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Cross-Reacting Material 197, a Specific Inhibitor of HB-EGF, and Its Anticancer Effects

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ABSTRACT

Genetic heterogeneity and metastasis remain the most challenging aspects of cancer treatment.Recent studies have introduced a mutant of diphtheria toxin,cross-reacting material197 (CRM197), as a new promising biological anticancer drug to improve cancer therapy in patients previously resistant to chemotherapy. Weak toxicity of CRM197accounts forthe stimulation ofcell apoptosis and the antitumor effect. Increasing evidence has indicated that the expression of heparin-binding epidermal growth factor-like (HB-EGF) growth factorincreases in most cancer cells, and CRM197 is its specific inhibitor.This study has focused on the structure, properties, and anticancer activity of CRM197.

Keywords: Cancer, Cross-reacting materials 197 (CRM197), Diphtheria Toxin (DT), Epidermal growth factor receptor (EGFR), Heparin-binding EGF-like growth factor (HB-EGF)

Diphtheria Toxin:

During the 1820s, corynebacterium diphtheriae was identifiedas apathogencausing a distinct form of sore throat (1). Visualization and isolation of the organism were performed in 1883 and 1884 by Klebs and Loeffler, respectively (1, 2). The DT gene expression is presented by lysogenic corynebacterium diphtheriae,which is regulated by the diphtheria toxin repressor (DtxR), a member of a family of metal ion-regulated repressors (1, 3, 4). Qianet al. reported that high levels of iron coupled with DtxRbind to the gene's operator and suppress the DT expression (3). Diphtheria toxin precursor includesa 26-amino acid signal sequence,and following cleavage by the processing enzyme, produces a 535-amino acids single-chain protein (58kDa). This structure has been discovered by Roux and Yersinin 1888 (5-8).Proteolytic cleavage of the toxin by trypsin results in the generation of two fragments, an N-terminal, enzymatically active fragment A of 193 aa (DTA, 21-kDa) involved in the inactivation of elongation factor 2 (EF2), and a C-terminal, fragment B of 342 aa (DTB, 37-kDa) which includes both the transmembrane (T) and the receptor-binding (R) domains (Fig. 1) (9-11).

DTA and DTB are connectedvia a 14-amino acid protease-sensitive loop and a disulfide bond between Cys168 and Cys201 (12). Murphy reported that the ADP-ribosyltransferase activity of the toxin is activated by proteolytic "nicking" of the α-carbon backbone at Arg193 in disulfide containing loop (13). DT binding to the human heparin-binding epidermal growth factor precursor (pro-HB-EGF) as a high-affinityreceptor for the toxinand to thespecific DT receptor is mediated by the R-domain (residues 432–535) (13-17). The T domain in the plasma membrane consists of nine helices organized in one amphipathic and two hydrophobic layers (12). These three layers form a pore across the endosomal membrane,in which the second layer stabilizes the structure (12).

Clathrin-mediated endocytosis is involved in the endocytosis of DT boundto its receptor and its internalization into early endosomal vesicles (18). Although protease furinis responsible for DTA and DTB domain cleavagein the endosomal vesicles,their linkage remains intact via a disulfide bond between Cys168 in DTA and Cys201 in DTB (19).Translocation of DTA from the endosome to the cytosol requires the insertion of the T domain into the endosomal membrane (20). Thus, the key protein components of clathrin on the vesicle membrane are substituted with some different protein components

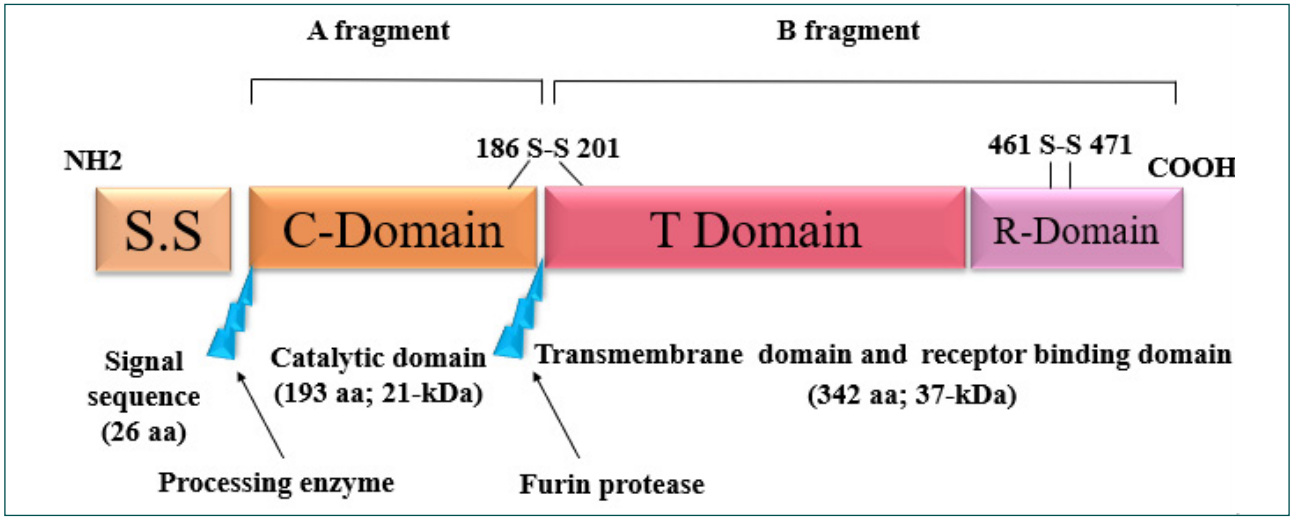

Figure.1. Diphtheria toxin structure. Diphtheria toxin synthesized in precursor form following the cleavage of a signal sequence by the processing enzyme has been observed in a Y-shaped molecule including A and B fragments. Fragment A includes the catalytic domain (C domain) of DT, cleaved by furin protease, and fragment B consists of two fragments, transmembrane (T) and receptor binding (R) domains.Two disulfide bonds have beendemonstrated in DT structure, Cys186 to Cys201, between fragments A and B and an internal disulfide bridge (Cys461 to Cys471) within fragment B (11,12).

such as ADP-ribosylation factor 1 (Arf-1), coat protein complex I (COPI), ras-related protein (Rab-5), and vacuolar H+-ATPase (V-ATPase) (20).The V-ATPase activity leads to acidification of endosomes, unfolding of Tdomain, insertion into the endosomal membrane, formation of 18–22Å cation-selective membrane pore, and finally, DTA translocation from endosometocytosol (12, 21). In this case,according to Murphy, the catalytic domain (C-domain) of diphtheria toxin located in fragment A is threaded through the pore by a process facilitated by a cytosolic translocation factor (CTF) complex. In asecond hypothesis, he suggested that the nascent chaperone-like activity of the partially unfolded Tdomain mediates the autonomous delivery of the C-domain across the membrane (13). The rate-limiting step for diphtheria toxin activation is reducing the disulfide bond between DTA and DTB that is catalyzed by thioredoxin reductases (TrxRs) or protein disulfide isomerase (PDI) (22). Following the delivery of DTA into the cytoplasm, activation of enzyme depends on refolding it and subsequently NAD^+-dependent ADP-ribosylation of EF-2, leading to the inhibition of protein synthesis and ultimately apoptosis (Fig. 2) (23).

The current study has focused on the structure, properties, and anticancer activity of CRM197.

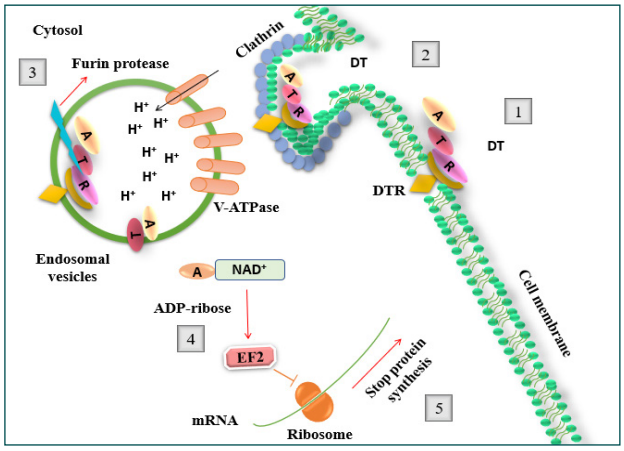

Figure.2.Mechanism of action of diphtheria toxin. DT binding toa receptor on the surface of sensitive cells (1) leads to internalization into early endosomal vesicles via clathrin-mediated endocytosis (2, 3). Following the DT insertion into endosomal vesicles, the key protein components of clathrin are substituted with different proteins such as V-ATPase.The V-ATPase activity provides the low endosomal pH essential for inserting the Tdomain into the endosomal membrane and the membrane pore formation. T domain helps in the delivery of the Adomain to the cytosol (3).Once the A domain is released into the cytoplasm of sensitive cells via interaction with NAD^+ (4) andtransformation of an adenosine diphosphate ribosyl (ADPR) moiety from NAD^+ to EF2, irreversibly terminates the protein synthesis (5). DT: diphtheria toxin, DTR: diphtheria toxin receptor (HB-EGF), V-ATPase: vacuolar H^+-ATPase, NAD; nicotinamide dinucleotide, EF2: elongation factor 2. The current study focuses on the structure, properties, and anticancer activity of CRM197.

Structure of CRM197

The nontoxic or partially toxic forms of diphtheria toxin, including CRMs 197, 228, and 176 with the same size as an intact toxin,results from the missense mutations (24). In addition, CRMs 30 and 45 with lower molecular weight than the native moleculeandan N-terminal fragmentmay resultfrom chain-termination mutations,according to Collier (24). In 1973,for the first time,Uchida et al.isolated these five mutantproteinsfrom C. diphtheriae strain C7(β) induced by ultraviolet.

CRM 40, another nontoxic form of DT toxin,with a C to T transition resulting inthe generation of (TAA) termination codon, lacks the last 149 carboxyterminal amino acid residues (approximately 17kDa) and the ability to bind to the cell surface (25). The nontoxic effect of CRM30 is due to the absence of binding and translocation domains, but its enzymatic activity remains intact (26, 27). Decreased binding capacity and enzymatic activity of CRM176 and CRM228 areattributed to the presence of several mutations and changes in the structure of Fragment A(26). The G/A transition in the DTA sequence leads to Gly52/Glu substitution and lack of enzymatic activity, perhaps due to alteration in the NAD^+ binding site (28). Different methods for structure analysis of CRM197 have revealedtwo main domains; fragment A or the catalytic domain and fragment B or the transmembrane domain,which contains two subdomains, an all-β-sheet structure that forms a subdomain for binding to the HB-EGF and anine α-helices which forms transmembrane subdomain for translocation into the target cell. Two S-S bonds have beenshown in the protein structure, the former (Cys186 to Cys201) links A and B fragments together,and the latter joins Cys461 to Cys471 within fragment B (29).

Diphtheria toxin and CRM197 interaction with cell surface

In addition to interaction with protein receptors, DT bindstothe negative charge of phosphate groups of phospholipids (28). In other words, even in the absence of a receptor, DT interacts with phospholipids with high affinity (30).

Due to alteration in the protonation state of the imidazole rings and carboxylic residues within both A and B, the pH ranges from neutrality to 5,and consequently, the conformational changes occur in the DT structure. Thus, pH has a vital role in DT translocation across the membrane. Papiniet al.showed that at neutral pH, DT interacts with the lipid bilayer surface only. However, at low pH, it binds to the hydrocarbon chains of phospholipids.Moreover, at low pH, CRM45 with only an N-terminal fragment is significantly less toxic than the native diphtheria toxin.These results suggest that the COOH-terminal segment of DT has a crucial role in interactions with cell receptors and the translocation of toxin across the membrane (30). Consistent with this, the importance of acidic conditions in penetration of diphtheria toxin into the cell has been reported by Sandviget al. They reported that protein synthesisis blockedat low pH.In cells exposed to DT at pH 4.5 for 10 min,protein productiondecreased as fast as in the cells exposed to the toxinwith1000 times concentration at neutral pH (31). In addition,Sandvig et al. hypothesized that in an acidic environment, the possible mechanism by which the DT enters into the cell is as follows; after enteringthe cell throughpinocytosis,the toxin breaks down to its constituent amino acidsin lysosomes,but some of them pass directly through vesicle membrane and escape from digestive lysosomal enzymesas soon as the vesicles become acidic. Low pH may also change the conformation of some protein structures in the plasma membrane, including receptors, or may induce opening different channelsand even conformational changes in DT.When the channels are opened, DT, hormones, and some growth factors flow into the cell, and significant amounts of ions and intracellular compounds leak out of the cell and ultimately lead to cell damage (31).

The CRM197 interaction with membrane lipids and cell surface receptors is stronger than DT (32). Another study has reported that both CRM197 and DT interact with diphtheria toxin receptors with the same affinity (28).

The partially nontoxic properties of CRM197 are due to similar DT or CRM197 B fragment effects that competitively inhibit the DT interaction with the cell surface receptor andenzymatically inactiveA fragment (27). Also, the permeability of CRM197 into the cell is temperature- and pH-independent, suggesting that CRM197 has an open conformationsimilar to the acidified DT, in which the R domain is separated from the C and T domains (33, 34).

Diphtheria toxin interaction with HB-EGF

DT interacts with HB-EGF precursorat the surface of various mammalian cell types (34). The specific DT receptor, mainly found in monkey Vero cells, was first isolated from the conditioned medium of macrophages and macrophage-like U937 cells (35). This particularreceptor on the cell surface is similar to proHB-EGF with an identical amino acid sequence (35).

For the first time, EGF was isolated from parotid glands of male mice and purified from human urine. Because of suppressive effects on gastric acid secretion in humans, it was termed urogastrone (36, 37). Epidermal growth factor (EGF), a53 aa protein (6 kDa) with three internal disulfide bonds,is secretedmainlyby monocytes, macrophages,vascular endothelial, and smooth muscle cells.The protein has a highaffinity forheparin,termedheparin-binding epidermal growth factor-like growth factor (HB-EGF) (38, 39). Moreover, HB-EGF, a member of the EGF family, is generated as a membrane-anchored precursor molecule (proHB-EGF).This precursor protein is cleaved by ADAM (which acts as adisintegrinand is a metalloprotease) in a process named ectodomain shedding leading to generation of soluble HB-EGF.ProHB-EGF cleavage is made by either matrix metalloproteases 7 (MMP7, expressed in tumor cells) at a position similar to that of ADAM or at a position in the N-terminal portion of the EGF-like domain (fig. 3) (40-44).

According to the previous studies, amino acid sequence within the EGF-like domain of the receptor (residues

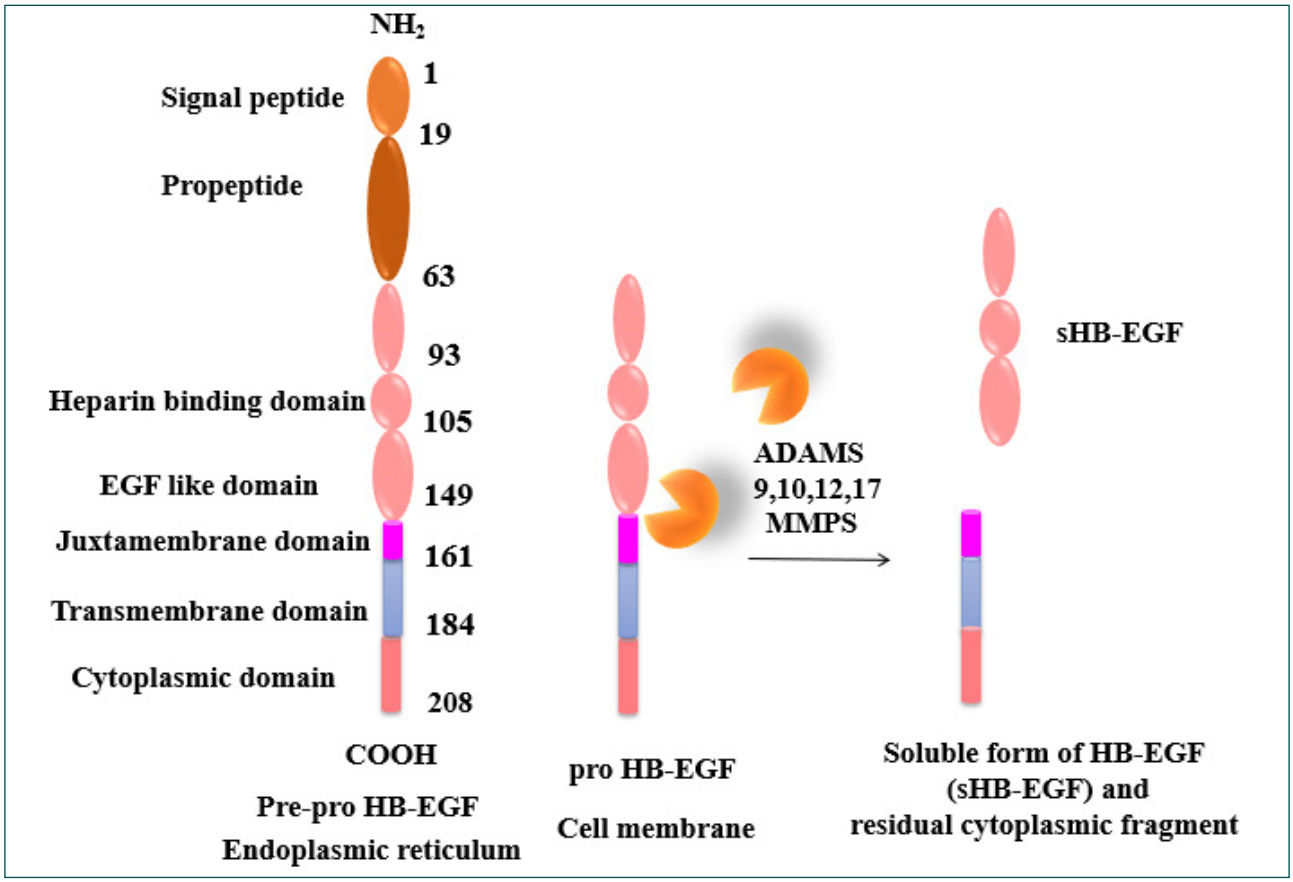

Figure.3. HB-EGF-like growth factor structure and ectodomain shedding of epidermal growth factor receptor (EGFR) ligand. Human HB-EGF-like growth factor is synthesized as a 208-amino acid membrane-anchored precursor molecule (pro-HB-EGF) cleaved by ADAM or MMPs to yield the mature protein or the soluble form of approximately 86 amino acids.ADAM: A disintegrin and metalloprotease, MMPs: matrix metalloproteases (45).

115-148) isspecifically essential for DT binding (27). In addition, ATP and other polyphosphates can inhibit DT toxicity by binding to a cationic region within the receptor-binding domain of the B-fragment, i.e., P-site (45). Based on this, Eidelset al. postulated that the P-site on the toxin molecule should be involved in both polyphosphate and receptorbinding. Also, highly cationic molecules such as histone, lysozyme, poly (L-ornithine), putrescine, spermidine, and spermine may inhibit DT frombinding toHB-EGF due to the similar effects of P-site (45). On the other hand, Brooke et al. reported that the negatively charged141Glu of the DT receptor and positively charged 516 Lys of diphtheria toxinhave the most critical role in crucialinteractions between the toxin and the receptor. Besides, the replacement of 141 Glu with His in the pro-HB-EGF binding site results ina significantreduction in-affinity binding and the toxicity of DT (46).

Because the DT binding to HB-EGF forms a complex with DRAP27, the monkey homolog of human CD9 antigen,and regulatesthe number of functional HB-EGF binding to the toxin. Thus, the 27-kD diphtheria toxin receptor-associated protein (DRAP27) is considered an essential factor in cell sensitivity to DT (16, 27).

Epidermal growth factor receptoris known as a protein tyrosine kinase receptor (PTKR). The receptors ofthe tyrosine kinase (RTK) family include four members: ErbB1 or (HER1), ErbB2 (Neu or HER2), ErbB3 (HER3), and ErbB4 (HER4) (47). Ligand binding leads to homo- or hetero-dimerization of the receptors and the kinase activity (48). EGFR signaling pathway results in the transition from the G1 phase of the cell cycle to the S phase and severaldifferent biological responsessuch as

migration, motility, proliferation, cellular differentiation, survival,and apoptosis (49). EGFR,firstly reported byPurba et al. (50), is a single-pass transmembrane protein consisting of an extracellular domain, a transmembrane domain, a juxtamembrane (JM) segment, a kinase domain, and a C-terminal regulatory tail.Various ligands such as epidermal growth factor (EGF), transforming growth factor-α (TGFA), amphiregulin (AREG), epigen (EPGN), betacellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin(EPR) have been known as activators for EGFR (51). The extracellular ligand-binding domain of EGFR has four motifs I–IV.The I (L1) and III (L2) members of the leucine-rich repeat superfamily are characterized by β-helix solenoid structure,whichinteracts with their ligands,whereas II (CR1) and IV (CR2) motifs contain cysteine-rich regions (50).This structure is followed by one transmembrane domain (25 aa) and an intracellular domain (about 550 aa) with 20 tyrosine residues which 12 of which are targeted for phosphorylation (52). Phosphorylated tyrosine residues serve as docking sites for soluble or membrane-anchored effector proteins and stimulate multiple signal transduction pathways, mediated by Ras/Raf/mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K)/Akt, and phospholipase C (fig. 4) (50).

CRM197 and anti-Cancer effect

EGFR-mediated signaling must be tightly regulated to provide proper cell growth, proliferation, differentiation, survival, or wound healing (53). EGFR signaling depends on the regulation of gene expression and liberation of their ligands (54). Excess EGFR signaling, increased production of ligands, overproduction of the EGFR protein, EGFR gene mutations,and lack of EGFR

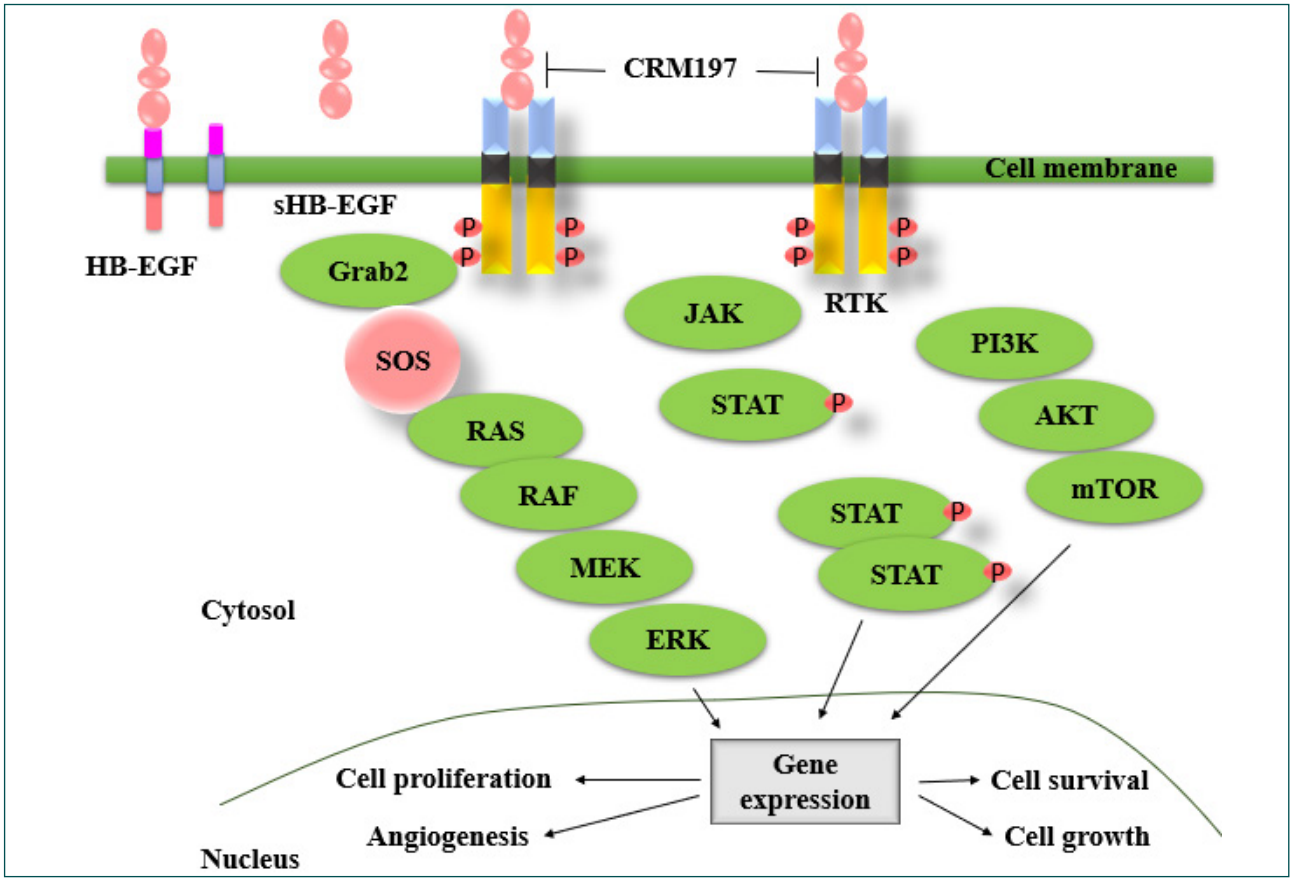

Figure.4. CRM197 as an inhibitor that abrogates the EGFR activation pathway. sHB-EGF: soluble Heparin-binding epidermal growth factor-likegrowth factor, RTK: receptor tyrosine kinase, EGFR: epidermal growth factor receptor, CRM197: cross-reacting material.

downregulation may lead to cancerdevelopment and progression (55, 56). CRM197 binds to HB-EGF, inhibits its mitogenic activity,and inhibits the ectodomain shedding of proHB-EGF (fig. 4) (40, 57). In addition totheppartiallytoxic nature of the molecule, CRM197 acts as an immunological adjuvant and carrier (58-64). Therefore, CRM197 may improve the efficacy of cancer therapywhen combined with chemotherapeutic drugs (65).

Like the intact DT, none of the EGFR ligands except for HB-EGF can interact with CRM197 (54). Therefore,HB-EGFsuppression leads to inhibition of metastasis, survival, cell adhesion, invasion, angiogenesis, and tumorigenicity of this growth factor (54).

The antitumor effects of CRM197 in the treatment of different cancer types are listedin table 1. Buzzi et al. investigatedthe anticancer effect of CRM197 on patients with various advanced tumors. According to theresults, subcutaneous injection of CRM197 accompanied by tolerable side effects such asinflammatory skin reaction, increased neutrophil count, and significantly enhanced serum levels of TNF-α (66). Yagiet al. assessedthe synergistic effects of paclitaxel as an antitumor chemotherapy drug and CRM197 as an inhibitor of HB-EGF on ovarian cancer (OC). The results of in vitro and in vivostudies suggested that resistance to paclitaxel can occur as follows:Although paclitaxel stimulates the proapoptotic signals such asc-Jun N-terminal kinase (JNK) and p38 MAPK,it activates antiapoptotic signals via enhancement of the ectodomain shedding of proHB-EGF and activation of the ERK and Akt signaling. After the ectodomain shedding of proHB-EGF, the carboxyl-terminal fragments of HB-EGF (HB-EGF-CTFs) translocate into the nucleus and trigger cell growth signals.CRM197 blocks the EGFR and subsequent ERK and Akt signaling and may suppress the HB-EGF-CTFs translocation into the nucleus (67).

Epithelial OCmetastasis occurs via multiple pathways such as the peritoneal cavity, blood, or lymphatic vessels termedtranscoelomic, hematogenous, or lymphatic metastasis,respectively (68, 69). The multi-step process in OC metastasisis divided into several distinct stages; (a) infiltration and survival of cancer cells in the peritoneal fluid, (b) adherence to the peritoneal surface, and finally (c) acquisition of potential for motility, angiogenesis, and invasion into the peritoneum to subsequent migration and proliferation in others organs resulting in colonization (70). Survival of individual ovarian tumor cells depends on the secretion of lysophosphatidic acid, HB-EGF, and vascular endothelial growth factor (VEGF) into the ascitic fluid. CRM197 has adverse effects on cell proliferation, growth, and survival in peritoneal fluid of patients with OC. CRM197 inhibits cell adhesion via integrins, protein tyrosine kinase 2 (PTK2),which is known as focal adhesion kinase (FAK), and EGF R (54, 71). CRM197 decreases theVEGF and interleukin 8 (IL- 8) gene expression significantly and reduces the metastatic potential of OC cells (72). Yotsumotoet al.reviewedthe efficacy of ligand-based targeting for the EGF system in cancer therapy. Theyreported thatalthough DT interactswiththe EGF domain of HB-EGF and inhibits cell proliferation, it is not considered an HB-EGF inhibitor because ofthe high toxicityeffectsagainst normal cells (73). In contrast,CRM197 interacts with HB-EGF more strongly than DT or at least as strong as a native molecule without significant side effects (35). Coadministration of CRM197 with paclitaxel blocks the gene expression of ERK and the Akt signaling pathway. It activates p38, c-Jun N-terminal kinases (JNK), and MAPK signaling pathways involved in cell proliferation and apoptosis. Therefore, combining chemotherapeutic drugs with CRM197 enhances apoptosis rate and inhibits cell proliferation (74).

According to Martarelli et al. (75), the therapeutic effect of CRM197 in adrenocortical carcinoma is attributed to its inhibitory effect on HB-EGF, its residual toxicity, and its potent properties in triggering the immune response. The role of HB-EGF-EGFR signaling in gastric cancer development and significant improvementinanticancer effect of coadministration of 5-FU, cisplatin, or paclitaxel with CRM197 was approved in both in vitro and in vivo experimentsperformed by Sanuiet al. (76). Consistent with these results,Yotsumotoet al. reported that HB-EGF was predominantly expressed in breast cancer, and CRM197 was introduced as both a specific inhibitor

Type of cancer	Drug dosage and type of injection	Human, animal or cell culture	The aims of a study / Results	REff
Various* advanced cancer	According to the patient's degree of immunological reactivity to DT/CRM197 (none, moderate, or high) three different dosages (1.7, 2.6, or 3.5 mg/day) of CRM197 injected subcutaneously in the abdominal wall, on alternate days, for 6 days.	outpatients	Anti-tumor effect of CRM 197 Different degree of inflammatory response and significant increase in circulating neutrophils and serum TNF-α concentration was observed.	Buzzi 2004
Ovarian cancer	10 nM–10 μM concentrations of paclitaxel and 1 μg/ml CRM197 for 24 hr	SKOV3 and SK-HB-1 cells	The effect of paclitaxel and CRM197 on cell proliferation The more effective suppressed SKOV3 cells viability was observed following paclitaxel co-administration with CRM197 than paclitaxel only.	Yagi 2009
	10 nM–1 μM concentrations of paclitaxel in the presence or absence of 1 μg/ml CRM197 for 48 hr	SKOV3 and SK-HB-1 cells	The effect of paclitaxel and CRM197 on apoptosis The effect of concurrent administration of paclitaxel and CRM197 on both SKOV3 and SK-HB-1 cells death was significantly higher than the paclitaxel only.	
	Dose-dependent administration of 5 mg/kg and 50 mg/kg of CRM197 in nude of mice	Tumorigenesis by SKOV3 or SK-HB-1 cells injection into mice	In vivo anti-tumor effects of CRM197 The tumor formation was partially inhibited following administration of 5 mg/kg CRM197 and completely inhibited following administration of 50 mg/kg CRM197.	
	Co-administration of 20 mg/kg and 10 mg/kg of paclitaxel in nude mice by	Tumorigenesis by SKOV3 or SK-HB-1 cells injection into mice	In vivo anti-tumor effects of paclitaxel Tumor formation was suppressed by SKOV3 cells (20 mg/kg), while this effect was not seen about SK-HB-1 cells (20 mg/kg), SKOV3 cells and SK-HB-1 cells (10 mg/kg).	
	Co-administration of 10 mg/kg paclitaxel and 5 mg/kg CRM197	Tumorigenesis by SKOV3 or SK-HB-1 cells injection into mice	In vivo anti-tumor effects of paclitaxel and CRM197 Tumor growth by both SKOV3 cells and SK-HB-1 cells Completely was suppressed.	
Adrenocortical carcinoma	1 mg/week CRM197 every week was injected intraperitoneally	Tumorigenesis by Subcutaneous injection of H95R and SW-13 cells into mice	Effect of CRM197 on tumor formation At presence of CRM197 tumor formation by H295R and SW-13 cells was significantly suppressed	Martarelli 2009

Adrenocortical carcinoma	For cell proliferation assays and cell cytotoxic assays CRM197 dissolved in DMSO at various concentrations: 0,1, 10 and 100 g/ml.	H295R and SW-13 cells	In vitro anti-tumor effect and cytotoxicity of CRM197	Martarelli 2009
			At a concentration higher than 1 g/ml of CRM197 the growth of tumor cells was considerably suppressed and at presence of 100 g/ml of CRM197 apoptosis was accrued.	
	CRM197 (0, 1, 10 and 100 g/ml)	H295R cells	Effects of CRM197 on cell migration and invasion	
			The cell invasion significantly was suppressed following the treatment with noncytotoxic doses of CRM197 and completely was inhibited at cytotoxic dosage of CRM197 (100 g/ml).	
	100 g/ml of CRM197 for 24 h	H295R and SW-13	CRM197 effect on apoptosis	
			Cell death was seen following treatment with CRM197 at both H295R and SW-13 cells	
		Tumor cell implanted in nude mice.	CRM197 effect on angiogenesis	
			Angiogenesis was suppressed in subcutaneous tumors.	
Gastric Cancer	Transfection of siRNA at concentration of 25 nM and incubation for 72 h for HB-EGF, amphiregulin and EGFR, and after incubation with CRM197 (1 μg/ml) and inhibitory antibody against AR (AR;10 μg/ml) and EGFR (10 μg/ml) and incubation for 72 h	NUGC3 and MKN28 cells	Investigation of the tyrosine phosphorylation of EGFR, ERK and AKT	Sanui 2010
			At presence of a siRNA, inhibitory anti-EGFR antibody or CRM197 separately, the EGFR, ERK and AKT phosphorylation in NUGC3 and MKN28 cells was decreased but no change in AR phosphorylation was observed at presence of amphiregulin siRNA. No alteration in EGFR, ERK and AKT phosphorylation was seen at presence the inhibitory anti- AR antibody. The significantly high rate of cell death was observed at presence of the CRM197 and inhibitory anti-EGFR antibody.	
	Different concentrations (0-10 μM) of 5-FU, cisplatin or paclitaxel	NUGC3 And MKN28 cells	5-FU, cisplatin or paclitaxel effect on concentration of HB-EGF and apoptosis	
			A significant increase in HB-EGF expression and apoptosis was observed following the treatment with paclitaxel at concentrations of 1 nM and 10 nM in compared to 5-FU and cisplatin in two types of cells.	

Gastric Cancer	1 µg/ml of CRM197 with different concentration (0-10 µM) of 5-FU, cisplatin or paclitaxel	NUGC3 And MKN28 cells	CRM197, 5-FU, cisplatin or paclitaxel effect on concentration of HB-EGF cell apoptotic rate	Sanui 2010
			The synergistic antitumor effect was reported between CRM197, 5-FU, cisplatin or paclitaxel.	
	CRM197 (50 mg/kg) or paclitaxel (10 mg/kg), or their combination was injected intraperitoneally each week for 6 weeks into mice	Tumorigenesis by Injection of NUGC3 And MKN28 cells into mice	Investigation of antitumor effects following co-administration of CRM197 and paclitaxel in xenografted mice	
			CRM197 alone significantly inhibited tumor growth. Partial suppression of NUGC3 cell growth and no inhibition of MKN28 cell growth reported following the treatment with paclitaxel. At presence the CRM197and paclitaxel tumor formation by both NUGC3 and MKN28 cells completely was suppressed.	
Breast cancer	1 µM of cetuximab or 100 nM of CRM197	BT549 and MDA-MB-231 cells	Anti-tumor effect of cetuximab or CRM197	Yotsumoto 2010
			Unlike the treatment with cetuximab, the significant higher rate of cell death and lower HB-EGF expression and EGFR, ERK and AKT activation was reported following the treatment with CRM197.	
	CRM197 (50 mg/kg) was injected intraperitoneally every day for 10 days after the tumor volume exceeded 100 mm3	Tumorigenesis by Injection of MDA-MB-231 cells into NOD/ SCID mice	Anti-tumor effect of CRM197	
			Completely tumor formation was suppressed	
	CRM197 (100 nM), trastuzumab (1 µM) trastuzumab (1 µM) plus recombinant human HB-EGF (rHB-EGF) (1 µg/ml)	SK-BR-3 and BT474 cells	HB-EGF is involved in trastuzumab-resistant signals	
			At presence of trastuzumab or CRM197 increase in cell death and decrease in HB-EGF expression was induced, but addition of HB-EGF suppressed the cell death following treatment with trastuzumab.	
Acute lymphoblastic leukemia T-ALL	10 µg/ml of CRM197 for 72 h	Jurkat E6-1 cells compared with untreated cells	CRM197 effect on HB-EGF expression and apoptosis in T-ALL cells	Kunami 2011
			Although CRM197 induces apoptosis and decreases HB-EGF protein expression in Jurkat E6-1 cells, no association was found between RNA expression and treatment with CRM197.	

Acute lymphoblastic leukemia T-ALL	25 nM doxorubicin and/or 10 µg/ml CRM197 for 72 hours	Jurkat E6-1 cells compared with untreated cells	Antitumor effects of doxorubicin co-administration with CRM197	Kunami 2011
			At presence of CRM 197 or doxorubicin separately the rate of apoptosis was 10.8±3.2% and 65.1±2.5%. respectively. Co-administration of CRM 197 and doxorubicin increased the rate of apoptosis by 75.6±2.4%.	
Glioma	5 mg/ml of cisplatin combined with 1mg/ml of CRM197	Glioma cell (U251) and human astrocytes (HA)	Effect of cisplatin and CRM197 on cell growth	Wang 2012
			The treatment with cisplatin and CRM 197 significantly blocked the U251 cells growth compared to HA cells.	
	No information has been mentioned	U251 cells	Effects of co-administration of cisplatin with CRM197 on apoptosis	
			Following the treatment with both cisplatin and CRM197 significantly cell apoptosis increased in compared to cisplatin alone.	
	No information has been mentioned	U251 cells	CRM197 suppressed the Akt phosphorylation induced by cisplatin	
			At presence the cisplatin Akt phosphorylation was increased, while treatment with either CRM197 or combination of cisplatin with CRM197 considerably blocked the Akt phosphorylation compared with the control group.	
	10 mmol/l of LY294002, 1 mmol/l of wortmannin, cisplatin combined with CRM197 and combination of cisplatin with CRM197 plus 10 mmol/l of LY294002 or 1 mmol/l of wortmannin		Anti-tumor effect of the combination of cisplatin with CRM197 plus LY294002 or wortmannin	
			Co-administration of cisplatin with CRM197 plus either LY294002 or wortmannin, Akt activation significantly blocked and cell death significantly induced compared to the co-administration of cisplatin with CRM197.	
Oral cancer	CRM197 at various concentrations (0, 0.5, 1.0, 1.5, and 2.0 µg/ml)	HSC3 and SAS cells	Effect of CRM197 on HSC3 cell growth	Dateoka 2012
			Cell growth at a concentration higher than 0.5 µg/ml of CRM197 significantly was inhibited.	

Oral cancer	2.0 µg/ml of CRM197 for 48 h	HSC3 cell vs. untreated cells	The effect of CRM197 on expression of HB-EGF	Dateoka 2012
			Decreased the HB-EGF mRNA expression was found in CRM 197 treated cells.	
	2.0 µg/ml of CRM197 for 48 h	HSC3 and SAS cells vs. Un-treatment cells	CRM197 effect on HSC3 and SAS cells invasion	
			At presence of CRM197 partially inhibited cell invasion was reported.	
	2.0 µg/ml of CRM197	HSC3 cells	CRM 197 effect on MMP-9 and VEGF expression	
			Significantly reduced MMP-9 expression and VEGF and MMP-9 activity was reported.	
	Groups were divided as follow; control (1), CRM197 1 mg/kg/day (2), CDDP 1 mg/kg/day (3), and CRM197 and CDDP 1 mg/kg/day (4)	Tumorigenesis by HSC3 cells implanted subcutaneously into female BALB/c nu/nu mice	CRM 197 effect on tumor formation	
			Partially inhibition of tumor growth was seen following the treatment with either CDDP (1 mg/kg/day) or CRM197 (1 mg/kg/day) and completely inhibition was seen at presence of coadministration of 1.0 mg/kg CDDP and 1.0 mg/kg CRM197.	
Neuroblastoma	10 µg/ml of CRM197 for 48h	SK-N-SH cells	CRM197 effect on apoptosis	Nam 2015
			The rate of cell apoptosis significantly increased in cells with high HB-EGF expression	
Triple-negative breast cancer	2 models of treatment were as follow; 1. the adjuvant therapy model CRM197 was injected intravenously at the same time as the subcutaneously injected MDA-MB-231 cells (1, 5, 10 and 50 mg/kg) 2. advanced cancer model CRM197 was injected after the tumor reached an estimated volume >100 mm3. (1, 5, 10 and 50 mg/kg)	Tumorigenesis by Subcutaneously injection of MDA-MB-231cells into NOD/SCID mice	Effect of CRM197 doses on tumor volume	Nam 2016
			The anti-tumor effect of CRM197 was more effective in the advanced cancer therapy model compared to the adjuvant therapy model.	
lung cancer	1 µg/ml of CRM197	RERF-LC-A1 and NCI-H1975 cells	CRM197 effect on apoptosis	Yotsumoto 2017
			Increased rate of cell death was reported.	
	1 mg/kg of CRM197 was administered daily for 10 consecutive days	Tumorigenesis by RERF-LC-A1 and NCI-H1975 cells injected subcutaneous into female BALB/c nu/nu mice	Anti-tumor effect of CRM197	
			Completely inhibition of tumor formation by RERF-LC-A1 cells, and significantly suppression by NCI-H1975 cells was demonstrated.	

Ovarian cancer (OC) or peritoneal cancer (PC)	(1.0, 2.0, 3.3, and 5.0 mg/m2) dose of CRM 197, intraperitoneal	Patients with advanced or recurrent OC or PC	CRM 197 effect on HB-EGF	Miyamoto 2017
			Reduced HB-EGF concentration in serum and abdominal fluid was observed. 8 of 11 patients completed treatment.	
Ovarian cancer	The A2780 cells and the SKOV3 cells that were infected with AdCRM197	A2780 cells vs. SKOV3 cells	Assessment the A2780 cells and SKOV3 cells sensitivity to AdCRM197	Dai 2018
			The A2780 cells were sensitive to AdCRM197, but the SKOV3 cells were resistant to AdCRM197	
	Wild type p53 A2780 cells and knockdown p53 A2780 cells were infected with AdCRM197	wild type p53 A2780 cells vs.	Cell sensitivity investigation to AdCRM197	
			Although the A2780 cells were sensitive, the knockdown p53 A2780 cells were resistance to AdCRM197.	
	p53 gene was restored in SKOV3 cells with Adp53 and SKOV3 cells and SKOV3-p53 cells infected with AdCRM197	SKOV3 cells vs. SKOV3-p53 cells	Assessment the cell sensitivity to AdCRM197	
			Only the SKOV3-p53 cells were sensitive to AdCRM197.	
	A2780 cells and A2780 cells with p53 knocked down infected with AdCRM197	A2780 cells vs. A2780 cells with p53 knocked down	The role of p53 in AdCRM197-induced apoptosis	
			Unlike the A2780 cells with p53 knocked down gene, caspase-dependent apoptosis was activated in A2780 cells.	
	A2780 cells and mutant EF2 (A2780-mEF2) cells were infected with AdCRM197.	wild type EF2 A2780 cells vs. the A2780- mutant EF2 cells	Effect of the EF2-ADP-ribosyl transferase activity of CRM197 in p53 activation	
			Unlike the A2780-mEF2 cells, apoptosis via p53 pathway was activated in wild type EF2 A2780 cells.	
	A2780 cells and A2780-mEF2 cells were infected with AdCRM197.	wild type EF2 A2780 cells vs. the A2780- mutant EF2 cells	Assessment the p53 and HB-EGF gene expression	
			The higher level of p53 and HB-EGF genes expression in AdCRM197-infected A2780 cells was reported, while no significant alteration of gene expression was observed in the AdCRM197-infected A2780-mEF2 cells.	
	The combined therapy of AdCRM197 plus Adp53 was used on A2780 xenograft tumors	Tumorigenesis by A2780 cells were injected into mice	Antitumor effect of the combined therapy with AdCRM197 plus Adp53	
			Reduced tumor volume and increased the cells death were reported in the mice that received combined therapy with AdCRM197 plus Adp53 in compared to that of the AdCRM197 group.	

Ovarian cancer	The combined therapy of AdCRM197 plus Adp53 was used on SKOV3 xenograft tumors	Tumorigenesis by SKOV3 were injected into mice	Antitumor effect of the combined therapy with AdCRM197 plus Adp53 to overcome resistance of SKOV3 xenograft tumors to AdCRM197 in vivo	Dai 2018
			Reduced tumor volume and increased the cells death were reported in the mice that received combined therapy with Ad-CRM197 plus Adp53 in compared to that of the AdCRM197 group.	

*Breast, Colon, Mouth, Kidney, Bladder, Ovary, Uterus, Prostate, Brain, neuroblastoma, and non–small cell lung cancer
Table 1. Anticancer effect of CRM197

of the EGFR signaling pathway and an effective drug in breast cancer patients resistant totrastuzumab (77).

The evidence has indicated that activation of PI3K/Akt signaling pathway via phosphorylation of 308Thr and 473Ser in Akt is involved in glioma progression, invasion, and angiogenesis (78-80). Wang et al.showed thatgrowth and apoptosis of human U251 glioma cells were induced after treatment withcisplatin combined with CRM197 plus LY294002 or wortmannin. Their research revealed that this activation occurred through Akt signaling pathway only in cisplatin-treated U251cells. They suggested that Akt activation in the cells exposed to cisplatin is due to cisplatin resistance. In contrast, inhibition of PI3K/Akt signaling pathway in CRM197-treated U251cells is done by inhibiting proteolytic cleavage of pro-HB-EGF and secretion of the soluble form of the receptor.This soluble form hasmitogenic and chemoattractant properties for various cell typesby activatingthe PI3K/Akt pathway.Moreover, they found that combining cisplatin, CRM197,andsome specific PI3K inhibitors such as LY294002 or wortmannin could significantly suppress the PI3K/Akt pathway leading to inhibition of U251 cell growth and inductionof apoptosis when compared to control groups (65). Dateokaet al.reportedthat CRM197 could suppress the invasiveness and motility of cultured oral cancer cell line through inhibition of HB-EGF and reduction in MMP-9 expression, which has a crucial role in VEGF produced by endothelial cells (81). The pivotal role of HB-EGF in neuroblastoma, triple-negative breast cancer, and OC was reported by Nam et al.These researchers introduced CRM197 as a safe and promising anticancer drug that targets HB-EGF (82-84). Yotsumoto reported that T790M mutation in exons 18-24 of EGFR gene,responsible for its tyrosine kinase activity, leads to induced resistance to pretreatment with small-molecule EGFR tyrosine kinase inhibitors (TKIs), stimulation of EGFR signaling, and increased expression of EGFR ligands such as HB-EGF in patients with lung cancer. So, the administration of CRM197 may be the best therapeutic approach in these patients (85).

To explain the role of CRM197 in apoptosis through the P53 pathway, Dai et al. recruitedtwo types of cells, A2780 cells with p53 wild-type gene, andp53 deleted SKOV3 cells, andthen treated them with adenovirus-mediated CRM197 (AdCRM197). In addition, P53 was knocked-down inA2780 cells by lentivirus-mediated RNA interference, restored in SKOV3 cells with Adp53, and treated with AdCRM197(86).Unlike the SKOV3 and A2780 cells with a deletion in gene encoding p53, caspase-dependent apoptosis was activated in A2780 and SKOV3 cells with the wild-type p53 gene. Although fora long time, until 2018, CRM197 had been considereda nontoxic variant of the diphtheria toxin,Dai et al. approved the crucial effect of weak EF2-ADP-ribosyl activity of CRM197 in the induction of apoptosis in OC cells through activation of the p53 pathway. With this aim, they treated A2780 cells with wild-type EF2 gene and A2780- mutant EF2 (A2780-mEF2) cells by AdCRM197. They demonstrated that cell death occursonly in A2780 cells. A2780-mEF2 cells areresistant to AdCRM197 and apoptosis.Thus, they hypothesized that the weak toxicity of CRM197 has led to induction of theADP-ribosylation of EF2 and cell death in A2780 cells with wild-typeEF2 gene. In addition, their results showed that p53 and HB-EGF expres-

sion were increased in AdCRM197 infected A2780 cells. But the expression of p53 and HB-EGF genes were inhibited in mutant EF2 A2780 cells,and no response was observed following treatment with AdCRM197.

DISCUSSION

Cancer has been considered one of the most significant causes of death around the world (87). Based on a report published in 2018, over half of the global cancer mortality has occurred amongthe Asian population (88). The survival rate of cancer patients remains poor despite impressive progress in cancer screening, diagnosis, and treatment (89, 90). More research must be conducted to investigate the problems related to cancer and its treatment. This review has focused on the role of CRM197 in the treatment of different types of cancers. Most of the studiesdemonstrated that treatment with CRM197 has a promising positive effect on inhibition of growth,angiogenesis,and metastasis and induction of apoptosis in various cancer types, such as oral, ovary, lung,prostate, breast, gastric, pancreatic, adrenocortical, and colon cancer,neuroblastoma, glioblastoma, acute lymphoblastic leukemia (ALL), leukemia, melanoma and solid tumors (Fig 5) (58, 62, 75, 76, 81, 83-85, 91-103).

Althoughoverexpression of EGFRin different types of cancers (104-106) has attracted the increasing interest of researchers as a therapeutic target, the drug resistance leading to poor outcome and severe complications during therapy is currently a problem (107). It has been shown thatthe coadministration of CRM197 with chemotherapeutic agents has a significantly stronger antitumor effect when compared to each of the agents separately (86, 108). In a clinical trial study performed by Miyamoto et al.,theantitumor effect of CRM197 via blockingHB-EGF in eleven Japanese patients with advanced or recurrent ovarian cancer (OC) or peritoneal cancer (PC) was approved (109). In addition, Miyamoto et al. compared the side effects of intraperitoneal injection of CRM197

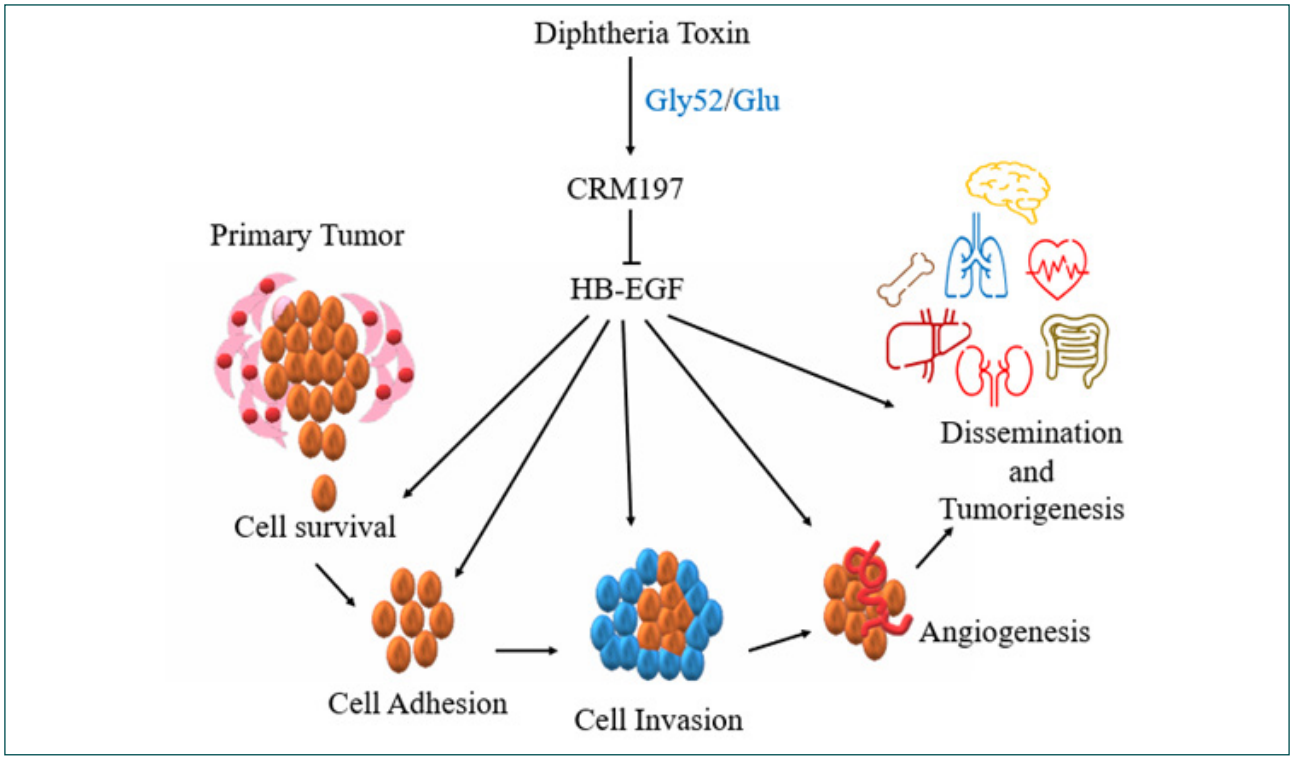

Figure.5. Mechanism of anticancer effect of CRM197; The G/A transition in the fragment A diphtheria toxin leads to Gly52/Glu substitution and CRM197 production. CRM197 can inhibit the HB-EGF-mediated signaling pathway, which leads to cell growth, invasion, proliferation, differentiation, survival, angiogenesis, and metastasis

with cisplatin.They observed that following 60–100 mg/m2 intraperitoneal (IP) injection of cisplatin, the plasma concentration of the drug was approximately 0.7–2.3 μg/mL.In contrast,after IP injection of 1–3.3 mg/m2of CRM197, the plasma concentration of this drug was undetectable.They suggested that the high molecular weight of CRM197 prevents its passing across the peritoneum, and due to the high level of HB-EGF in the peritoneum,CRM197 easily interacts with itand enters into the lymphatic capillaries(109).Another clinical trial study showed that administration of CRM197 via the subcutaneous route is safe and relatively well tolerated. Other side effects of DT/CRM197, including skin irritation in the injection sites and a flu-like syndrome with fever, were observedonly in patients with hypersensitivity (66). In addition, CRM197 is one of the best carriers for stimulating the immune response in cancer immunotherapy. To assess the immune response, Globo H (GH), a hexasaccharideoverexpressed in a variety of cancer cells, was attached to different carriers such as keyhole limpet hemocyanin, CRM197, tetanus toxoid, and BSA combined with an adjuvant and were injected into mice. Huang et al.reported that combination of GH-CRM197 adjuvant with α-galactosylceramide C34 induces the highest production of antibodies with IgG-dominant. These antibodies act very specific and selective against cancer cells that overexpressed GH and the GH-related epitopessuch as breast cancer cells (110). Interestingly,-Dai et al. revealed that the weak toxicity of CRM197 and ADP-ribosylation of EF2 caused p53 pathway activation, but in the A2780 cells carrying the EF2 mutant gene, p53 expression reduced, and the cells were resistant to Ad-CRM197 significantly (86).

It hasbeen reported that the overexpression of HB-EGF and membrane-type matrix metalloproteinase-1 (MT1-MMP) is correlated with different types of malignancies(111, 112). MT1-MMP expression leads to extracellular matrix degradation and facilitates tumor cell invasion and growth (113). Also, Naohikoet al. demonstrated that concurrent overexpression of MT1-MMP and HB-EGF in gastric cancer cells resulted in HB-EGF cleavage and enhanced cell proliferation (57). The increased production of soluble HB-EGF is associated with MT1-MMP processing found in malignant ascites in patients with metastatic ovarian carcinoma (114). Therefore, the administration of CRM197 with MT1-MMP inhibitors might provide an effective treatment for metastatic carcinoma (57).

Although many significant advances have taken placein this respect,many vital questions remain unanswered. While most studies have introduced CRM197 as a safe antitumor drug, further clinical trial studies should be conducted to confirm that long-term treatment with this agent has no serious adverse consequences.This review reveals that CRM197 induced cell apoptosis through the activation of the p53 pathway. However, to the best of our knowledge, there has been no study on the effects of CRM197 on the expression of other tumor suppressor genes or the functions of other proteins.

CONCLUSION

This review indicates that CRM197 binding to HB-EGF and soluble HB-EGF inhibits the mitogen-activated protein kinase (MAPK) and protein kinase B (Akt) signaling pathwaysand suppressescell proliferation, survival, and tumorigenicity. CRM197 inhibits angiogenesis, invasion, and metastasis through silencing the genes of protein tyrosine kinase 2 (PTK2), vascular endothelial growth factor (VEGF), and interleukin 8 (IL-8). The toxicity of CRM197 depends on activating the p53 pathway in cancer cells carrying a wild-type elongation factor 2 (EF2) gene. The combination of chemotherapeutic drugs and CRM197 enhances the effectiveness of cancer treatment. CRM197 as an ideal carrier protein induces the highest production of antibodies with specific and selective functions against cancer cells.

The weak or lack of enzymatic activity of CRM197 compared with diphtheria toxin decreases the survival of cancer cells via overexpression of HB-EGF, inhibiting HB-EGF's interaction with EGFR, or by residual enzymatic activity in cells transfected with CRM197. Regarding the positive anticancer effect of CRM197 on different types of cancer cells, appropriate clinical trial studies are required to investigate the efficacy of its combination with other therapies such as surgery and radiotherapy.

Further studies in gene therapy aimed atrestoring the defective tumor suppressor genescombined with targeted drug delivery methods such as injecting virus-mediated CRM197 to human cancer cells are needed to confirm the precise anticancer effect of CRM197 and assessthe life-threatening side effects..

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Abbreviations:

HB-EGF: Heparin-binding epidermal growth factor-like growth factor
CRM197 cross-reacting materials 197
EGFR: epidermal growth factor receptor
DtxR: diphtheria toxin repressor
DT: diphtheria toxin
EF2: elongation factor 2
Cys: Cysteine
Arf-1: ADP-ribosylation factor 1
COPI: coat protein complex I
Rab-5: ras-related protein
V-ATPase: vacuolar H+-ATPase
CTF: cytosolic translocation factor
TrxRs: thioredoxin reductases
PDI: protein disulfide isomerase
ADPR: adenosine diphosphate ribosyl
Gly: Glycine
Glu: Glutamic acid
CTF: cytosolic translocation factor
TrxRs: thioredoxin reductases
PDI: protein disulfide isomerase
ADPR: adenosine diphosphate ribosyl
Gly: Glycine
Glu: Glutamic acid
ADAM: a disintegrin and metalloproteinases
MMP: matrix metalloproteases
Lys: lysine
His: histidine
DRAP27: diphtheria toxin receptor-associated protein
CD9: cluster of differentiation 9
PTKR: protein tyrosine kinase receptor
RTK: receptors of tyrosine kinase
JM: juxtamembrane
TGFA: transforming growth factor-α
AREG: amphiregulin
EPGN: epigen
BTC: betacellulin
EPR: epiregulin
aa: amino acid
MAPK: mitogen-activated protein kinase
PI3K/Akt: phosphoinositide-3-kinase/protein kinase B
sHB-EGF: soluble Heparin-binding epidermal growth factor-like growth factor
RTK: receptor tyrosine kinase
TNF-alpha: tumor necrosis factor alpha
JNK: Jun N-terminal kinase
HB-EGF-CTFs: carboxyl-terminal fragments of HB-EGF
VEGF: vascular endothelial growth factor
PTK2: protein tyrosine kinase 2
FAK: focal adhesion kinase
IL- 8: interleukin 8
5-FU: 5-Fluorouracil
Thr: Threonine
Ser: Serine
TKIs: tyrosine kinase inhibitors
mEF2: mutant EF2
ALL: acute lymphoblastic leukemia
OC: ovarian cancer
PC: peritoneal cancer
ip: intraperitoneal
GH: Globo H
BSA: bovine serum albumin
SSEA3: stage-specific embryonic antigen-3
MT1-MMP: membrane-type matrix metalloproteinase-1

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