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Review Article

Pharmacological mechanisms of norcantharidin against hepatocellular carcinoma

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Abstract: Norcantharidin (NCTD) is a water-soluble synthetic small molecule drug that has been approved by the Chinese FDA for the treatment of cancer in China. Among these NCTD-treated cancers, hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide and one of the most extensively studied. Research over the past few decades has made great strides in understanding how NCTD induces mitotic arrest, anti-proliferation, anti-metastasis, apoptosis and cytotoxic autophagy or autophagic cell death in HCC. In this article, we review recent progress in the application of NCTD for the treatment of HCC, with emphasis on the pharmacological mechanism of NCTD against hepatocellular carcinoma. The accumulated results show that NCTD has the ability to induce mitotic arrest, anti-proliferation, anti-metastasis, apoptosis and cytotoxic autophagy or autophagic cell death in HCC by down-regulating the expression of ISG15, MMP-9, u-PA, Mcl-1 and the accumulation of regulatory T cells, up-regulating the expression of FAM46C, miR-214 and the expression and phosphorylation of $p21^{Cip1/Waf1}$ and CDC25C, and by inhibiting the c-Met-mTOR and JAK/STAT3 signaling pathways, reversing the methylation of RASSF1A gene, and activating TRAIL-R2/DR5 signal transduction.

Keywords: Norcantharidin (NCTD), anti-hepatocellular carcinoma, anti-proliferation, apoptosis, anti-metastasis, cytotoxic autophagy, mechanisms

Introduction

Norcantharidin (NCTD, exo-7-oxabicylo-[2.2.1] heptane-2,3-dicarboxylic anhydride) is a water-soluble synthetic small molecule (**Figure 1**) and is FDA approved in China for the treatment of cancer [1]. It is a demethylated analogue of cantharidin (CTD, 7-oxabicyclo-[2.2.1] heptane-2,3-dicarboxylic acid) (**Figure 1**) [2]. CTD is a natural compound isolated from 1,500 species of medicinal insect called the blister beetle (mylabris phalerata Pallas) [3]. In China, the use of mylabris (specifically, the dried body of the Chinese blister beetle) as a traditional medicine can be dated back more than 2,000 years, and today it is still used in traditional Chinese medicine [2]. The most important medicinal effect of CTD is its anticancer activity [2, 4, 5]. However, the use of CTD has limits due to its toxicity to the kidney, gastrointestinal tract and urinary tract.

In order to address the toxicity of CTD, NCTD was synthesized for use in cancer therapy [2, 6]. At present, there is limited information on the toxicological effects of NCTD and little data on its safety evaluation [7]. A few sporadic studies reported that NCTD can markedly inhibit lymphocyte proliferation stimulated by mitogen canavalin or *lipopolysaccharide (*LPS) in vitro, and inhibit the mixed lymphocyte reaction (MLR) of mice in vivo in a dose-related manner [8], as well as induce dose and circadian time-dependent transient leucocytosis in normal mice [9], stimulate the bone marrow production of white cells [6], suppress plasma vascular endothelial growth factors (VEGFs) levels of tumor-bearing mice [10], cause hypoglycemia by interfering with glucose metabolism [11] and is genotoxic to human cultured lymphocytes as measured by sister chromatid exchanges (SCEs) assay [12]. In addition, serum meta-

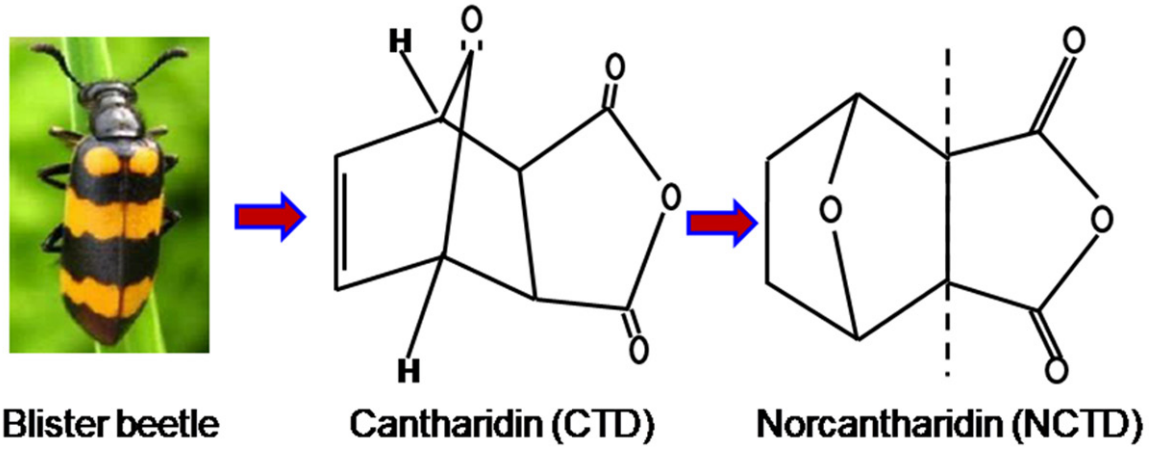

Figure 1. The molecular formula of CTD and NCTD. NCTD (a molecular formula of $C_8H_8O_4$ and with formula weight of 168.15 g/mol) is the demethylated analog and the low-cytotoxicity derivative of CTD with antitumor properties. (CTD = Cantharidin, 7-oxabicyclo-[2.2.1] heptane-2,3-dicarboxylic acid; NCTD = Norcantharidin, exo-7-oxabicylo-[2.2.1] heptane-2,3-dicarboxylic anhydride).

bolic panel revealed dysregulation of metabolic analytes that suggest mainly liver damage, and to a lesser extent, damage in renal tissue [7, 13]. However, further systematic studies of the side effects or adverse reactions of NCTD is absolutely necessary to clarify the toxicity of NCTD for clinical application.

Like CTD, NCTD has been shown to be an effective anticancer drug and has been used in China for many years [2, 4]. Several studies have shown that NCTD can effectively inhibit the proliferation of and/or induce apoptosis of various types of cancer cells, such as multiple hepatocellular cancer cells [14-27], neuroblastoma cells [6], glioma cells [28], medulloblastoma cells [29], cholangiocarcinoma cells [30], colorectal cancer cells [1, 31-33], osteosarcoma cells [34, 35], GCTB (giant cell tumor of bone) stromal cells [36], prostate cancer cells [37], bladder cancer cells [38], breast cancer cells [39], nonsmall cell lung cancer (NSCLC) cells [40], human renal cell carcinoma cells [41-43], pancreatic cancer cells [44], and also suppress tumor growth and/or cancerometastasis, as well as prolong survival in animal models in vivo, including of hepatocellular carcinoma (HCC) [18, 22-24, 45, 46], medulloblastoma (intra-cerebellum tumors in orthotopicxenograft nude mice) [32], cholangiocarcinoma, colorectal cancer, osteosarcoma, prostate cancer, OSCC *(oral squamous cell carcinoma)* and breast cancer [29, 34, 37, 39]. However, the underlying mechanism of its action is still not fully understood.

Among these NCTD-treated cancers, HCC is the third leading cause of cancer-related death worldwide [22, 47] and is one of the most extensively studied. Accumulated data show that NCTD can induce mitotic arrest and antiproliferation, apoptosis, cytotoxic autophagy or autophagic cell death and inhibit epithelial-mesenchymal transition (EMT) and cell invasions in vitro in BEL7402 [17], H22 [18], HCCLM3 [21], Hep3B [14, 16, 25], HepG2 [14, 17, 19, 20, 22, 23], Huh7 [14, 15, 17, 48], MHCC97-H [22, 25, 47], MHCC97-L [25], SK-Hep-1 [24], and/or SMMC-7721 [17, 20, 21, 24, 47], as well as inhibit angiogenesis and growth of liver cancer tumors in mice with HCC [18, 23], repress the growth of human HepG2 cell graft tumors and prolong host survival in nude mice in vivo. Here, we review recent progress made in studies on the application of NCTD in the treatment of HCC, with emphasis on the pharmacological mechanism of NCTD against hepatocellular carcinoma.

Mechanisms underlying anti-hepatocellular carcinoma effect of NCTD

Downregulating ISG15 (Interferon-stimulated gene 15) expression

ISG15 is one of the proteins induced by type 1 IFNs (interferons), which plays an important role in innate immunity against viral and bacterial infections [49]. It is an ubiquitin-like protein that conjugates to many cellular proteins via isopeptide bonds [50]. ISG15 is expressed and

conjugated in IFN- and LPS (lipopolysaccharide)-stimulated cells, suggesting that ISG15 binding may be an important component in mediating innate immune responses [51]. Specific changes in the ISG15 pathway have been found in bladder cancer [52], prostate cancer [53], breast cancer [54], colorectal cancer [55], nasopharyngeal carcinoma [56] and oral squamous cell carcinoma [48, 57].

ISG15 has also been shown to be highly expressed in HCC tissues and multiple HCC cell lines [58]. Detection of ISG15 expression shows that ISG15 is significantly over-expressed in pairs of HCC and adjacent non-tumor tissues, as well as Hep3B, Huh7, MHCC97-H, MHCC97-L HCC cells [25]. The expression of ISG15 is significantly correlated with the degree of differentiation, tumor metastasis, and survival in patients with HCC. ISG15 promotes the proliferation and migration of HCC cells by maintaining the stability of survivin protein via isolation of X-linked apoptosis inhibitors (XIAP) with survivin interactions [25, 58]. Down-regulation of ISG15 by SiRNA inhibits the growth of xenograft tumors and prolongs the life span of tumor bearing mice [58]. High expression of ISG15 is an intrinsic feature of HCC and a trigger for tumorigenesis and metastasis [25, 58].

Down-regulation of ISG15 by transfection with small interfering RNA can improve the sensitivity of HCC cells to NCTD [25]. NCTD significantly up-regulates the expressions of Bax, caspase-3 and caspase-9, and down-regulates the expression of Bcl-2, while down-regulated ISG15 significantly increases the effect of NCTD on the expression of these apoptosis-related proteins in HCC cells [25]. In addition, overexpression of ISG15 significantly inhibits the expressions of Bax, caspase-3 and caspase-9, and promotes the expression of Bcl-2. Furthermore, NCTD could induce a significant decrease in cell proliferation and colony formation of IsG15-overexpressed HCC cells, reversing the tumor promoting effect of ISG15-overexpression [25]. These findings imply that the anti-tumor effect of NCTD is in part mediated by down-regulation of ISG15 in HCC cells in vitro.

Repressing c-Met-mTOR signaling pathway

Mesenchymal epithelial transition factor (c-Met), a unique disulfide-linked heterodimer, acts as a receptor tyrosine kinase for hepatocyte growth factor (HGF) [59]. Abnormal activation of c-Met signaling is responsible for the growth, progression, invasion, and poor prognosis of various tumor types, including HCC [60]. c-Met overexpression is often observed in HCC patients [61]. Binding of HGF to c-Met induces homodimerization and autophosphorylation of the receptor, leading to downstream phosphorylation of various key signaling proteins, including mammalian target of rapamycin (mTOR) [62].

It has been found that NCTD is able to induce cell death, as reflected in reduced cell viability, by triggering high levels of autophagy in human MHCC-97H (97H) and HepG2 HCC cells in vitro, and significantly reducing tumor growth in HepG2 tumors in vivo [22]. NCTD-induced cytotoxic autophagy correlates with a reduction in the phosphorylation status of both c-Met and m-TOR. By inhibiting the c-Met/mTOR signaling pathway, NCTD enhances cytotoxic autophagy, thereby reducing the activity of HCC and inhibiting the growth of HCC tumors [22]. Therefore, inhibition of the c-Met signaling pathway may represent a therapeutic approach for the treatment of HCC [22, 63].

Downregulating accumulation of regulatory T cells

Recruitment of regulatory T cells (Tregs) is one of the mechanisms of immune system dysfunction, which is a characteristic of the HCC tumor microenvironment [64]. Tregs have been shown to reduce host anti-tumor response, and the presence of Tregs in peripheral blood and tumor microenvironment is associated with poor prognosis in HCC patients [65]. The frequency of Tregs in HCC patients is higher [66, 67]. Knockdown of TGF-β1 (transforming growth factor-β1) reduces the number of Tregs and metastatic nodules in mice, suggesting that HCC cells induce Tregs production by secreting TGF-β1 [67]. In HCC patients, increased Tregs are associated with CD8 T-cell damage and poor survival, implying that Tregs may play a key role in controlling CD8+ cytotoxic T-cell activity, thereby promoting HCC progression [66].

It has been reported that Ganoderma lucidum polysaccharide extract (GLPS) significantly inhibits HCC growth in hepatoma-bearing mice by

down-regulating the accumulation of Tregs and eliminating the inhibitory effect of Tregs on the proliferation of effector T cells (Teffs) [68]. In addition, NCTD (+CLSO, Coixlacryma-jobi seed oil) exhibits strong antitumor effects on human HepG2 and HepG2/ADM cells in vitro in terms of cytotoxicity and induction of apoptosis, and significantly inhibits tumor formation in vivo in Hepal1 hepatoma-bearing mice [23]. NCTD (+CLSO) effectively down-regulates the increase of CD4+, CD25+ T cells in co-culture of HepG2 or HepG2/ADM cells with peripheral blood mononuclear cells (pmcs) from healthy donors in vitro, and the amount of Tregs in HePAL-1 hepatoma-bearing mice, as well as down-regulates the expression of FoxP3 (Forkhead Box P3), CTLA-4 (cytotoxic T lymphocyte-associated antigen-4) and Tregs-associated cytokines (TGF-β and IL-10) in serum of tumor-bearing mice in vivo [23]. These results suggest that NCTD enhances anti-tumor activity by inducing apoptosis and inhibiting HCC growth via down-regulating accumulation of regulatory T cells [23]. Therefore, targeting the number and function of Tregs may be an effective approach for HCC therapies.

Suppressing JAK/STAT3 signaling

JAK2 (Janus kinase 2), a member of the JAK family of protein tyrosine kinases, plays a variety of functions in carcinogenesis [69]. STAT3 (signal transducer and activator of transcription 3) is considered to be a critical transcriptional activator of cell survival genes and cell cycle, and its phosphorylation may be related to HCC tumor progression [70]. It has been reported that activation of the JAK2/STAT3 pathway is associated with the facilitated tumorigenesis, proliferation, invasion and migration, and/or EMT induced by lncRNAs (Long non-coding RNA) [71], accelerated invasion by NLRC3 (Nucleotide-binding domain, leucine-rich repeat family with a caspase activation and recruitment domain 3) [72], promoted cell proliferation, migration and/or invasion induced by B7-H3 (B7-homologue 3, a recently identified immunoregulatory protein) [73], CEP55 (centrosome protein 55 kDa) [74], IL-8 (interleukin 8) [75] and IL-9 (interleukin 9) [76]; as well as miRNA-21 (microRNA-21-5p)-mediated short term recurrence [77] and AKT (protein kinase)-dependent tumor progression by IL-17 (interleukin-17) [78] in hepatocellular carcinoma both in vitro and in vivo.

In contrast, inhibition of JAK2/STAT3 signaling pathway has been shown to be one of the important mechanisms underlying the anti-tumor effects of Icaritin [79], ANGPTL1 (angiopoietin-like protein) [80], antrodia camphorata mycelia [81], FOIs (fused oxazepino-indoles) [82], quercetin [83], and scutellarin [84] in hepatocellular carcinoma. Both NCTD [21] and CTD [85] have been found to play an anti-hepatocellular carcinoma role by inhibiting the JAK2/STAT3 signaling pathway. Observation of HCC patient samples, nude mouse models, and HCC cell lines (HepG2, Hep3B, and SMMC-7721 cells) suggests that CTD is a novel inhibitor of EphB4 (erythropoietin-producing hepatoma B class 4, a promoter of oncogenesis and tumor development and progression), while JAK2 and STAT3 are novel downstream signaling targets of EphB4 [85]. CTD can bind to EphB4, resulting in inhibition of EphB4 at the mRNA and protein levels, subsequently down-regulating the JAK2/STAT3 signaling pathway. Therefore, inhibition of CTD on HCC development by repressing the JAK2/STAT3 pathway is an EphB4-dependent process [85]. NCTD has been shown to be capable of significantly inhibiting IL-6-induced EMT and cell invasiveness by suppressing the JAK/STAT3 signaling pathway in a recent study using cell line models (HCC cell lines: HCCLM3 and SMMC-7721) [21]. These findings support the notion that targeting the JAK2/STAT3 signaling pathway may also be a useful and effective approach for HCC therapy.

Up-regulating FAM46C (family-with-sequence-similarity-46C)

The FAM46 protein is encoded in all known animal genomes, belongs to the nucleotide transferase (NTase) folding superfamily, and is classified as an active non-canonical poly(A) polymerase [86]. All four human FAM46 paralogs (FAM46A, FAM46B, FAM46C, FAM46D) are thought to be involved in several diseases [86]. It has been reported that FAM46C, a member of the FAM46 family, is a potential marker and tumor suppressor for multiple myeloma [87]. Point mutations in FAM46C have been associated with short progression-free survival and reduced overall survival in multiple myeloma cases. FAM46C deletion may promote cell survival, while overexpression induces substantial cytotoxicity in multiple myeloma [87]. The FAM46 gene is also believed to be involved in the development of other major

malignancies, including lung, colorectal, hepatocellular, head and neck, urothelial, endometrial and renal papillary cancers and melanoma [86].

NCTD could significantly enhance the expression of FAM46C in colorectal cancer tissues and cells where the expression of FAM46C is reduced [33]. Both NCTD and over-expression of FAM46C can increase caspase 3 cleavage and apoptosis, inhibit glycolysis, and decrease the expression of tumor-specific pyruvate kinase M2 and phosphorylated ERK, while FAM46C knockout can significantly weaken the effect of NCTD [33]. These observations indicate that FAM46C may be an intermediate factor of NCTD promoting apoptosis and inhibiting glycolysis in colorectal cancer cells. The expression of FAM46C in HCC tissues is also decreased, and is significantly lower than that in normal liver tissues [24]. Several studies have shown that NCTD could significantly enhance the expression of FAM46C in HCC tissues in vivo [24] and SMMC-7721 and MHCC-97H HCC cells in vitro [47].

In mice in vivo, NCTD and overexpression of FAM46C significantly inhibit diethylnitrosamine (DEN)-induced HCC [24]. In HCC cell lines in vitro, FAM46C overexpression inhibits cell proliferation and increases G2/M phase cell population and apoptosis rate, resulting in significant decreases in Ras expression, MEK1/2 phosphorylation and ERK1/2 phosphorylation in SMCC-7721 and SK-Hep-1 cells [24], as well as suppressing cell migration and invasion by inhibiting TGF-β/Smad signaling pathway and EMT process in SMMC-7721 and MHCC-97H cells [47]. These anti-proliferation, anti-metastasis and pro-apoptotic effects of NCTD were significantly weakened by knockdown of FAM-46C [24, 47]. This suggests that the anticancer function of NCTD in HCC is mediated in part by up-regulation of FAM46C. The results also suggest that FAM46C may play a cancer-suppressing role not only in colorectal cancer and multiple myeloma, but also in HCC.

Reversing the methylation state of RASSF1A gene

RASSF1A (Ras-associated domain family 1A) is one of the tumor suppressor proteins [88, 89]. The RASSF1A promoter is highly hypermethylated in lung, breast, ovarian, and thyroid carcinomas [90, 91]. Abnormal promoter methylation is the basic mechanism of inactivation of tumor suppressor genes in cancer [88]. Reinserting RASSF1A into different cancer cell lines inhibits tumor development both in vivo and in vitro [90, 91]. RASSF1A has many physiological functions, such as regulating cell cycle, microtubule stability and controlling cell apoptosis [89] and also plays important roles in tumor occurrence and development [92]. Studies have shown that widespread inactivation of RASSF1A in liver cancer is necessary for disease progression; therefore, severe loss of RASSF1A may promote metastasis and recurrence of dormant liver cancer [93].

Inactivation of RASSF1A due to methylation is a common event, and hypermethylation of RASSF1A can be used as a marker for HCC [88, 89, 94]. RASSF1A protein deletion or abnormal downregulation and promoter hypermethylation plays an important role in the occurrence, development, and metastasis of liver cancer [94]. In human HepG2 cell lines, NCTD significantly inhibits cell proliferation and RASSF1A methylation levels, and increases RASSF1A mRNA and protein levels in a dose-dependent manner [19]. NCTD inhibits cell proliferation by reversing the methylation state of RASSF1A gene and up-regulating the expression of RASSF1A in HepG2 cells.

Inhibiting Mcl-1 transcriptional expression

Myeloid cell leukemia-1 (Mcl-1) protein is an important pro-survival member of the B-cell lymphoma 2 (Bcl-2) protein family, which plays a critical role in the regulation of the intrinsic pathway of apoptosis and is associated with the progression and drug resistance of multiple cancers [95]. Therefore, effective and selective inhibition of Mcl-1 induced apoptosis has become a widely accepted anticancer strategy [96]. Multiple studies have shown that Mcl-1 is generally highly expressed in HCC and other malignant tumors [97].

NCTD significantly reduces the levels of Mcl-1 in human kidney [41] and prostate cancer cells [37]. In HCC cell lines, NCTD inhibits the expression of Mcl-1 by transcriptional repression to enhance cytoplasmic release of cytochrome C, the cleavage of caspase-9 and caspase-3 induced by ABT-737 (a small molecule cell-permeable Bcl-2/Bcl-xL antagonist). NCTD can

induce apoptosis and enhance the inhibitory effect of ABT-737 on cell proliferation by activating the mitochondrial apoptosis signaling pathway; thus, enhancement of ABT-737 can induce apoptosis of hepatoma cells. Therefore, NCTD combined with ABT-737 can overcome the drug resistance of ABT-737 and enhance the therapeutic efficacy of ABT-737 in the treatment of human HCC [17, 20].

Increasing miR-214 expression

It has been reported that miR-214 (microRNA-214) is one of the most significantly downregulated miRNAs in the patients with HCC [98]. Kaplan-Meier analysis shows that the 3-year survival rate of the high miR-214 expression group is increased compared with that of the low miR-214 expression group [61.70% (29/47) vs. 30.00% (12/40); χ^2 = 6.928; P = 0.008] after TACE (transcatheter arterial chemoembolization) treatment [99], indicating that high expression of miR-214 is associated with high survival rate. As a tumor suppressor, MiR-214 plays a substantial role in inhibiting HCC tumorigenesis by suppressing its target genes, including β-catenin [98], HDGF (hepatoma-derived growth factor) [100], FGFR1 (fibroblast growth factor receptor 1) [101], lncRNA PVT1 (long noncoding RNAPVT1) [102], PDK2 (pyruvate dehydrogenase kinase 2) and PHF6 (plant homeodomain finger protein 6) [103].

All these target genes of MiR-214 have been demonstrated to be over-expressed in HCC and contribute to the pathophysiology of various cancers, including HCC [104-106]. NCTD is shown to drastically impair tumor growth in mice with liver cancer by significantly increasing miR-214 expression and then inhibiting β-catenin expression, which is associated with increased anti-tumor activity of TAMs (tumor-related macrophages) [18]. The inhibition of β-catenin expression by NCTD could be reversed by miR-214 inhibitors, and H22 cell survival and invasion could be inhibited by conditioned medium derived from TAMs or transfected with pre-miR-214 in HCC mice treated with NCTD. In addition, NCTD has been found to induce changes in the HCC microenvironment, which manifest as decreased M2 polarization to M1 polarization and reduced numbers of CD4+/CD25+Foxp3 T cells [18]. These findings reveal the novel role of NCTD in inhibiting HCC by regulating macrophage polarization through miR-214.

Activating TRAIL-R2/DR5 signal transduction

Death receptors (DRs) are members of the TNF (tumor necrosis factor) receptor superfamily and are characterized by a cytoplasmic region known as the “death domain” that enables the receptor to initiate cytotoxic signaling when it is involved by a homologous ligand [107]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an important cytokine that can induce apoptosis [108]. The death receptor pathway is one of the main biochemical pathways that induce apoptosis [16], and apoptosis can be initiated by binding extracellular death signaling proteins (including TRAIL) to their homologous DRs (including TRAIL-R2/DR5) [109]. TRAIL has been considered as an agent for cancer treatment because of its ability to induce apoptosis in transformed cells [110], and DR5 as an important target for development of anti-cancer drugs against triple-negative breast cancer (TNBC) [111]. In human brain tumors, the TRAIL-DR5 system not only acts as an inducer of apoptotic cell death, but also acts as a sensor for pro-inflammatory and angiogenic signals [112].

In hepatoma cell line Hep3B, NCTD is found to inhibit cell proliferation in a dose- and time-dependent manner [16], consistent with findings reported in the same cell [14]. Low concentrations of NCTD induces cell cycle arrest in G_2M phase, while high concentrations of NCTD induced cell cycle arrest in G_0G_1 phase [16], observations similar to those reported by other studies of NCTD-induced cell cycle arrest at G_2M phase in glioblastoma cells and colorectal cancer [31]. In addition, pretreatment with inhibitors of caspase-3 or caspase-10 could almost completely inhibit DNA fragmentation in NCTD-treated Hep3B cells, while inhibitors of caspase-2, caspase-8 and caspase-9 could not [16]. These results indicate that NCTD induces apoptosis mainly by activating caspase-3 and caspase-10 [16]. Furthermore, although TRAIL-R1/DR4 and TRAIL-R2/DR5 proteins are both expressed in Hep3B cells treated with NCTD, only TRAIL-R2/DR5 neutralizing antibody could block the NCTD-induced apoptosis event of Hep3B cells. This indicates

that TRAIL-R2/DR5, but not TRAIL-R1/DR4, is involved in the NCTD-induced apoptosis pathway of Hep3B cells [16]. These results suggest that NCTD may inhibit Hep3B cell proliferation through cell cycle arrest at G2M or G0G1 phase, and induce cell-apoptosis through TRAIL/DR5 signaling by activating caspase-3 and caspase-10 [16].

Inhibiting MMP-9 and u-PA expression

Matrix metalloproteinases (MMPs) are a family of Zn^{2+}-dependent and Ca^{2+}-containing endoproteases comprising of 23 members in humans [113]. MMPs play important roles in cell regeneration, programmed death, angiogenesis, and many other essential tissue functions, and are also involved in normal development and pathological processes such as EMT [114]. MMP-9, also known as gelatinase B, is one of the most studied MMPs in the pathogenesis of EMT in HCC [114]. Overexpression of MMP-9 in HCC leads to increased lymph node invasion, and promotes metastasis, poor differentiation, disease recurrence, and overall poor prognosis [115]. MMP-9 is thought to be a consistent marker of progression and is associated with invasion and metastasis of HCC [116]. Urokinase plasminogen activator (uPA) is an extracellular matrix (ECM)-degrading protease involved in cancer invasion and progression [117]. Overexpression of uPA is associated with poor prognosis in many cancers, including breast, lung, and ovarian cancers [118], and is also associated with aggressiveness, metastasis and higher mortality rate in patients with HCC [119]. This protease is considered to be a clinically relevant biomarker for HCC patients receiving curative resection [119].

Degradation or decomposition of ECM by protease is a key step in tumor invasion or migration. This leads to the separation of stroma between cells, which promotes the migration of cancer cells and eventually leads to metastasis [120]. In human mesangial cells, NCTD was found to inhibit the expression of MMP-9 [121]. In colorectal cancer CT26 cells, NCTD has been shown to down-regulate the expression of MMP-9 by inhibiting the transcriptional activity of Sp1 (promoter specific transcription factor) [32]. In HCC (Huh7) cells, NCTD substantially inhibits several basic steps of metastasis, including cell invasion and migration of HCC cells, MMP-9 and u-PA activity and protein levels, as well as ERK1/2 phosphorylation, NF-kappa B content, and nuclear factor DNA-binding activity [15]. This implies that NCTD inhibits the expression of MMP-9 and u-PA by phosphorylating ERK1/2 and NF-kappaB signaling pathways, suggesting that NCTD may be a strong candidate for the development of drugs to prevent cancer metastasis [15].

Enhancing $p21^{Cip1/Waf1}$ and CDC25C expression and phosphorylation

$p21^{cip1/waf1}$ is a well-known and effective universal CDK (cyclin-dependent kinase) inhibitor [122]. In addition to its role in cell cycle regulation, including mitosis, p21 is involved in differentiation, cell migration, cytoskeletal dynamics, apoptosis, transcription, DNA repair, reprogramming of induced pluripotent stem cells, autophagy, and the onset of aging through p53 dependent and independent pathways [123]. P21 can act as either a tumor suppressor or a tumor promoter, depending largely on the cellular environment, its subcellular localization, its role in inducing/inhibiting apoptosis, and post-translational modification [123]. Due to its functional “duality” [122, 124] and its “essentially unstructured” characteristics [125], there is still a long way to go in revealing the various faces of p21 in normal and malignant cells [123]. CDC25C (Cell Division Cycle 25C) is a cyclin of the specific phosphatase family, which activates the cyclin B1/CDK1 complex in cells to enter mitosis, regulates the G2/M process, and plays an important role in the regulation of checkpoint proteins in case of DNA damage [126, 127]. The regulation of CDC25C in the cell cycle is influenced by a variety of signaling pathways [126, 127]. The change of CDC25C expression is closely related to the tumor occurrence and development, which can be used as a potential target for cancer therapy [40].

In human hepatoma cell lines HepG2, Hep3B, and Huhuh-7, NCTD is found to inhibit proliferation, induce mitostatic arrest, and then progress to apoptosis, enhancing expression and phosphorylation of $p21^{Cip1/Waf1}$ and CDC25C (but not the expression of Bax or Bad), phosphorylation of Bcl-2 and Bcl-X(L), and significantly decreasing the expression of p53 protein [14]. In addition, treatment with NCTD can induce activation of Caspase-9 and Caspase-3,

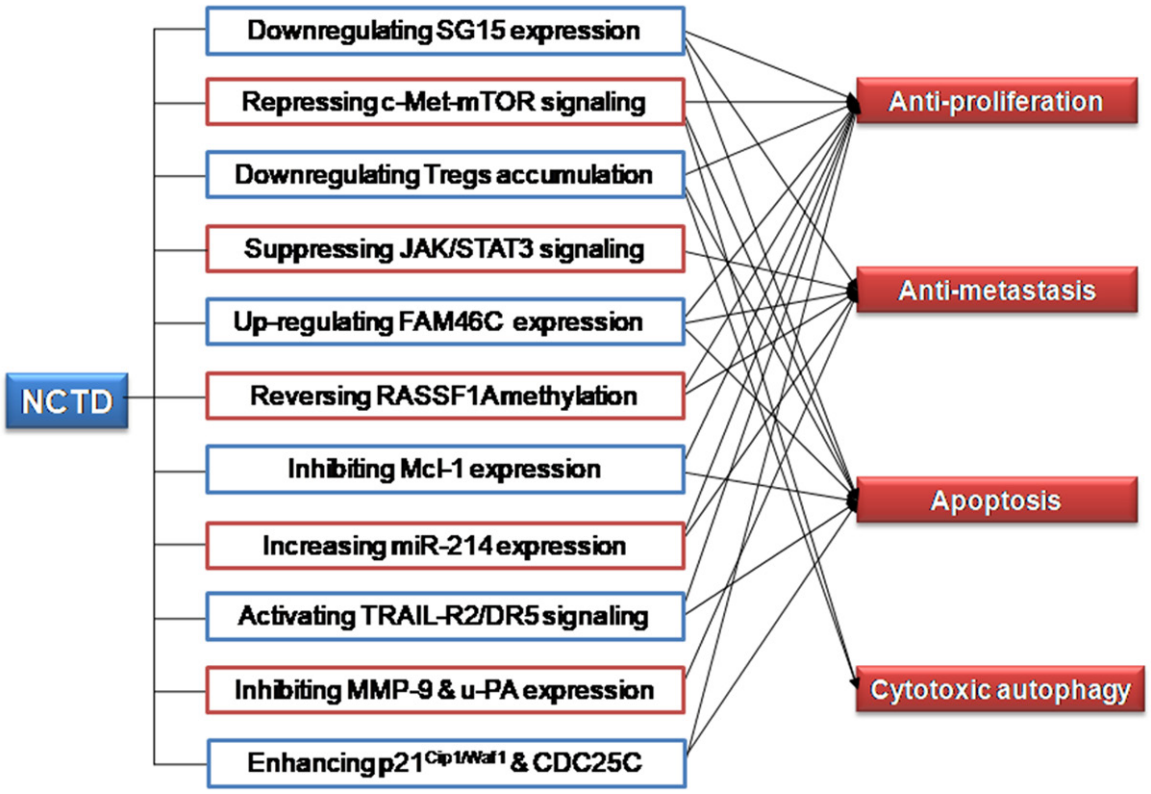

Figure 2. Mechanisms underlying anti-hepatocellular carcinoma effect of norcantharidin. ISG15: Interferon-stimulated gene 15; c-Met: mesenchymal-epithelial transition factor; mTOR: mammalian target of rapamycin; Tregs: Regulatory T cells; JAK: Janus kinase; STAT3: signal transducer and activator of transcription 3; FAM46C: family-with-sequence-similarity-46C; RASSF1A: Ras-associated domain family 1A; Mcl-1: Myeloid cell leukemia-1; miR-214: microRNA-214; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; DR5: Death receptor 5; MMP-9: Matrix metalloproteinase-9; u-PA: Urokinase plasminogen activator; $p21^{Cip1/Waf1}$: a universal CDK (cyclin-dependent kinase) inhibitor; CDC25C: Cell Division Cycle 25C.

leading to DNA fragmentation and morphological features of apoptosis, while pretreatment with the broad-spectrum caspase inhibitor z-VAD-fmk significantly inhibits NCTD-induced caspase-3 activity and cell death [14]. These results suggest that NCtD-induced mitotic arrest (M-phase cell-cycle arrest) may be due in part to increased expression and phosphorylation of $p21^{Cip1/Waf1}$ and CDC25C, while NCTD-induced apoptosis is at least partially related to increased expression of $p21^{Cip1/Waf1}$, phosphorylation of Bcl-2 and Bcl-X(L), and activation of caspase-9 and caspase-3 in these human hepatoma cells [14].

Concluding remarks

Research over the past few decades has made great strides in understanding of how NCTD plays a role against hepatocellular carcinoma. The accumulated results show that NCTD has the ability to induce mitotic arrest and anti-proliferation, anti-metastasis, apoptosis and cytotoxic autophagy or autophagic cell death in HCC by down-regulating the expression of ISG15, MMP-9, u-PA, Mcl-1 and by suppressing accumulation of regulatory T cells, up-regulating the expression of FAM46C, miR-214 and the expression and phosphorylation of $p21^{Cip1/Waf1}$ and CDC25C, inhibiting the c-Met-mTOR and JAK/STAT3 signaling pathways, reversing the methylation of RASSF1A gene, and by activating TRAIL-R2/DR5 signal transduction (**Figure 2**). Our recent studies have shown that NCTD can reduce iron content in cells by inhibiting the IL-6/JAK2/STAT3 signaling pathway and then down-regulating hepcidin expression, which may be a new mechanism that explains the anti-cancer effect of NCTD [128, 129].

It should be noted, however, that most of the data on the mechanism of NCTDS against hepatocellular carcinoma, as reviewed in the

preceding paragraphs, come from in vitro cell studies and very little come from clinical studies. There is still a long way to go in uncovering a detailed acocunt of the mechanisms by which NCTD protects against hepatocellular carcinoma. Further research is absolutely needed, especially relating to clinical studies.

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Disclosure of conflict of interest

None.

Abbreviations

ABT-737, A small-molecule cell-permeable Bcl-2/Bcl-xL antagonist; AKT, A serine-threonine protein kinase; ANGPTL1, Angiopoietin-like protein; B7-H3, B7-homologue 3; Bcl-2, B-cell lymphoma 2; CDC25C, Cell Division Cycle 25C; CDK, Cyclin-dependent kinase; CEP55, Centrosome protein 55 kDa; CLSO, Coixlacryma-jobi seed oil; c-Met, Mesenchymal-epithelial transition factor; CTD, Cantharidin; CTLA-4, Cytotoxic T lymphocyte-associated antigen-4; DEN, Diethylnitrosamine; DRs, Death receptors; EMT, Epithelial-mesenchymal transition; EphB4, Erythropoietin-producing hepatoma B class 4; FAM46C, Family-with-sequence-similarity-46C; FGFR1, Fibroblast growth factor receptor 1; FOIs, Fused oxazepino-indoles; FoxP3, Forkhead box P3; GCTB, Giant cell tumor of bone; GLPS, Ganodermalucidum polysaccharide extract; HCC, Hepatocellular carcinoma; Hep3B (and BEL7402, H22, HCCLM3, HepG2, Huh7, MHCC97-H; MHCC97-L, SK-Hep-1, SMMC-7721), HCC cells; HDGF, Hepatoma-derived growth factor; HGF, Hepatocyte growth factor; IL-8, Interleukin 8; IL-9, Interleukin 9; IL-17, Interleukin-17; lncRNAs, Long non-coding RNA; ISG15, Interferon-stimulated gene 15; JAK2, Janus kinase 2; LPS, Lipopolysaccharide; Mcl-1, Myeloid cell leukemia-1; miRNA-21, MicroRNA-21-5p; miR-214, MicroRNA-214; MMPs, Matrix metalloproteinases; MMP-9, Gelatinase B; mTOR, Mammalian target of rapamycin; NCTD, Norcantharidin; NLRC3, Nucleotide-binding domain, leucine-rich repeat family with a caspase activation and recruitment domain 3; NTase, Nucleotidyltransferase; OSCC, Oral squamous cell carcinoma; $p21^{cip1/waf1}$, A universal CDK (cyclin-dependent kinase) inhibitor; PBMCs, Peripheral blood mononuclear cells; PDK2, Pyruvate dehydrogenase kinase 2; PHF6, Plant homeodomain finger protein 6; PVT1, Long noncoding RNAPVT1; RASSF1A, Ras-associated domain family 1A; Sp1, Promoter-specific transcription factor; STAT3, Signal transducer and activator of transcription 3; TACE, Transcatheter arterial chemoembolization; TAMs, Tumor-associated macrophages; Teffs, Effector T cells; TGF-β1, Transforming growth factor-β1; TNBC, Triple-negative breast cancer; TNF, Tumor necrosis factor; TRAIL, Tumor necrosis factor-related apoptosis-inducing ligand; Tregs, Regulatory T cells; uPA, Urokinase plasminogen activator; XIAP, X-linked inhibitor of apoptosis.

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