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Review
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Modulation of Reactive Oxygen Species to Overcome 5-Fluorouracil Resistance

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Abstract

5-Fluorouracil (5-FU) remains to be an important chemotherapeutic drug for treating several cancers when targeted therapy is unavailable. Chemoresistance limits the clinical utility of 5-FU, and new strategies are required to overcome the resistance. Reactive oxygen species (ROS) and antioxidants are balanced differently in both normal and cancer cells. Modulating ROS can be one method of overcoming 5-FU resistance. This review summarizes selected compounds and endogenous cellular targets modulating ROS generation to overcome 5-FU resistance.

Key Words: Reactive oxygen species, Cancer, Resistance, 5-Fluorouracil

INTRODUCTION

Despite the introduction of targeted anticancer therapy, 5-fluorouracil (5-FU) remains an important chemotherapeutic drug for treating several cancers, including colorectal, breast, and gastric cancer. 5-FU's cytotoxic mechanism involves the inhibition of thymidylate biosynthesis or the misincorporation of fluorinated nucleotides into newly synthesized DNA or RNA (Longley *et al*., 2003). It can be effective in the treatment of cancer when targeted therapy is unavailable. As described in previous studies, the development of prodrugs such as capecitabine has improved the limitation of 5-FU due to poor oral absorption (Pazdur *et al*., 1998). Furthermore, combination chemotherapy improved 5-FU's anticancer effect, as demonstrated by FOLFOX (folinic acid, 5-FU, and oxaliplatin) and FOLFIRI (folinic acid, 5-FU, and irinotecan) (Souglakos *et al*., 2006). Combining chemotherapeutics with different mechanisms could overcome the heterogeneity of tumor cells and decrease the development of resistance (Frei *et al*., 1998). Nevertheless, the overall response rate remains less than 50% (Mehrzad *et al*., 2016) due to the cells being resistant to chemotherapy.

Studies have been conducted to elucidate the 5-FU resistance mechanism described elsewhere (Blondy *et al*., 2020). The generation of reactive oxygen species (ROS) frequently correlates with the induction of apoptosis in many cancer cells; modulation of ROS may be one mechanism by which cancer cells avoid the cytotoxicity induced by 5-FU (Mates and Sanchez-Jimenez, 2000). For example, in human lung carcinoma cells NCI-H1299, the expression of reactive oxygen modulator 1 (Romo1) is elevated, and the cellular level of ROS is high. At the same time, tumor cells maintain high levels of antioxidant enzymes and antiapoptotic Bcl-2 family proteins, most likely to reduce oxidative stress (Hwang *et al*., 2007). This implies that cancer cells prefer a high level of ROS while keeping the protective mechanisms running to minimize the unwanted toxicity of ROS.

ROS have a versatile role in cancer cell biology (Liou and Storz, 2010). When elevated, ROS are thought to act as mitogens, inducing cancer cell proliferation (Torres and Forman, 2003). DNA damage from oxidative stress may lead to mutations that can either activate oncogenes or inactivate tumor suppressor genes (Wei, 1992). ROS production is minimal in normal cells, and antioxidant functions effectively remove ROS (Fig. 1A). Increased ROS production is frequently observed in cancer cells with a poor prognosis (Kumar *et al*., 2008). Cancer cells maintain a relatively high level of ROS, likely due to the tumor-promoting effects of ROS such as angiogenesis (Ushio-Fukai and Nakamura, 2008), metastasis (Nishikawa, 2008), and proliferation (Juhasz *et al*., 2017). As shown in Fig. 1B, cancer cells increase the level of antioxidant systems in response to elevated levels of ROS to protect

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themselves from oxidative stress (Gorrini *et al*., 2013). Many anticancer drugs, including 5-FU, induce high levels of ROS to exert cytotoxic effects. Cancer cells adapt to the escalated ROS level by expressing even more antioxidant systems (Fig. 1C) (Liu *et al*., 2016b). When there is insufficient protection from high levels of ROS, cancer cells may not survive (Fig. 1D).

In this study, we summarized our understanding of natural and synthetic compounds (Table 1) and identified possible cellular targets involved with the modulation of cellular ROS levels to overcome 5-FU resistance.

NATURAL/SYNTHETIC COMPOUNDS THAT MODULATE ROS TO OVERCOME 5-FU RESISTANCE

Metal chelators

Tetrathiomolybdate, a copper-chelating drug, was initially developed as an anticopper and antiangiogenic agent to treat Wilson's disease (Brewer *et al*., 1991). Interestingly, it enhances the activity of the anticancer drug doxorubicin, a DNA intercalator in ovarian cancer cells (Kim *et al*., 2011). Tetrathiomolybdate increased the cytotoxicity of doxorubicin at subcytotoxic levels, likely by targeting antioxidant enzymes such as

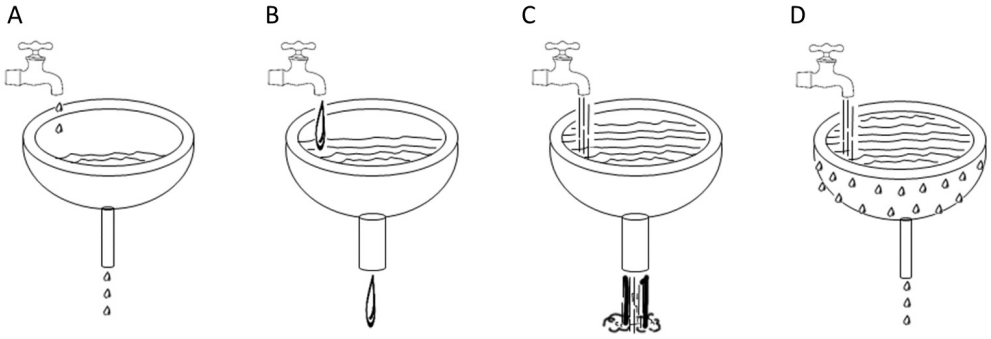

Fig. 1. Balance between ROS production and antioxidant function. (A) ROS (water in the figure) are produced by various mechanisms (drawn as a water tap), and antioxidant function (drawn as a drain) effectively removes them, allowing physiological ROS levels to remain low. (B) Increased ROS generation is frequently observed in cancer cells, and cancer cells increase the level of antioxidant functions accordingly. The cellular level of ROS increases but not to toxic levels. (C) Even higher antioxidant function accompanies ROS overproduction when cancer cells adapt to chemotherapy. (D) Decrease of antioxidant function may result in cellular toxicity.

Table 1. Selected compounds increasing ROS generation to overcome 5-FU resistance

Compound	Cell/tissue type	Effects/Mechanisms
tetrathiomolybdate	Ovarian cancer cells	Stress-mediated apoptosis↑, activation of JNK and p38 MAPK↑
TPEN	Colon cancer HCT116 cells	Mitochondrial membrane potential (MMP)↓
apigenin	Hepatocellular carcinoma cells	Mitochondrial apoptosis↑
Polyphenolics from quince	Colon cancer cells LS174	NF-κB activation↓, cell cycle progression↓, angiogenesis↓
kaempferol	Colon cancer cells LS174	Activation of STAT3↓, angiogenesis↓
shikonin	Gastric cancer SGC-7901	Translocation of AIF and Endo G into nucleus
proanthocyanidin	Breast cancer MDA-MB-231 cells	G2/M cell cycle arrest↑, MMP↓
B63 (curcumin analog)	Gastric cancer cells SGC-7901 etc.	Expression of Thioredoxin reductase 1↓
dimethoxycurcumin	Colon cancer cells SW480, SW620	Expression of Bax and cyt C↑, expression of Bcl-2↓
Sanguisorba officinalis L. radix	Colorectal cancer cells RKO, HCT116	Bax/Bcl-2 disruption↑, autophagy↑
manuka honey	Colon cancer cells HCT116	Expression of EGFR, HER2, Akt and mTOR↓
emodin	Breast cancer MCF7 cells	Expression of E2F1 and NRPARP↓
gypenoside	Colorectal cancer cells SW-480,SW-620 and Caco2	DNA damage induction↑, expression of p53↑
tubeimoside-I	Colorectal cancer cells SW480, SW620, HCT116, and RKO	Activation of AMPK↑
oridonin	Colorectal cancer cells HCT115	Activation of JNK/c-Jun pathway↓
Coptis herb extracts	Lung cancer A549 cells	ROS↑
mahanine	Colorectal cancer cells HCT116, SW480	Expression of PTEN and p53 in nucleus↑
caffeine	Liver cancer cells HepG2, HLF, Huh7, etc.	Cleavage of PARP↑, expression of Bcl-2 and Bcl-xL↓
selenocysteine	Skin cancer cells A375	Activation of ERK/Akt signaling↓
allicin	Liver cancer cells SK-Hep-1, BEL-7402	ROS↑, MMP↓
3-bromypyruvate	Liver cancer cells SNU449, Hep3B	ROS↑, MMP↓

copper/zinc–superoxide dismutase (SOD). Furthermore, by generating ROS, tetrathiomolybdate increased the cytotoxicity of several anticancer drugs, including 5-FU and mitomycin C (Kim *et al*., 2012). The production of ROS induced by tetrathiomolybdate resulted in the activation of stress-mediated apoptosis, JNK, and p38 mitogen-activated protein kinase (MAPK), which increased cytotoxicity.

N,*N*,*N′*,*N′*-tetrakis-[2-pyridylmethyl]-ethylenediamine (TPEN) was reported to have a cancer-specific copper chelation mediated cytotoxicity (Fatfat *et al*., 2014). Additionally, TPEN treatment resulted in the excessive generation of ROS via the formation of the TPEN-copper complex, leading to cytotoxicity in human colon cancer HCT116 cells. Evidently, elevated copper levels may be important in maintaining the proper level of ROS generation in cancer cells, whereas intracellular copper levels are crucial to maintaining the proper level of ROS generation in cancer cells (Gupte and Mumper, 2009). Furthermore, although cellular copper levels may be a target for cancer treatment, it remains to be seen whether a copper-chelating drug can help overcome 5-FU resistance.

Phenolic compounds

Interestingly, several antioxidant compounds promote the production of ROS in cancer cells. Although more research is needed to elucidate the precise mechanisms, these antioxidant compounds are thought to modulate ROS generation and increase the cytotoxicity of 5-FU. Phenolic compounds refer to diverse natural products such as flavanols, flavonols, chalcones, tannins, curcuminoids, etc. Their antioxidant function is usually attributed to the phenolic ring structure (Cai *et al*., 2006). The following sections list several phenolic compounds (Fig. 2) that have been reported to have synergistic cytotoxicity when combined with 5-FU or to be cytotoxic to 5-FU resistant cancer cells.

Apigenin is a flavonoid compound found in common fruits and vegetables that exhibits anti-inflammatory, antioxidant, and anticancer activity (Shukla and Gupta, 2010). Research revealed that apigenin cotreatment with 5-FU at a subtoxic level demonstrated synergistic cytotoxicity in treating hepatocellular carcinoma (HCC) cells *in vitro* and *in vivo* (Hu *et al*., 2015). Moreover, the ROS level was increased, and the mitochondrial apoptotic pathway was activated, indicating that apigenin has a pro-oxidant function. Although it remains to be seen whether apigenin is cytotoxic to 5-FU resistant cancer cells, apigenin, which is well-known for its antioxidant activity, appears to also demonstrate some pro-oxidant activity.

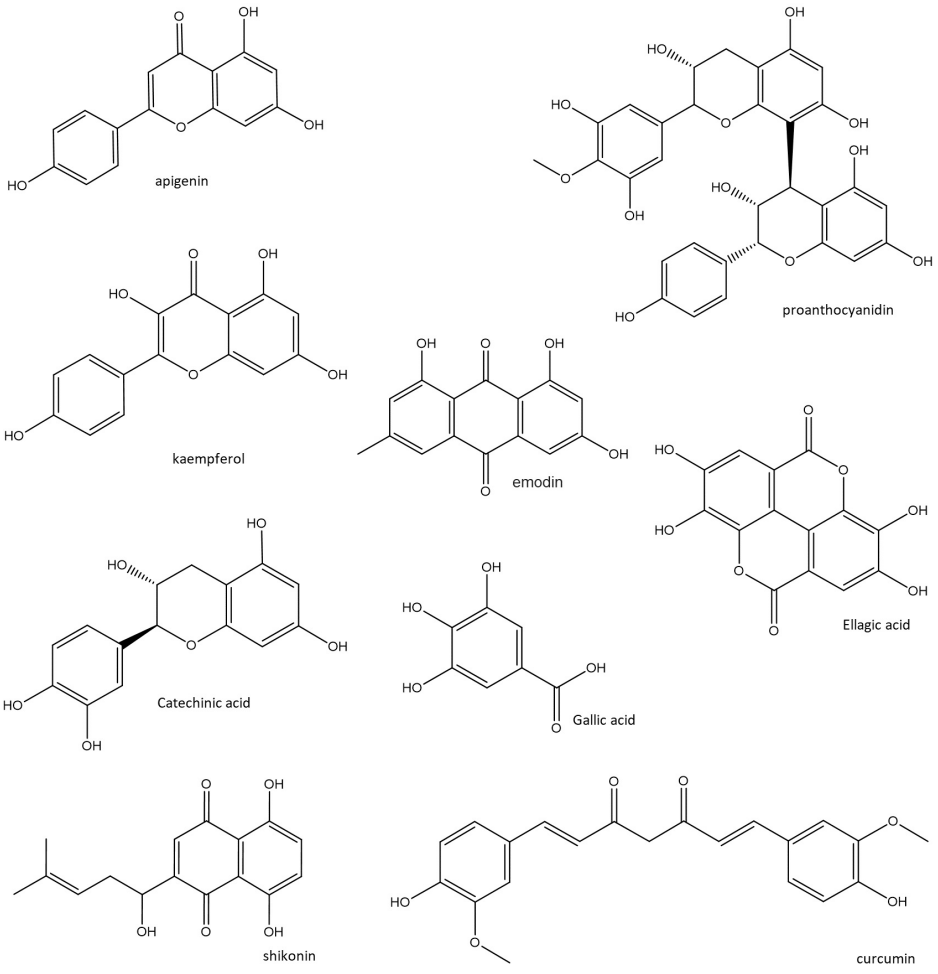

Fig. 2. Phenolic compounds.

The polyphenolic extract from quince (*Cydonia oblonga* Miller) has shown antiproliferative effects in kidney and colon cancer cells (Carvalho *et al*., 2010). A Tunisian research group reported that quince peel polyphenolic extract induced ROS production, and the cytotoxic effect of 5-FU was increased in human colon adenocarcinoma LS174 cell (Riahi-Chebbi *et al*., 2015). Although the potential expansion of the cellular work to a preclinical level requires further study, it is worth noting that ROS generation may be linked to the cytotoxicity of 5-FU. Riahi-Chebbi *et al*. (2019), conversely, reported that kaempferol, another phenolic compound derived from quince, inhibited the production of ROS while exhibiting the same cytotoxicity as other phenolic compounds and was effective even in 5-FU resistant colon cancer cells. This intriguing result cautions us not to assume that a decrease in ROS levels is cytoprotective, as other mechanisms may simultaneously be responsible for cytotoxicity.

Shikonin, a naphthoquinone derivative found in the shikonin plant (*Lithospermum erythrohizon*), is known for its cytotoxicity and anti-inflammatory activity (Chen *et al*., 2002). Similarly, Liang *et al*. (2016) studied the antitumor activity of shikonin on gastric cancer. They observed that shikonin induced ROS generation and enhanced the 5-FU sensitivity *in vitro* and *in vivo*. In addition to the mitochondria-mediated apoptotic pathway, they detected the caspase-independent nuclear translocation of the apoptosis-inducing factor and endonuclease G from mitochondria.

Proanthocyanidin compounds from white fig *Ficus virens* (Chen *et al*., 2017b) and *Uncaria rhynchophylla* (Chen *et al*., 2017c) have been shown to have cytotoxic activity on human breast cancer MDA-MB-231 cells. Proanthocyanidins increased cellular ROS and the mitochondrial apoptotic pathway, and synergistic cytotoxicity was observed when proanthocyanidins were combined with 5-FU. Surprisingly, the cytotoxic effect appeared to be cancer cell-specific, and proanthocyanidins alleviated intestinal mucositis in 5-FU-treated rats (Chen *et al*., 2017b).

Curcumin, a polyphenolic compound frequently found in curry powders, has long been considered an antioxidant (Ak and Gulcin, 2008). Several studies, however, have reported the generation of ROS by curcumin analogs. Researchers created B63, a curcumin analog, as an anticancer agent and discovered that B63 induced ROS-mediated paraptosis in gastric cancer cells (Chen *et al*., 2019). They showed the inhibition of thioredoxin reductase 1 (TrxR1) by B63 *in vitro*, and the overexpression of TrxR1 negated the proparaptotic activity of B63. Their findings indicate that TrxR1 is a target of B63 and that B63 effectively suppressed the growth of 5-FU-resistant gastric cancer cells. Similarly, dimethoxycurcumin increases ROS production in colon cancer cells, allowing it to exert cytotoxic activity against colon cancer SW480 and SW620 cells when combined with 5-FU (Zhao *et al*., 2017).

A Chinese research group studied the water extract of *Sanguisorba officinalis* L. radix, for its anticancer activity on human colorectal cancer HCT116 and RKO cells (Liu *et al*., 2016a). They demonstrated that treating cells with the extract and 5-FU significantly increased ROS generation and that cotreatment increased 5-FU cytotoxicity. Moreover, they reported an increase in autophagy-related markers, light chain LC3, and p62, besides ROS generation, implying that the generation of ROS is not the only explanation for the synergism between *Sanguisorba officinalis* L. radix and 5-FU. The study demonstrated that gallic acid, catechinic acid, and ellagic acid, three main constituents of *Sanguisorba officinalis* L. radix, are responsible for the synergistic activity.

Manuka honey, a type of honey collected from the manuka tree *Leptospermum scoparium*, has antioxidant, anti-inflammatory, and anticancer properties (Afrin *et al*., 2018b). Reports describe the synergistic cytotoxicity of manuka honey on human colon cancer HCT116 and LoVo cells when combined with 5-FU (Afrin *et al*., 2018a). Manuka honey, a polyphenol-rich natural product, suppressed cell survival signals in HCT116 and LoVo cells while inducing pro-apoptotic signals and ROS production. Furthermore, the combined treatment reduced the activity of antioxidant enzymes such as SOD, catalase, glutathione peroxidase, glutathione reductase, and the expression of Nrf2, SOD, catalase, and HO-1, resulting in increased cell death due to oxidative stress.

Emodin, a natural anthraquinone compound, has antiproliferative activity in human breast cancer MCF7 cells (Huang *et al*., 2007). In a later study, tests were conducted to determine whether low-dose emodin could potentiate the activity of 5-FU in MCF7 cells (Zu *et al*., 2018). Findings revealed that emodin increased 5-FU-induced apoptosis in breast cancer cells by generating ROS. Surprisingly, researchers observed cellular senescence after 5-FU treatment with emodin, which they believe was caused by the upregulation of cyclin-dependent kinase inhibitors and the downregulation of E2F1 and the notch-regulated ankyrin repeat protein (NRARP) protein. Their findings suggested that NRARP is a critical target for inducing cellular senescence.

Polycyclic compounds and alkaloids

Several polycyclic compounds and alkaloids (Fig. 3) have been investigated for their role in producing ROS in cancer cells. For instance, gypenosides are triterpenoid saponin compounds whose potential use in cancer treatment has been documented (Ahmad *et al*., 2019), and they are thought to have potentiated 5-FU's cytotoxicity (Kong *et al*., 2015). Results showed that p53 and ROS generation mediates the synergism between gypenosides and 5-FU to exert anticancer activity. Additionally, the triterpenoid saponin compound, tubeimoside-I, isolated from *Rhizoma Bolbostemmatis*, has exhibited antitumor activity in various types of tumors (Yu *et al*., 1994). Yan *et al*. (2019) discovered that combining 5-FU and tubeimoside-I suppressed the growth of colorectal cancer SW480, SW620, HCT116, and RKO cells in a synergistic manner, whereas tubeimoside-I induced cellular ROS and the activation of AMPK, resulting in cytotoxic autophagy.

Oridonin, a diterpenoid from the medicinal herb *Rabdosia rubescens*, exhibits antitumor activity (Li *et al*., 2011). Studies assessed oridonin's anticancer effect in colorectal cancer HCT15 cells and compared the 5-FU resistant HCT15 cells and sensitive cells (Zhang *et al*., 2019). To exert its cytotoxicity, oridonin induced the generation of ROS in both cells and the activation of the JNK/c-Jun pathway. Notably, cotreatment with N-acetylcysteine reversed JNK/c-Jun pathway activation, indicating that ROS generation mediates JNK/c-Jun pathway activation. Although oridonin activated apoptosis in colorectal cancer cells, it appears to activate necroptosis in renal carcinoma 786-O cells (Zheng *et al*., 2018). Cotreatment of oridonin and 5-FU showed synergistic cytotoxicity, probably through separate mechanisms, and notably, the same compound showed a different mechanism of action.

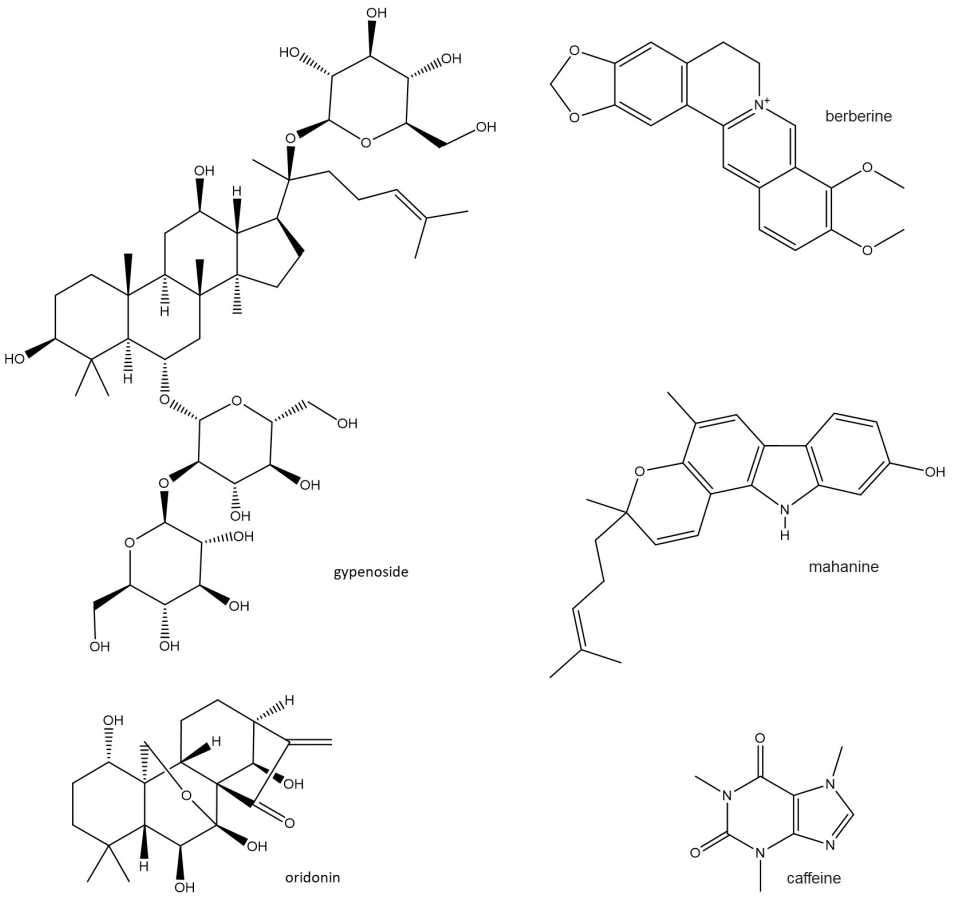

Fig. 3. Polycyclic compounds and alkaloids.

The anticancer effects of the *Coptis* herb extracts and the major alkaloid component berberine have been well-reported, and their cytotoxic effects have been detected in various cancer cell lines (Tang *et al*., 2009). Furthermore, *Coptis* extract showed cytotoxicity when combined with 5-FU in human lung cancer A549 cells (He *et al*., 2012). The cytotoxicity of either *Coptis* extract or berberine was associated with an increase in ROS generation in a dose-dependent manner, and when combined with 5-FU, the anticancer effect was enhanced.

Mahanine, an alkaloid from the curry leaf plant (*Murraya koenigii*), has exhibited various biological activities (Ramsewak *et al*., 1999). Das *et al*. (2014) showed the synergistic enhancement of cytotoxicity of 5-FU when mahanine was used together in human colorectal cancer HCT116 and SW480 cells. Interestingly, the synergistic effect was observed irrespective of p53 status, i.e., both $p53^{wt}$ and $p53^{null}$ cells were sensitive to mahanine in combination with 5-FU. Although the precise mechanism is unknown, mahanine induced ROS production and led to the accumulation of PTEN and p53 in the nucleus. The increased production of ROS appears to be linked to the activation of tumor suppressor proteins PTEN and p53, resulting in increased cytotoxicity of 5-FU.

Caffeine, a food ingredient found in coffee and tea, slows the growth of liver cancer cells, including HepG2, HLF, Huh7, and PLC/PRF/5 (Okano *et al*., 2008). Many studies report a synergistic effect of caffeine and cisplatin in various cancers, such as the human endometrial cancer cell line RL95-2 (Lin *et al*., 2021). Recently, Wang *et al*. (2019) reported that the antitumor activity of 5-FU was enhanced by cotreatment of caffeine in HCC HepG3 and SMMMC cells. They discovered that combining 5-FU and caffeine inhibited HCC cell growth and induced apoptosis by increasing ROS production.

Role of other small molecules in ROS production

As described below, reports suggest that other small molecules (Fig. 4) may modulate ROS generation in cancer cells. First, selenocystine is the oxidation product of selenocysteine, which has a diselenide bond connecting two amino acids. It induces apoptosis in human cancer cells such as A375, HepG2, and MCF7 by increasing ROS production (Chen and Wong, 2009). Fan *et al*. (2013) investigated whether selenocystine cotreatment could increase the cytotoxicity of 5-FU in human melanoma A375 cells. They observed significant selenocystine-induced DNA damage mediated by ROS production and the inactivation of the extracellular-signal-regulated kinase (ERK) and Akt signaling pathways, resulting in anticancer synergism. Furthermore, the induction of ROS-mediated apoptosis in melanoma cells by 3,3′-diselenodipropionic acid, a selenocysteine derivative, is another example of potentially overcoming anticancer drug resistance (Cao *et al*., 2014).

Allicin, a compound in garlic, has drawn considerable attention as an antimicrobial antioxidant (Chan *et al*., 2013). Zou

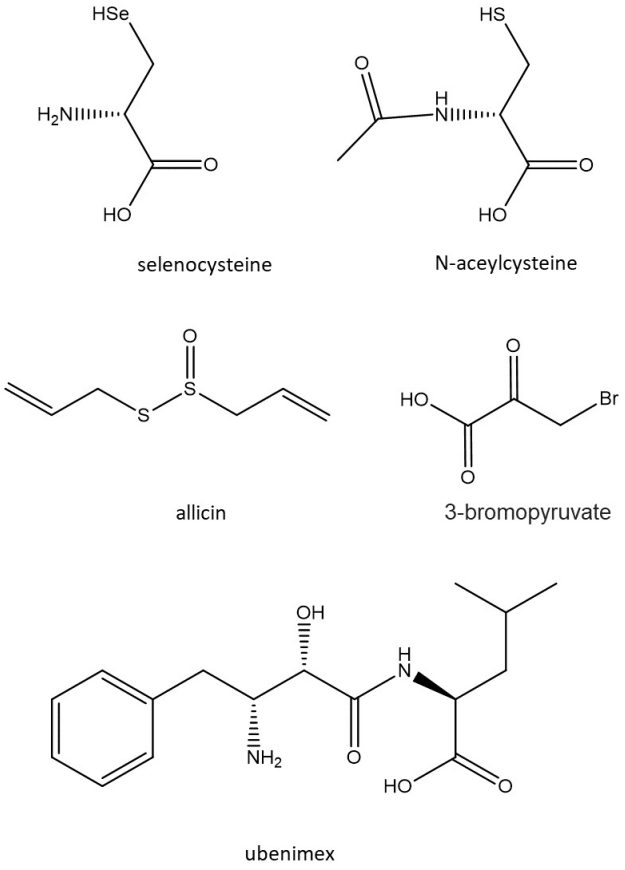

Fig. 4. Other small molecules.

et al. (2016) tested whether the anticancer activity of 5-FU in human HCC SK-Hep-1 and BEL-7402 cells and in nude mice increased with allicin and 5-FU cotreatment. They discovered that cotreatment with allicin increased ROS production and sensitization of HCC cells to 5-FU. The synergistic effect was reversed by N-acetylcysteine treatment, indicating that the anticancer activity is mediated by ROS generation. Their study also demonstrated that cotreatment with allicin and 5-FU significantly inhibited the growth of HCC xenograft tumors in nude mice; although commonly thought to be an antioxidant, allicin increased ROS generation when combined with combined 5-FU.

3-Bromopyruvate is an inhibitor of hexokinase (Ko *et al*., 2001), the key enzyme of glycolysis. The researchers reported that 3-bromopyruvate induced the ROS-mediated cell death of hepatoma SNU449 and Hep3B cells (Kim *et al*., 2008). Upon treatment with 3-bromopyruvate, both cell lines underwent necrosis and apoptosis in an ATP depletion-dependent manner due to increased intracellular ROS and the disruption of mitochondrial function. Furthermore, the combination of 3-bromopyruvate and 5-FU inhibited tumor growth *in vivo* and *in vitro* (Chong *et al*., 2017).

ENDOGENOUS CELLULAR TARGETS TO OVERCOME 5-FU RESISTANCE

Nuclear factor erythroid 2-related factor 2 (Nrf2)

The transcription factor Nrf2 mediates antioxidant response (Moi *et al*., 1994). Nrf2 exists in the cytoplasm as the Nrf2-Keap1 complex in the absence of oxidative stress. The cellular level of Nrf2 is kept low by continuous degradation via the ubiquitin–proteasome system, which is mediated by Keap1, the Nrf2 key repressor (Zhang, 2006). Several cysteine residues of Keap1 are modified when exposed to oxidative stress, resulting in the dissociation of the Nrf2-Keap1 complex. Nrf2, which is released by Keap1, enters the nucleus and binds to the DNA in the antioxidant response element (ARE) region to regulate the expression of several genes involved in antioxidant function, such as glutamate-cysteine ligase catalytic subunit (Solis *et al*., 2002), thioredoxin reductase (Soriano *et al*., 2009), and heme oxygenase-1 (HO-1) (Jarmi and Agarwal, 2009). When expressed, these antioxidants may impart some degree of protection to cells under oxidative stress. Overexpression of Nrf2 in gastric cancer serves as a prognostic marker for 5-FU resistance, lending credence to Nrf2's prosurvival role (Hu *et al*., 2013). Similarly, Nrf2 has a role in developing 5-FU resistance in colon cancer HT-29 cells (Akhdar *et al*., 2009). Kang *et al*. (2014) discovered hypomethylation of Nrf2 promoter CpG islands in 5-FU resistance colorectal cancer SNU5/5-FUR cells compared with nonresistant cancer cells, indicating that Nrf2 upregulation led to 5-FU resistance.

Besides its antioxidant function, Nrf2 regulates the expression of drug-metabolizing enzymes and drug transporters, resulting in a decrease in 5-FU efficacy (Bai *et al*., 2016). A team of researchers reported that 2′,4′-dihydroxy-6′methoxy-3′,5′-dimethylchalcone, an inhibitor of Nrf2/ARE pathway, could reverse 5-FU resistance in HCC BEL-7402 cells by inhibiting the 5-FU efflux (Wei *et al*., 2018).

ROS/mitogen-activated protein kinases pathway

JNK, c-Jun N-terminal kinase, belongs to MAPKs. The function of JNK is related to both cell survival (Wu *et al*., 2019) and death (Dhanasekaran and Reddy, 2008). Based on the stimuli, JNK signaling can be either prosurvival or pro-apoptotic, and the signaling pathway is not directly linked to the cytotoxic effect of 5-FU. It appears that either activation or inactivation of the proper signaling pathway could place an additional burden on cells treated with 5-FU, potentially increasing 5-FU cytotoxicity.

Compared with differentiated and chemosensitive pancreatic cancer stem cells, the JNK signaling pathway is activated in pancreatic cancer stem cells (Okada *et al*., 2014; Suzuki *et al*., 2015). Researchers established that the JNK signaling pathway is activated in the pancreatic cancer stem cells (Suzuki *et al*., 2015). Pretreatment of cells with SP600125, a JNK inhibitor, resulted in the sensitization of the cells to 5-FU and gemcitabine. The cytotoxic effects of these chemotherapeutics were accompanied by an increase in ROS production. Furthermore, the use of N-acetylcysteine, a free radical scavenger, reduced the intracellular level of ROS and allowed the cells to remain resistant to 5-FU; this is an example of the detrimental use of an antioxidant in chemotherapy. The synergistic cytotoxicity of 5-FU and the compounds mentioned above, tetrathiomolybdate (Kim *et al*., 2012) and oridonin (Zhang *et al*., 2019), is associated with the generation of ROS and the

activation of JNK. 5-FU cytotoxicity appears to be enhanced by oxidative stress and JNK activation, potentially overcoming 5-FU resistance.

Similarly, coronarin D, a diterpene compound derived from grapes, has anticancer activity. *Zingiberaceae* (Bailly, 2020) is involved with the activation of JNK signaling and ROS generation, as shown in human nasopharyngeal cancer cells (Chen *et al*., 2017a). It was recently reported that coronarin D induces the apoptosis of 5-FU resistant human oral cancer cells. The cytotoxicity is related to the JNK signaling pathway (Hsieh *et al*., 2020).

Besides JNK signaling, activation of p38 MAP kinase is linked to 5-FU cytotoxicity. According to Xie *et al*. (2016), the overexpression of nicotinamide N-methyltransferase causes a decrease in ROS levels, and the inactivation of p38 signaling is involved in 5-FU resistance in colorectal cancer SW480 cells. Moon *et al*. (2020) reported that the activation of p38 by yeast extract resulted in the antitumor effect on 5-FU resistant colorectal cancer SNU-C5 cells.

Nevertheless, the role of the ERK pathway in 5-FU sensitivity appears to be prosurvival. Kim *et al*. (2016) discovered ERK overexpression in SUNC5/FUR cells, resistant to 5-FU. Furthermore, sensitization was achieved by transfecting 5-FU resistant cells with siRNA against ERK. The role of aluminum chloride in inducing 5-FU resistance was further investigated, revealing that ERK activation facilitated the survival of HCC HepG2 cells during 5-FU treatment (Li *et al*., 2019a). By contrast, U0126, an ERK inhibitor, reversed aluminum chloride-induced 5-FU resistance; it also remains unclear whether ERK activation is required for cells to remain resistant to 5-FU. A Japanese group, for example, reported a 5-FU resistant human squamous carcinoma UM-SCC-23 cell line that activated both ERK and Akt signals (You *et al*., 2009). Nevertheless, U0126 could not reverse the resistance, whereas Akt inhibition was. Furthermore, Wang *et al*. (2017b) reported that 5-FU resistance caused by ADAM12 overexpression increased phosphorylated Akt but not phosphorylated ERK in breast cancer SKBR3 MDA-MB-231 cells.

PI3K/Akt pathway

Phosphatidylinositol 3-kinase (PI3K)/Akt pathway is considered one of the key signaling pathways that confers cancer cells' resistance to chemotherapy (Liu *et al*., 2020). Researchers reported the constitutive activation of Akt signaling in 5-FU resistant squamous carcinoma UM-SCC-23 cells (You *et al*., 2009). By contrast, the inhibition of Akt signaling was noticed when the synergistic cytotoxicity of 5-FU was observed by the cotreatment of several compounds, including violacein (Kodach *et al*., 2006), curcumin (Zhang *et al*., 2017), and kaempferol (Li *et al*., 2019b). Recent reports state that celecoxib, a COX-2 inhibitor, induced apoptosis by inhibiting Akt in 5-FU resistant gastric carcinoma AGS cells (Choi *et al*., 2021). The inhibition of COX-2 is thought to have resulted in the downregulation of Akt and the induction of apoptosis. MK-2206's direct inhibition of Akt also increases the cytotoxicity of 5-FU in gastric cancer SGC-7901 and MKN45 cells (Jin *et al*., 2016). Hence, it remains to be confirmed whether MK-2206 could be used to treat 5-FU resistant cells.

Autophagy pathway

Autophagy, a self-eating process involving the autophagosome (Glick *et al*., 2010), has two states, either prosurvival or prodeath, depending on the mode of activation and which cells are affected. Initially, the prosurvival role of autophagy was reported because inhibition of autophagy was associated with increased cytotoxicity of 5-FU in human colon cancer colon26 and HT29 cells *in vitro* and *in vivo* (Li *et al*., 2010). The prosurvival role of autophagy was again shown in HCT116 $p53^{-/-}$ cells (Sui *et al*., 2014) and human HCC Bel-7402 cells (Wang *et al*., 2017a). The involvement of prosurvival autophagy in 5-FU resistance has been demonstrated for several cellular components such as TSPAN9 (Qi *et al*., 2020) and claudin-1 (Tong *et al*., 2019). Zhang *et al*. (2017) reported synergistic cytotoxicity from the combination of curcumin and 5-FU, which included a reduction in prosurvival autophagy mediated by AMPK and Unc-51 Like Autophagy Activating Kinase 1 (ULK1). By contrast, several compounds have been shown to increase the cytotoxicity of 5-FU by inducing prodeath autophagy. β-Elemene, a sesquiterpene compound found in various plants, induces prodeath autophagy in 5-FU resistant colorectal cancer HCT116 $p53^{-/-}$ cells (Zhang *et al*., 2020). Similarly, when combined with 5-FU, withaferin-A, a natural product with a steroidal lactone structure, induces endoplasmic reticulum stress-mediated autophagy (Alnuqaydan *et al*., 2020) in CRC cells (SW480, HT29, HCT116).

Aminopeptidase N (CD13)

Aminopeptidase N, also known as CD13, is a cell-surface-anchored zinc peptidase with various functions, including peptide cleavage, endocytosis, and signaling (Mina-Osorio, 2008). It was initially identified as a cell surface marker CD13 for myeloid leukemia cells (Sakai *et al*., 1987), and the signaling function of aminopeptidase N appears to be independent of enzyme activity. Aminopeptidase expression has been linked to a poor prognosis and angiogenesis in cancer cells such as nonsmall cell lung cancer (Tokuhara *et al*., 2006), pancreatic carcinoma (Ikeda *et al*., 2003), and colon cancer (Hashida *et al*., 2002). When substrates or inhibitors bind to CD13, the conformation of the dimeric CD13 structure changes, resulting in signal transduction (Xu *et al*., 1997), and CD13 protects cells from apoptosis by reducing ROS-induced DNA damage.

Ubenimex, also known as bestatin, is a dipeptide compound produced by actinomycetes (Umezawa *et al*., 1976). It specifically blocks and antagonizes CD13 (Look *et al*., 1989). Haraguchi *et al*. (2010) reported that ubenimex or a CD13-neutralizing antibody inhibited CD13 in hepatocellular cancer HuH7 cells. They revealed that combining 5-FU and ubenimex increased ROS production and improved liver cancer therapy. Additionally, Dou *et al*. (2017) investigated the therapeutic potential of BC-02, a conjugation compound of ubenimex and 5-FU, in the successful inhibition of the growth and self-renewal of liver cancer stem cells. Similarly, Sun *et al*. (2015) reported that 4cc, a synthetic inhibitor of aminopeptidase N, could increase 5-FU cytotoxicity by generating ROS in human liver cancer HCC cells. It remains to be seen whether CD13 inhibitors can be used to treat cancer cells other than HCC.

CONCLUSION

In this study, we have compiled a list of compounds that can be used alone or combined with 5-FU to modulate ROS generation. Although the precise mechanisms underlying their cytotoxicity remain unknown, several endogenous cellular tar-

gets have been identified, as described above. These compounds and cellular targets could help develop new strategies for combating 5-FU resistance.

CONFLICT OF INTEREST

The authors claim no conflicts of interest.

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