curr Top Med Chem.* 2016;16(28):3365-3378. doi:10.2174/13892002166616050613012

16. Mallepalli S, Gupta MK, Vadde R. Neuroblastoma: an updated review on biology and treatment. *Curr Drug Metab.* 2019;20(13):1014-1022. doi:10.2174/13892002216619122610231

17. Gu Y-Y, Chen M-H, May BH, et al. Matrine induces apoptosis in multiple colorectal cancer cell lines in vitro and inhibits tumour growth with minimum side effects in vivo via Bel-2 and caspase-3. *Phytomedicine.* 2018;51:214-225. doi:10.1016/j.phymed.2018.10.004

18. Hong X, Zhong L, Xie Y, et al. Matrine reverses the Warburg effect and suppresses colon cancer cell growth via negatively regulating HIF-1α. *Front Pharmacol.* 2019;10:1437. doi:10.3389/fphar.2019.01437

19. Wang Y, Chen Q, Shi C, Jiao P, Gong Z. Mechanism of glycyrrhizin on ferroptosis during acute liver failure by inhibiting oxidative stress. *Med Med Rep.* 2019;20(5):4081-4090. doi:10.3892/mmr.2019.10660

20. Mou Y, Wang J, Wu J, et al. Ferroptosis, a new form of cell death: opportunities and challenges in cancer. *J Hematol Oncol.* 2019;12(1):34. doi:10.1186/s13045-019-0720-y

21. Zhu S, Zhang Q, Sun X, et al. HSPA5 regulates ferroptotic cell death in cancer cells. *Cancer Res.* 2017;77(8):2064-2077. doi:10.1158/0008-5472.CAN-16-1979

22. Bai T, Lei P, Zhou H, et al. Sigma-1 receptor protects against ferroptosis in hepatocellular carcinoma cells. *J Cell Mol Med.* 2019;23(11):7349-7359. doi:10.1111/jcmm.14594

23. Zhu S, Zhang Q, Sun X, et al. HSPA5 regulates ferroptotic cell death in cancer cells. *Cancer Res.* 2017;77(8):2064-2077. doi:10.1158/0008-5472.CAN-16-1979

24. Seibh TM, Proneth B, Conrad M. Role of GPX4 in ferroptosis and its pharmacological implication. *Free Radic Biol Med.* 2019;133:144-152. doi:10.1016/j.freeradbiomed.2018.09.014

25. Chen Y, Hu S, Mu L, et al. Slc7a11 modulated by POU2F1 is involved in pigmentation in rabbit. *Int J Mol Sci.* 2019;20(10):2493. doi:10.3390/ijms20102493

26. Kopplu P, Zhang Y, Zhuang L, Gan B. Amino acid transporter SLC7A11/sCT at the crossroads of regulating redox homeostasis and nutrient dependency of cancer. *Cancer Commun.* 2018;38(1):12. doi:10.1186/s40880-018-0288-x

27. Lang X, Green MD, Wang W, et al. Radiotherapy and immunotherapy promote tumoral lipid oxidation and ferroptosis via synergistic repression of Slc7a11. *Cancer Discov.* 2019;9(12):1673-1685. doi:10.1158/2159-8290.CD-19-0338

28. Liu T, Jiang L, Tavana O, Gu W. The deubiquitylase OTUB1 mediates ferroptosis via stabilization of Scl7a11. *Cancer Res.* 2019;79(8):1913-1924. doi:10.1158/0008-5472.CAN-18-3037

29. Dodson M, Castro-Portrugos R, Zhang DD. NRF2 plays a critical role in mitigating lipid peroxidation and ferroptosis. *Redox Biol.* 2019;23:101107. doi:10.1016/j.redox.2019.101107

30. Wang J, Shanmugam A, Markand S, Zorrell E, Ganapathy V, Smith SB. Sigma 1 receptor regulates the oxidative stress response in primary retinal Müller glial cells via NRF2 signaling and system xc(-), the Na(+) independent glutamate-cystine exchanger. *Free Radic Biol Med.* 2015;86:25-36. doi:10.1016/j.freeradbiomed.2015.04.009

31. Chen D, Fan Z, Rauh M, Buchfelder M, Eyupoglu IY, Sasaki N. ATF4 promotes angiogenesis and neuronal cell death and confers ferroptosis in a xCT-dependent manner. *Oncogene.* 2017;36(40):5593-5608. doi:10.1038/onc.2017.146

32. Bai T, Liang R, Zhu R, Wang W, Zhou L, Sun Y. MicroRNA-214-3p enhances erastin-induced ferroptosis by targeting ATFS in hepatoma cells. *J Cell Physiol.* 2020;235(7-8):5637-5648. doi:10.1002/jcp.29496

33. Kawabata H. Transferrin and transferrin receptors update. *Free Radic Biol Med.* 2019;133:46-54. doi:10.1016/j.freeradbiomed.2018.06.037

34. Herbison CE, Thorstensen K, Chua ACG, et al. The role of transferrin receptor 1 and 2 in transferrin-bound iron uptake in human hepatoma cells. *Am J Physiol Cell Physiol.* 2009;297(6):C156-7-C1575. doi:10.1152/ajpcell.00649.2008

35. Ooko E, Saeed MEM, Kadioglu O, et al. Artemisinin derivatives induce iron-dependent cell death (ferroptosis) in tumor cells. *Phytomedicine.* 2015;22(11):1045-1054. doi:10.1016/j.phymed.2015.08.002