Title: The analog of cGAMP, c-di-AMP, activates STING mediated cell death pathway in estrogen-receptor negative breast cancer cells

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Abstract

Immune adaptor protein like STING/MITA regulate innate immune response and plays a critical role in inflammation in the tumor microenvironment and regulation of metastasis including breast cancer. Chromosomal instability in highly metastatic cells releases fragmented chromosomal parts in the cytoplasm, hence the activation of STING via an increased level of cyclic dinucleotides (cDNs) synthesized by cGMP-AMP synthase (cGAS). Cyclic dinucleotides 2' 3'-cGAMP and it's analog can potentially activate STING mediated pathways leading to nuclear translocation of p65 and IRF-3 and transcription of inflammatory genes. The differential modulation of STING pathway via 2' 3'-cGAMP and its analog and its implication in breast tumorigenesis is still not well explored. In the current study, we demonstrated that c-di-AMP can activate type-1 IFN response in ER negative breast cancer cell lines which correlate with STING expression. c-di-AMP binds to STING and activates downstream IFN pathways in STING positive

metastatic MDA-MB-231/MX-1 cells. Prolonged treatment of c-di-AMP induces cell death in STING positive metastatic MDA-MB-231/MX-1 cells mediated by IRF-3. c-di-AMP induces IRF-3 translocation to mitochondria and initiates Caspase-9 mediated cell death and inhibits clonogenicity of triple-negative breast cancer cells. This study suggests that c-di-AMP can activate and modulates STING pathway to induce mitochondrial mediated apoptosis in estrogen-receptor negative breast cancer cells.

Introduction

The crosstalk between tumor cells, infiltrating immune cells and stroma in breast cancer tumor microenvironment (TME) provides an optimal niche for the growth and proliferation of cancer cells [1]. Hypoxic TME of solid tumors promotes the clonal evolution of the cancer cells which leads to the progression of the tumor [2]. Hypoxic TME can also induce necrotic cell death leading to the release of intrinsic danger- associated molecular patterns (DAMPs), which can activate innate immune response [3]. The activation of the innate immune system and its regulation during tumorigenesis is emerging [4] however, its role in the acquisition of tumorigenic phenotype, its physiological and chemical modifiers are not well understood. Our previous reports demonstrated that innate immune regulators are uniquely positioned at mitochondria which in turn links the inflammatory pathways and metabolism, hence playing an important role in the metabolic adaption of tumor cells [5]. STING (Stimulator of interferon gene) is also known as MITA, MPYS, TMEM 173 is localized at the ER/mitochondria contact site and is a major regulator of the type I immune response. Interestingly, STING is differentially expressed in ER/PR positive and negative breast cancer patients, therefore can differentially regulate inflammatory cell death [6]. The implication of increased level of STING in triple-negative breast cancer cells and association with metastasis and resistance to cell death is

not well understood. The release of nuclear DNA in cytoplasm, either in highly proliferating cells or stressed solid TME is sensed by cyclic GMP-AMP synthase (cGAS) which adds phosphate bond between the 2 and 3 carbons of GMP and AMP nucleotide [7], hence synthesis of 2' 3'-cGAMP, a ligand for the innate immune receptor STING. The binding of 2' 3'-cGAMP to STING activates downstream signalling and recruits TBK-1 which phosphorylates the inflammatory transcription fac- tors IRF-3 and p65 [8], leading to increased level of type-I IFN, and other chemokines [9]. Type-I IFNs are pleiotropic cytokines and are known for their anti-tumor effects [10]. In the tumor microenvironment (TME), type-I IFN may stimulate dendritic cells maturation, and activation of cyto- toxic T-lymphocytes and enhancement of memory T-cell survival which are characteristics of anti-tumor cytokines [11]. Breast cancer metastasis and TME are majorly associated with type-1 immune response. The lack of type -1 immune response helps tumor cells to escape from the immune-mediated anti-tumor response [12]. Decreased infiltration of CD8+ T lymphocyte in TME is associated with poor prognosis in cancer [13]. Activation of the IFN pathway in tumor cells recruits CD8+ T lymphocytes and enhances anti-tumor immunogenic response [14]. Hence stimulation of the cGAS-STING-IFN pathway has become a central target for the development of anticancer therapeutics [11]. The cDNs produced by intracellular pathogens can also activate STING-mediated innate immune and inflammatory response [15]. Since cGAMP is an endogenous molecule hence its level may be altered by several feedback mechanisms hence its signaling strength may determine its potential outcome in different pathological conditions [16, 17]. Therefore the potential of other cDNs molecules for activation of cGAS/STING/IFN pathway should be investigated further for an anti-tumorigenic response. c-di-AMP produced by *Listeria monocytogenes* in host cells activates type-1 immune response via STING and IRF-3 [18]. Similarly, intracellular infection of *Chlamydia* synthesizes cyclic di-AMP (c-di-AMP), which also acts as a ligand for STING that activates IFN responses during infection [19]. Though cyclic di-nucleotides of prokaryotic origin can effectively stimulate STING signaling and activate the down- stream pathway [20], their potential as modulators of tumor cell survival and death mediated by STING pathway had not been explored. In the current study, we demonstrate that bacterial origin c-di-AMP, the analogue of cGAMP, activates STING

mediated type-1 immune response and cell death in estrogen- receptor negative breast cancer cells. Moreover, the c-di-AMP induced cell death directly correlates with STING expression in breast cancer cells. c-di-AMP treated cells show caspase activation and PARP cleavage in STING expressing cell line independent of cGAS expression. Further c-di-AMP induces IRF-3 translocation to mitochondria to induce cell death.

Materials and methods

Cells line maintenance and culture

MCF-7, T-47D, ZR75, BT-474, MDA-MB-231 purchased

from ATCC. MCF-7 cultured in EMEM media while T-47D, ZR75 and BT-474 maintained in RPMI medium. MX-1 obtained from CLS, Germany and cultured in F12K media. MDA-MB-231 was cultured in Leibovitz's L-15 media (HI-MEDIA, India). The media used were supplemented with 10% FBS (Life Technologies, USA) and 1% penicillin, streptomycin, and neomycin (PSN) antibiotic mixture (Life Technologies, USA). Cells were incubated at 37 °C, 5% CO_2 in specified media. All cell lines were checked for myco- plasma contamination by Universal Mycoplasam detection kit ATCC.

Plasmids and reagents

STING cloned in pCMV6 ENTRY plasmid was gift from Dr. Hong Bing Su (Wuhan University China), p65-shRNA and control shRNA were received from Dr. Edurne Berra Ramírez (Gene Silencing Platform, CICbioGUNE, Derio, Spain). STING-shRNA and IRF-3-shRNA were a gener- ous gift from Dr. Peter Chumakov (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences). Primary antibody against STING was purchased from Proteintech, USA, cGAS and HRP-conjugated secondary anti-rabbit and anti-mouse antibodies were purchased from Thermo Scientific, USA. Antibodies against PARP, Caspase 9, Caspase 3, and, NF-kB p65 were purchased from Cell signalling, Inc., USA, 2' 3'-cGAMP and c-di-AMP from Sigma, Prestoblue™ cell viability reagent from Invitrogen, USA and Caspase 3/7

luciferase reporter activity kit was purchased from Promega, USA.

Generation of cGAS-sgRNA clones

cGAS-sgRNA clones were generated using protocol described by Ran et al. [21]. The guide-RNAs targeting the first exon of cGAS was designed using GPP sgRNA Designer tool (Broad institute) [21]. sg-RNA-top and sg- RNA-bottom strands were synthesized as described earlier [21]. Synthesized oligos were annealed and cloned into BbsI

-linearized pSpCa9(BB)-2A-Puro (PX459) V2.0 vector. cGAS-sgRNA clones were transformed into competent Stbl3

E. coli strain and transformants were screened by colony PCR using U6 sequencing primer and sg-RNA-bottom. Posi- tive clones were finally confirmed by Sanger sequencing.

cGAS: 5′caccg**AGACTCGGTGGGATCCATCG**′3.

5′aaacCGATGGATCCCACCGAGTCTc′3

Transfection

MCF-7, T-47D, ZR75, BT-474, MX-1 and MDA-MB-231

transfected with X-treamGENE (Sigma, USA) using manufacturer's protocol

Quantitative analysis of gene expression

Total RNA was isolated using Tri Reagent (Life Technologies, USA) and was reverse transcribed to synthesize cDNA using Transcriptor First Strand cDNA synthesis kit (Roche, Germany) or SuperScript VILO cDNA Synthesis Kit (Life Technologies, USA) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Premix Ex Taq TM (Takara, Japan) or SYBR mix (Life Technologies, USA) or Applied Biosystems as per manufacturer's instructions. Specific primers of the genes are listed below.

STING: Fwd 5′-CGCCTCATTGCCTACCAG-3′; Rev, 5′-ACATCGTGGAGGTACTGGG-3′;

cGAS: Fwd 5′-GGGAGCCCTGCTGTAACACTTCTT AT-3′;

Rev 5′-CCTTTGCATGCTTGGGTACAAGGT-3′; β-Actin: Fwd   5′-TCGTGCGTGACATTAAGGGG-3′;

Rev 5′-GTACTTGCGCTCAGGAGGAG-3′; GAPDH: Fwd   5′-AGAAGGCTGGGGCTCATTTG-3′;

Rev 5′AGGGGCCATCCACAGTCTTC 3′.

Western blot

Cells were plated at a density of 4.5×10^5 cells/well in the six-well plate and transfected with indicated expression plasmid or shRNAs/sgRNA using X-treamGENE (Sigma, USA). After 48 h of transfection, the cells were harvested, washed with ice-cold PBS and lysed in buffer A (150 mM NaCl, 30 mM Tris–Cl, 10% Triton X-100, 10% Glycerol, $1 \times$ Protease Inhibitor (Roche, Germany). The equal pro- tein was loaded and resolved on 11% SDS-PAGE. Protein was electro blotted on PVDF membrane at 110 V for 1 h at 4 °C. The membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) or 5% BSA (BSA (Sigma-Aldrich, USA), 0.1% Tween-20 in TBS-0.02 M Tris–Cl, 0.15 M NaCl) for 1 h at room temperature. The membrane was incubated overnight with a specific primary antibody. After incubation, the membrane was washed three times with TBS-T (TBS containing 0.1% Tween-20) for 10 min and incubated with a secondary antibody at room temperature for 1 h. The membrane was washed three times with TBS-T and the signal was visualized by using EZ-ECL

chemiluminescence detection kit for HRP (Biological Indus- tries, Israel) by exposing it to UVTEC gel documentation system.

IFN-β and NF-κB luciferase assay

MCF-7, MX-1, BT-474and MDA MB 231 were seeded at density of 1×10^5 cells in 24 well-plated, next day co-transfected with IFN-β firefly luciferase or NF-kB firely luciferase using gene X-tream GENE (Sigma) and treated with 200 μM c-di-AMP for 24 h IFN-β or NF-kb activity measured using as per manufacturer's instructions (Promega, USA).

Caspase 3/7 activity assay and Caspase 3/7 green assay for microscopy

The activity was performed using Caspase-Glo® 3/7 Assay kit (Promega, USA) or Caspase-Glo® 8 Assay kit (Promega, USA). Cells were plated at the density of 4×10^4 cells per well in 96 well in white clear-bottom plates and transfected with indicated expression plasmids or shRNAs and respective controls. Caspase-Glo® 3/7 (10 μl) reagent was added to each well and luminescence was measured with a Centro LB 960 Luminometer (Berthold Technologies, Germany). Caspase 3/7 green assay for microscopy performed as per manufacturer protocol (Thermofisher, USA).

Cell growth inhibition assay and clonogenic assay

All cell lines were seeded at a density of 5000 cells/well in 96 well plates and treated with different concentrations of c-di-AMP for 4 days. At the end of treatment, cell viability was measured using Presto blue cell viability reagent (Invitrogen, USA). The colonogenic assay has been performed as described previously [22].

Molecular docking and cellular thermal shift assay

a) Binding of c-di-AMP with STING was further analysed by Molecular Docking using Maestro ver11.9, Schrodinger Suite. Crystal structure of STING [4KSY (Homo sapiens)] with a bound ligand having resolution 1.88 Å was retrieved from Protein Data Bank and prepared using the Protein Preparation Wizard and minimized using OPLS3e force field. All the heteroatoms and water molecules were removed except the conserved water molecules within 5 Å and the hydrogen atoms were added. Each structure was minimized for all-atom constrained minimization using Ligprep module with OPLS3e force field

b) *Cellular thermal shift assay* MDA-MB 231 cells were lysed in HBSS via three freeze–thaw cycles in liquid nitrogen. Total protein was quantified and an equal protein (Cell lysate) was aliquoted into PCR tubes and incubated with and without 30 μg/ml c-di-AMP for 1 h. After incubation suspension was transferred into PCR tubes subjected to specific temperature treatment for 3 min. Precipitated protein was separated using centrifugation at 17000×g for 20 min at 4 °C then supernatant of each sample was collected and analyzed by western blot.

Fluorescent microscopy

MDA-MB-231 cells were seeded in an optical bottom dish. After overnight incubation, the cells were imaged using NIKON (Japan) Eclipse Ti2-E inverted fluorescent microscope.

Results

c-di AMP induces STING mediated IFN pathway in breast cancer cell lines

We investigated if c-di-AMP can activate the STING path- way in breast cancer cells and modulate the IFN pathway. Firstly, we monitored the expression of STING in ER-positive and negative breast cancer cells. The analysis of transcript level of cGAS by RT-qPCR (Fig. 1b) showed ubiquitous expression in both ER-positive (MCF-7, T-47D, ZR75) as well as negative breast cancer cell lines (MDA- MB- 231,

BT-474 and MX-1). In consonance with RT-qPCR data, western blotting showed the expression of cGAS protein in all selected breast cancer cells. Interestingly, we found that STING mRNA expression was lower in ER- positive breast cancer cell lines (MCF-7, T-47D, ZR75) compared to ER- negative breast cancer cell lines (MDA- MB- 231, BT-474 and MX-1) (Fig. 1a). Gene expression correlation using TCGA database showed negative correlation between STING and estrogen receptor. The *r* value is represented in different color in different types of breast cancer (Fig. 1c). In agreement with RNA expression, protein expression of STING was undetectable in ER-positive breast cancer cell lines (MCF-7, T-47D and ZR75), whereas, its expression was high in ER-negative breast cancer cell lines (MDA- MB- 231, BT-474, MX-1) (Fig. 1d). Further, we analyzed whether the STING-IFN pathway is intact in breast cancer cell lines. MCF-7 cells (cGAS +ve/ STING –ve) and MDA-MB-231 (cGAS +ve/STING +ve) were treated with c-di-AMP and monitored the activation of both NF-kB and IFN pathway using luciferase assay. Interestingly c-di-AMP showed no activation of both NF-kB and IFN pathways in MCF-7 whereas it activated both path- ways in MDA-MB-231 cells (Fig. 1e, f). In further experiments, we selected MDA-MB-231 as STING positive cell line and MCF-7 as STING negative cell line. This further suggests that the STING pathway is intact in MDA-MB- 231(cGAS +ve/STING +ve) and is inhibited in MCF-7 cells (cGAS +ve/STING –ve) by downregulation of STING.

c-di-AMP binds to STING

As c-GAMP is known to bind to STING hence we hypothesized its analog c-di-AMP may also bind to STING expressed in human cells. To understand the binding of c-di- AMP as compared to other dinucleotides with STING, we performed docking of c-di-AMP with STING. The pyrimidine ring of purine forms two hydrogen bonds with Arg238 of STING. The amino group on the pyrimidine ring forms the hydrogen bond with Val239 which is bridged through the conserved water molecule to Ser241 and the oxygen of phosphate group forms the hydrogen bond and salt bridge with Arg238. Further amino group on the pyrimidine ring of purine forms a hydrogen bond with Tyr 167 and Ser

241. The oxygen of phosphate forms the hydrogen bond with Arg238. The imidazole ring of purine shows

the pi–pi interaction with Tyr167 and the oxygen atom of phosphate forms the hydrogen bond with Arg238. Interestingly, the purine ring of c-di-GMP shows the pi–pi interaction with Tyr167 and the oxygen atom of phosphate forms the hydro- gen bond with Ser162. In di-nucleotide, the amino group on the pyrimidine ring of purine forms a hydrogen bond with Ser241 same as molecule c-di-AMP. The conserved water molecule forms the hydrogen bond with Ser241 and Val239 and oxygen atoms of phosphate form two hydrogen bonds and a salt bridge with Arg238. Apart from these interactions, the molecules also have hydrophobic interactions with Tyr167, Tyr240, Val239, Tyr163 and Leu159 similar to the bound ligand (Fig. 2a–d). The docking score of c-di-AMP and c-di-GMP was found comparable and near to the docking score of the co-crystal ligand. Further, we used cellular thermal shift assay which measures the thermal stability of a target protein and the binding of a ligand to the protein causes an increase in protein melting temperature, hence quantitative measure of binding of a ligand. The natural ligand 2, 3'-cGAMP synthesized by cGAS binds to STING and stabilizes it as reported previously [23]. We used a similar method to detect c-di-AMP binding to STING. We observed that binding of c-di-AMP stabilized STING protein with increasing temperature whereas unbound STING denatured fast (Fig. 2f) suggesting the binding of c-di-AMP to STING.

c-di- AMP activates cell death in ER/PR negative breast cancer cell lines

We and others have previously reported that STING can act as a tumor suppressor by sensitizing the cells to TNF-α induced cell death pathway [24]. We assessed whether c-di- AMP can activate the STING pathway and induce cell death in breast cancer cells. MCF-7 (ER-positive) breast cancer cell line, having an undetectable expression of STING, treated with c-di-AMP showed no effect on cell survival and caspase 3/7 activity (Fig. 3a). MX-1, BT-474, MDA- MB- 231 (ER-negative), STING positive breast cancer cell lines showed a significant decrease in cell survival and increased caspase 3/7 activity (Fig. 3b–d). We analysed if c-di-AMP can activate the apoptotic cell death in breast cancer cells by monitoring PARP cleavage by western blotting. The band of 89 kDa corresponding to the cleaved subunit of PARP was observed in MDA-MB-231 and MX-1 cells whereas no band was detected in MCF-7 cells (Fig. 3e). The representative images

of the fourth day after treatment of c-di-AMP (Fig. 3f) suggest that c-di-AMP inhibits the proliferation of STING positive tumor cells and induces apoptosis.

STING is essential for c-di-AMP induced cell death

As we observed here that c-di-AMP inhibited cell proliferation, we further characterized the role of STING in the initiation of the cell death pathway. STING was knockdown using shRNA in MDA-MB-231 and BT-474. After the knockdown of STING, cells were treated with c-di-AMP and monitored cellular viability caspase 3/7 activity. We observed that c-di-AMP treatment showed a significant reduction in cell viability in MDA-MB-231 and BT-474 cells, (Fig. 4a, d) which was rescued after STING knock-down both in MDA-MB-231 and BT-474 cells. Similarly, the caspase-3/7 activity increased significantly in c-di-AMP treated MDA-MB-231 cells and BT-474 cells (Fig. 4b, e). The knockdown of STING in MDA-MB-231 and BT-474 rescued cell proliferation and Caspase-3/7 activity of both the cell types. The knockdown of STING (Fig. 4c, f) was confirmed by western blotting. These experiments strongly suggest that STING is essential for c-di-AMP mediated cell death in MDA-MB-231 and BT-474 cells.

IRF-3 is indispensable for c-di-AMP induced apoptosis in ER-negative breast cancer cells

The implication of c-di-AMP regulated NF-kB and IFN pathway for induction of cell death in breast cancer cells is not understood hence we explored the implication of these pathways in cell death. MDA-MB-231 and MX-1 cells were transfected with p65 and IRF-3 shRNA to inhibit NF-kB and IFN pathway respectively. cGAS, p65 and IRF-3 were knocked out using CRISPR/Cas-9 sgRNA/shRNA and its role in the regulation of cell death was monitored. The knockdown of cGAS, p65 and IRF-3 was confirmed by western blotting (Fig. 5a, b). The knockdown of p65 showed no significant change in cell death both in c-di-AMP treated MDA-MB-231 and MX-1. Interestingly, the knockdown of IRF-3 significantly enhanced the cell survival in c-di-AMP treated MDA-MB-231 and MX-1 cells as observed. Simi- larly, IRF-3

knockdown also inhibited caspase activity in c-di-AMP treated MDA-MB-231 and MX-1 cells (Fig. 5e, f). We also analyzed the role of cGAS in the regulation of cell death, hence we knockdown cGAS and monitored cell death. The knockdown of cGAS in both cell lines showed no sig- nificant change in c-di-AMP induced cell death suggesting that cGAS acts upstream and is dispensable. These results suggest that IRF-3 is indispensable for c-di-AMP induced STING-mediated apoptosis in triple-negative breast cancer cells.

c-di-AMP induces IRF-3 translocation to mitochondria and induce the mitochondrial-mediated intrinsic pathway of apoptosis

The above experiment strongly suggests the indispensable role of IRF-3 in c-di-AMP induced cell death in STING positive MDA-MB-231 and MX-1 cells. It had been observed previously that IRF-3 activates mitochondrial-mediated apoptosis in viral infected cells [25]. IRF-3 plays a critical role in STING-mediated apoptosis via mitochondrial cytochrome c release [26]. We also monitored the subcellular localization of IRF3-GFP both in MCF-7 and MDA-MB-231 in the presence/absence of c-di-AMP. IRF-3-GFP showed diffused cytoplasmic localization both in MCF-7 and MDA-MB-231. Interestingly c-di-AMP treated cells show distinct puncta of IRF-3-GFP 3 both in MX-1 and MDA-MB-231 cells. The increased numbers of IRF-3 puncta in IRF-3-GFP positive cells were observed in the presence of c-di-AMP as com- pared to untreated in both MDA-MB-231 cells and MX-1 (Fig. 6a). The co-localization of IRF-3 at mitochondria was checked by coexpressing IRF-3-GFP and mt-RFP constructs in MDA-MB-231 cells. Colocalization was measured by fluorescence microscopy in the presence and absence of c-di-AMP. We observed that localization of IRF-3 to mitochondria was significantly increased in c-di-AMP treatment condition in MDA-MB-231 cells (Fig. 6b).

Hence, we further analyzed if c-di-AMP induces mitochondrial-mediated apoptosis by measuring PARP cleavage and caspase-9 activation (Fig. 6c) Treatment of MDA- MB-231 and MX-1 cells with c-di-AMP cells showed the band of 89 kDa corresponding to a cleaved subunit of PARP predominantly in both cells whereas it was not observed in untreated cells. Further, we monitored the cleavage of Caspase-3 in similar

conditions. The cleaved subunit of 17 kDa subunit was predominantly observed in c-di-AMP treated both MDA-MB-231 cells and MX1 cells (Fig. 6d). This was further confirmed by increased caspase 3/7 green fluorescence activity in both cells showing enhanced apoptotic cell death (Fig. 6e). Cas- pase-9 is activated upstream of executioner Caspase-3. Interestingly the cleaved subunit of 35 and 37 kDa was observed in c-di-AMP treated both MDA-MB-231 cells and MX1 cells. To further monitor the effect of c-di- AMP on the clonogenic ability of both MDA-MB-231 and MX-1 cells we performed a colony-forming assay in presence of c-di-AMP. The clonogenic ability of both MDA-MB-231 and MX-1 cells significantly decreased in presence of c-di-AMP (Fig. 6f). Overall, these results show that c-di-AMP enhances IRF-3 translocation to mitochondria and induces mitochondrial-mediated intrinsic pathway of apoptosis, inhibiting the clonogenic ability of STING positive breast cancer cells.

Discussion

Highly proliferating tumor cells show a high level of chromosomal instability which has becomes the hallmark of cancers of different origins [27]. Previous reports from our lab and others had shown that ER-positive breast cancer cells are STING negative and using TCGA database we found negative gene expression correlation between *TMEM173* (STING) and *ESR1* (estrogen receptor) in breast cancer patients. This strongly suggests that ER expressing breast cancer cells have low expression of STING [28], whereas highly proliferative cells ER/PR/Her-2 negative, MDA-MB-231 and MX1 cells, are STING positive [24]. It has been observed that chromosomal instability promotes errors in chromosome segregation which leads to micronuclei formation and leakage of genomic DNA into the cytosol [9–14]. This leads to the activation of the cGAS–STING cytosolic DNA-sensing pathway and downstream noncanonical NF-κB [29] signaling leading to proliferation and metastasis. Cyclic dinucleotide, cGAMP, is an endogenous high-affinity ligand for the adaptor protein STING [2]. The synthesis of the cGAMP analog like c-di-AMP in bacteria is an interesting phenomenon and modulate the STING pathway during infection however its potential in regulating these pathways and modulation of cell death in breast cancer cells has not been well

studied. We observed that c-di-AMP can activate NF-κB and IFN pathways in breast cancer cells depending upon the presence of STING. It was observed that ER-positive cells are low STING or negative expressing cells and ER-negative cells show high STING positivity. The evidence here suggests that STING is eliminated or down-regulated during the early stage of the growth hormone ER/PR positive cells where some cells retained STING and became growth hormone-independent [30]. This supports the emerging hypothesis of clonal evolution of the cells which positively determines the tumor growth and metastasis by clonal amplification and retaining the gene expression at the different stages of cancer. The basal STING activation is essential for cell proliferation and metastasis by promoting non-canonical activation of NF-kB [31]. This hypothesis is in consonance with earlier reports where STING positive cells showed enhanced proliferation, brain metastasis cells and chemoresistance in breast cancer cells and lung cancer cells [32]. Previously it had been observed that cGAMP can be transferred from the metastatic cells to brain astrocytes through cellular GAP junctions [29]. The intactness of the type-I IFN pathway and NF-kB may play an essential role in tumor cells which can be therapeutically targeted. STING-NF-kB and/IRF-3 pathway is intact in triple-negative breast cancer as compared to ER/PR- positive cells as observed in this study and can be differentially modulated by c-di-AMP. This is an interesting characteristic of aggressive breast cancer cells which can be further investigated and can be exploited therapeutically. Interestingly sustained activation of the STING pathway by c-di-AMP shows the activation of cell death specifically in ER/PR negative cells and not in ER-positive cells where the IRF-3 pathway seems to be essential for the cell death pathway. Interestingly, c-di-AMP induces translocation of IRF-3 to mitochondria and activation of mitochondrial path- way in ER-negative cells, which is in consonance with the earlier observation of IRF-3 translocation to mitochondria during viral infection induced apoptosis [25]. This pathway is although independent of the DNA binding ability and is dependent upon mitochondrial localization [33]. We also observed that mitochondrial functions are compromised leading to the activation of Caspase-9 mediated Caspase-3 activation in MDA-MB-231 cells and MX-1 cells. This path- way is not activated in MCF-7 and other ER-positive cells.

MCF-7 is also IRF-3 positive cells however ER/PR positive cells are either less/negative STING, this suggests that a critical level of STING is required for c-di-AMP induced cell death in breast cancer cells. The sustained activation of STING mediated pathways with fewer side effects is a requirement that may activate the immunogenic cell death pathway [34] specifically in triple-negative breast cancer cells which are highly proliferative and metastatic and show high-level chromosomal instability. It had been observed previously due to chromothripsis and formation of micro nuclei, there is a release of DNA in cytoplasm leading to cGAS/cGAMP/STING activation however no type-I IFN activation [35]. However release of DNA leads to activation of the non-canonical NF-kB pathway which may be determined by the strength of cGAMP induced signaling and which is enzymatically regulated and determine the outcome. Therefore the sustained activation of this pathway by cGAMP analog-like c-di-AMP which may induce IRF3 translocation to mitochondria and induce intrisic pathway of apoptosis is of therapeutic importance for immunogenic cell death in solid tumors. There is no direct comparison of cGAMP with cDNs however isolated studies suggest cGAMP rather than therapeutic beneficial it may further support growth [36, 37].

This study provides a rationale for screening the bacterial origin cyclic dinucleotides which may activate the intrinsic cell death pathway and may activate the immunogenic cell death pathway. Therefore, the bacterial-derived c-di-AMP and other analogues should be screened for their potential as combinatorial therapy with DNA damaging agent for therapeutic potential in breast cancer cell lines and patient- derived xenograft model. Previously, the potential of c-di- GAMP increased the expression levels of maturation mark- ers CD80/CD86 and MHC-II on DCs isolated from spleens of 4T1 tumor-bearing mice, which is important for the presentation of tumor-associated antigens (TAAs) and activation of TAA-specific T cells and can cause tumor regression. The potential of c-di-AMP and other bacterial-derived nucleotide and human origin di-nucleotide should be explored for the potential in antigen presentation and tumor cell-intrinsic cell death mechanism and should be exploited for therapeutic potential.

Declarations

Conflict of interest The authors declare no conflict of interest.

References

1. Soysal SD, Tzankov A, Muenst SE (2015) Role of the tumor microenvironment in breast cancer. Pathobiol J Immunopathol Mol Cell Biol 82(3–4):142–152

2. Sormendi S, Wielockx B (2018) Hypoxia pathway proteins as cen- tral mediators of metabolism in the tumor cells and their microen- vironment. Front Immunol. https://doi.org/10.3389/fimmu.2018. 00040/full

3. Sachet M, Liang YY, Oehler R (2019) The immune response to secondary necrotic cells. Apoptosis. https://doi.org/10.1007/ s10495-017-1413-z

4. Woo S-R, Corrales L, Gajewski TF (2015) Innate immune recog- nition of cancer. Annu Rev Immunol 33:445–474

5. Weinberg SE, Sena LA, Chandel NS (2015) Mitochondria in the regulation of innate and adaptive immunity. Immunity 42(3):406–417

6. Singh K, Sripada L, Lipatova A, Roy M, Prajapati P, Gohel D et al (2018) NLRX1 resides in mitochondrial RNA granules and regulates mitochondrial RNA processing and bioenergetic adapta- tion. Biochim Biophys Acta Mol Cell Res 1865:1260–1276

7. Li A, Yi M, Qin S, Song Y, Chu Q, Wu K (2019) Activat- ing cGAS-STING pathway for the optimal effect of cancer immunotherapy. J Hematol Oncol. https://doi.org/10.1186/ s13045-019-0721-x

8. Corrales L, McWhirter SM, Dubensky TW, Gajewski TF (2016) The host STING pathway at the interface of cancer and immunity. J Clin Invest 126(7):2404–2411

9. Basit A, Cho M-G, Kim E-Y, Kwon D, Kang S-J, Lee J-H (2020) The cGAS/STING/TBK1/IRF3 innate immunity pathway main- tains chromosomal stability through regulation of p21 levels. Exp Mol Med 52(4):643–657

10. Medrano RFV, Hunger A, Mendonça SA, Barbuto JAM, Strauss BE (2017) Immunomodulatory and antitumor effects of type I interferons and their application in cancer therapy. Oncotarget 8(41):71249–71284

11. Lu C, Klement JD, Ibrahim ML, Xiao W, Redd PS, Nayak-Kapoor A et al (2019) Type I interferon suppresses tumor growth through activating the STAT3-granzyme B pathway in tumor-infiltrating cytotoxic T lymphocytes. J Immunother Cancer 7(1):157

12. Fuertes MB, Woo S-R, Burnett B, Fu Y-X, Gajewski TF (2013) Type I interferon response and innate immune sensing of cancer. Trends Immunol 34(2):67–73

13. Maimela NR, Liu S, Zhang Y (2018) Fates of CD8+ T cells in tumor microenvironment. Comput Struct Biotechnol J 22(17):1–13

14. Zhu Y, An X, Zhang X, Qiao Y, Zheng T, Li X (2019) STING: a master regulator in the cancer- mmunity cycle. Mol Cancer. https://doi.org/10.1186/s12943-019-1087-y

1. Andrade WA, Firon A, Schmidt T, Hornung V, Fitzgerald KA, Kurt-Jones EA et al (2016) Group B streptococcus degrades cyclic-di-AMP to modulate STING-dependent type I interferon production. Cell Host Microbe 20(1):49–59

2. Ma F, Li B, Liu SY, Iyer SS, Yu Y, Wu A, Cheng G (2015) Posi- tive feedback regulation of type I IFN production by the IFN- inducible DNA sensor cGAS. J Immunol (Baltimore, Md.: 1950)

194(4):1545–1554. https://doi.org/10.4049/jimmunol.1402066

3. Ablasser A, Hur S (2020) Regulation of cGAS- and RLR-medi- ated immunity to nucleic acids. Nat Immunol 21:17–29

4. Archer KA, Durack J, Portnoy DA (2014) STING-dependent type I IFN production inhibits cell-mediated immunity to listeria monocytogenes. PLoS Pathog. https://doi.org/10.1371/journal. ppat.1003861

5. Barker JR, Koestler BJ, Carpenter VK, Burdette DL, Waters CM, Vance RE et al (2013) STING-dependent recognition of cyclic di- AMP mediates type I interferon responses during chlamydia tra-chomatis infection. mBio. https://doi.org/10.1128/mBio.00018-13

6. Ahn J, Barber GN (2019) STING signaling and host defense against microbial infection. Exp Mol Med 51(12):1–10

7. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F (2013) Genome engineering using the CRISPR-Cas9 system. Nat Protoc 8(11):2281–2308

8. Tomar D, Singh R, Singh AK, Pandya CD, Singh R (2012) TRIM13 regulates ER stress induced autophagy and clono- genic ability of the cells. Biochim Biophys Acta—Mol Cell Res 1823(2):316–326

9. Huber KVM, Olek KM, Müller AC, Soon Heng Tan C, Bennett KL, Colinge J et al (2015) Proteome-wide small molecule and metabolite interaction mapping. Nat Methods 12(11):1055–1057

10. Bhatelia K, Singh A, Tomar D, Singh K, Sripada L, Chagtoo M et al (2014) Antiviral signaling protein MITA acts as a tumor sup- pressor in breast cancer by regulating NF-κB induced cell death. Biochim Biophys Acta. https://doi.org/10.1016/j.bbadis.2013.11. 006

11. Chattopadhyay S, Sen GC (2017) RIG-I-like receptor-induced IRF3 mediated pathway of apoptosis (RIPA): a new antiviral pathway. Protein Cell 8(3):165–168

12. Sun F, Liu Z, Yang Z, Liu S, Guan W (2019) The emerging role of STING-dependent signaling on cell death. Immunol Res 67(2–3):290–296

13. Vargas-Rondón N, Villegas VE, Rondón-Lagos M (2017) The role of chromosomal instability in

cancer and therapeutic responses. Cancers. https://doi.org/10.3390/cancers10010004

14. Li T, Fu J, Zeng Z, Cohen D, Li J, Chen Q, Li B, Liu XS (2020) TIMER2.0 for analysis of tumor-infiltrating immune cells. Nucleic Acids Res. https://doi.org/10.1093/nar/gkaa407

15. Chen Q, Boire A, Jin X, Valiente M, Er EE, Lopez-Soto A, Jacob LS, Patwa R, Shah H, Xu K, Cross JR, Massagué J (2020) Car- cinoma–astrocyte gap junctions promote brain metastasis by cGAMP transfer. Nature. https://doi.org/10.1038/nature18268

16. Zhang M, Lee AV, Rosen JM (2017) The cellular origin and evolu- tion of breast cancer. Cold Spring Harb Perspect Med. https://doi. org/10.1101/cshperspect.a027128

17. Dunphy G, Flannery SM, Almine JF, Connolly DJ, Paulus C, Jøns- son KL et al (2018) Non-canonical activation of the DNA sensing adaptor STING by ATM and IFI16 mediates NF-κB signaling after nuclear DNA damage. Mol Cell 71(5):745-760.e5

18. Su T, Zhang Y, Valerie K, Wang X-Y, Lin S, Zhu G (2019) STING activation in cancer immunotherapy. Theranostics 9(25):7759–7771

19. Yanai H, Chiba S, Hangai S, Kometani K, Inoue A, Kimura Y et al (2018) Revisiting the role of IRF3 in inflammation and immunity by conditional and specifically targeted gene ablation in mice. Proc Natl Acad Sci 115(20):5253–5258

20. Gulen MF, Koch U, Haag SM, Schuler F, Apetoh L, Villunger A et al (2017) Signalling strength determines proapoptotic functions of STING. Nat Commun 8(1):1–10

21. Bakhoum SF, Ngo B, Laughney AM, Cavallo JA, Murphy CJ, Ly P, Shah P, Sriram RK, Watkins TBK, Taunk NK, Duran M, Pauli C, Shaw C, Chadalavada K, Rajasekhar VK, Genovese G, Venkatesan S, Birkbak NJ, McGranahan N, Lundquist M, LaPlant Q, Healey JH, Elemento O, Chung CH, Lee NY, Imielenski M, Nanjangud G, Pe'er D, Cleveland DW, Powell SN, Lammerd- ing J, Swanton C, Cantley LC (2018) Chromosomal instability drives metastasis through a cytosolic DNA response. Nature 553(7689):467–472. https://doi.org/10.1038/nature25432

22. Chen Q, Boire A, Jin X, Valiente M, Er EE, Lopez-Soto A, Jacob L, Patwa R, Shah H, Xu K,

Cross JR, Massagué J (2016) Carcinoma-astrocyte gap junctions promote brain metastasis by cGAMP transfer. Nature 533(7604):493–498. https://doi.org/10. 1038/nature18268

23. Kwon J, Bakhoum SF (2020) The cytosolic DNA-sensing cGAS- STING pathway in cancer. Cancer Discov 10(1):26–39

Figure legends:

Fig. 1 c-di-AMP induces STING mediated IFN response in breast cancer cell lines. **a** Relative expression of STING in ER-positive (MCF-7, ZR75, T-47D) and ER-negative breast cancer cell lines (BT-474, MX-1 MDA-MB-231). **b** Relative expression of cGAS in ER-positive (MCF-7, ZR75, T-47D) and ER-negative breast cancer cell lines (BT-474, MX-1 MDA- MB-231). **c** Pearson correlation (*r*) between *TMEM173* and *ESR1* using TCGA database. **d** Western blot rescued cell proliferation and Caspase-3/7 activity of both the cell types analysis of cGAS and STING proteins in breast cancer cell lines. **e**, **f** MCF-7 [STING (–ve)], MDA-MB-231 [STING (+ve)] breast cancer cell lines were transfected with IFN-β and NF-κB reporter constructs and treated with c-di-AMP (200 μM) for 24 h and IFN-β and NF-κB activity was measured. Data represent mean fold change compared to control (n = 3, mean ± SD); *P < 0.05, **P < 0.01 and ***P < 0.001, based on a Student's t-test

Fig. 2 c-di-AMP binds to STING directly. **a** Protein (PDB: 4KSY). Protein (STING) is represented in the wire model and ligands (c-di- AMP) are shown in a ball and stick model where the color represents atoms (Carbon-green, Nitrogen-blue, Oxygen-red, Hydrogen-white, Phosphorus-pink). The dotted lines indicate the protein–ligand inter- actions (Yellow- hydrogen bond, Pink- salt bridge, Blue- pi–pi inter- action). **b–d** Shows the ligand interactions of molecule A, B and C with the protein (PDB: 4KSY) respectively. Protein is represented in the wire model and ligands are shown in the ball and stick model where the color represents atoms (carbon-green, nitrogen-blue, oxy- gen-red, hydrogen-white, phosphorus-

pink). The dotted lines indicate the protein–ligand interactions (yellow- hydrogen bond, Pink- salt bridge, Blue- pi–pi interaction). **e** Respective dinucleotide and their Docking score. **f** Cellular thermal Shift assay was performed using MDA-MB-231 lysate incubated with c-di-AMP and western blotting was performed (Color figure online)

Fig. 3 c-di-AMP activates cell death in ER-negative breast cancer cell lines: Breast cancer cell lines treated with c-di-AMP (200 μM) and cell survival was monitored by cell viability assay and Cas- pase3/7 activity (n = 3, mean ± SD). **a** MCF-7, **b** MDA-MB-231, **c** BT-474; **d**; MX-1 n = 3, mean ± SD). **e** MCF-7, MDA-MB-231, MX-1 treated with c-di-AMP (200 μM) for 24 h and PARP cleavage was analysed by western blotting. **f** MCF-7, BT-474, MDA-MB-231 and MX-1 treated with c-di-AMP (200 μM) and analysed by phase contrast microscopy image captured on the fourth day.*P < 0.05, **P < 0.01,***P < 0.001 and ***P < 0.0001 based on a Student's t test

Fig. 4 STING is essential for c-di-AMP induced cell death: **a**, **d** BT-474 and MDA-MB-231 were transfected with STING shRNA and treated with c-di-AMP (200 μM) for 4 days and cell survival was ana- lysed (n = 3, mean ± SD). **b**, **e** BT-474 and MDA-MB-231 were trans- fected with STING shRNA and treated with 200 μM c-di-AMP for 24 h and Caspase 3/7 activity was measured (n = 3, mean ± SD). **c**, **f** Western blot was performed to show the level of STING knockdown in BT-474 and MDA-MB-231 breast cancer cell lines.*P < 0.05, **P < 0.01 and ***P < 0.001, based on a Student's t test

Fig. 5 c-di-AMP induces IRF-3 mediated apoptosis in STING posi- tive, ER- negative breast cancer cells. cGAS, p65 and IRF-3 were knockdown MDA-MB-231 (**a**) and MX-1 (**b**) breast cell lines. MDA- MB-231 (**c**) and MX1 (**d**) with cGAS, p65 and IRF-3 knockdown were treated with c-di-AMP (200

μM) and cell viability was meas- ured by presto blue (n = 3, mean ± SD), cGAS, p65 and IRF-3 knock-down MDA-MB-231 and MX-1 were knockdown in breast cell line and treated with c-di-AMP 200 μM and caspase 3/7 activity measured (n = 3, mean ± SD). **e** MDA-MB-231, **f** MX-1 cells were knockdown with IRF-3 and treated with c-di-AMP (200 μM) after 24 h capse3/7 activity measured *P < 0.05, **P < 0.01 and ***P < 0.001, based on a Student's t-test

Fig. 6 c-di-AMP activates STING mediated mitochondrial apopto- sis **a** MDA-MB-231 and MX-1 STING expressing cell lines treated with c-di-AMP (200 μM) for 24 h and IRF-3 puncta was analysed using fluorescent microscope. **b** MDA-MB-231 cells were transfected with IRF-GFP and mt-RFP constructs and their colocalization was measured using fluorescent microscopy in presence and absence of c-di-AMP. **c**, **d** Western blotting was performed in MDA-MB-231 and MX-1 STING expressing cell lines by treating with c-di-AMP 200 μM after 24 h to measure cleaved PARP, Caspase 9 and cleaved caspase 3. **e** STING expressing cell lines MDA-MB-231 and MX-1 were treated with c-di-AMP (200 μM) for 24 h and caspase 3/7 green activity was measured using microscopy. **f** MDA-MB-231 and MX-1 cells were treated with c-di-AMP (200 μM) and the colony-forming assay was performed
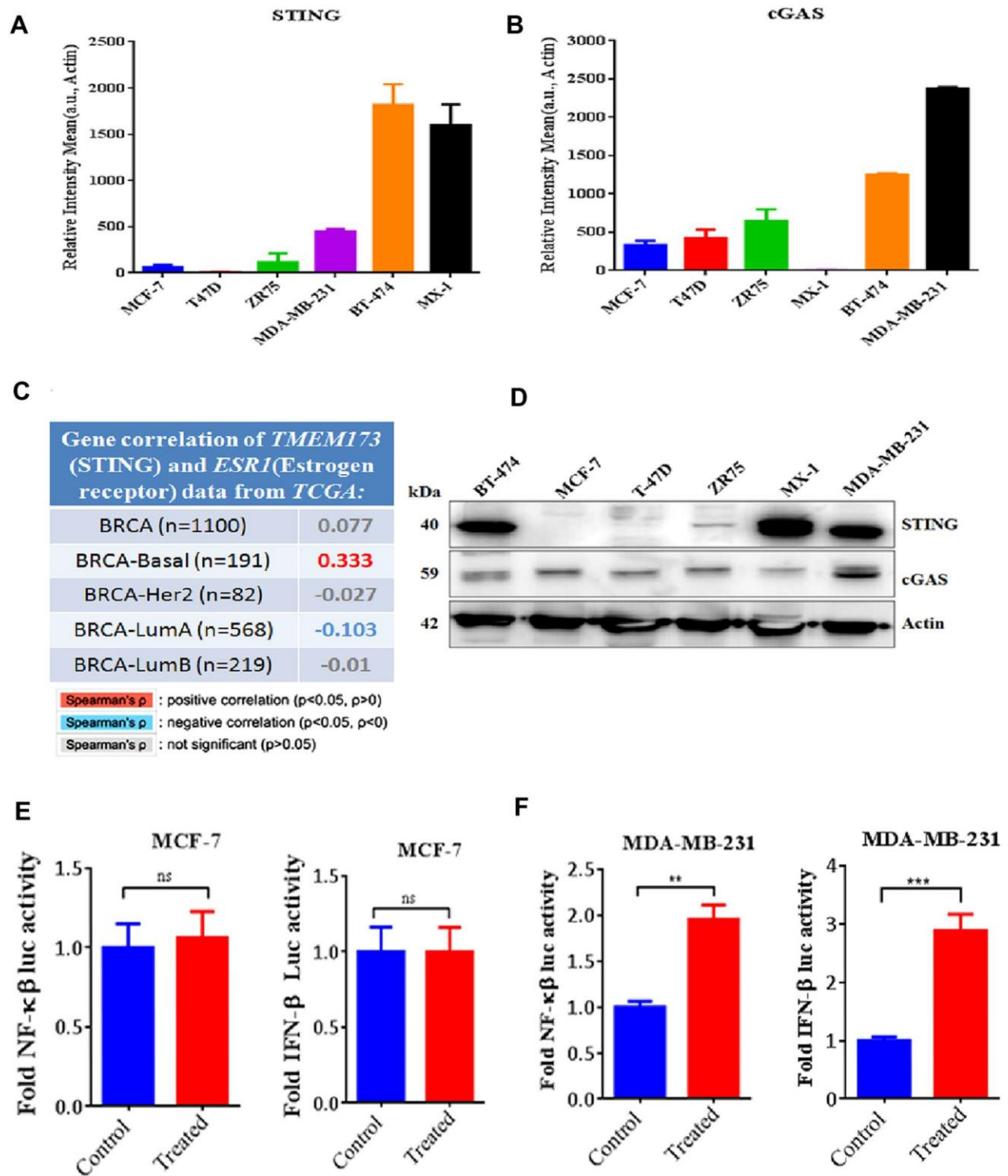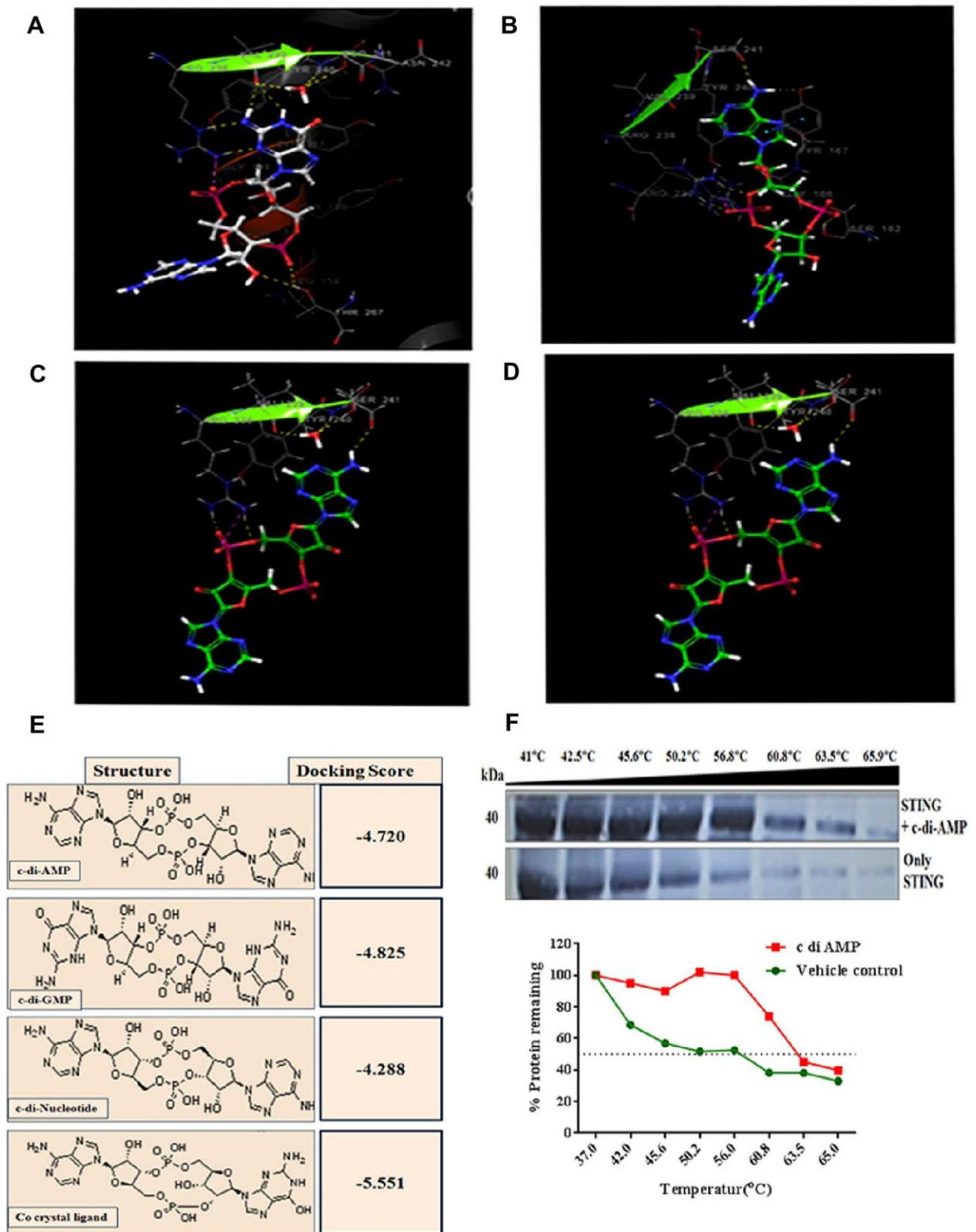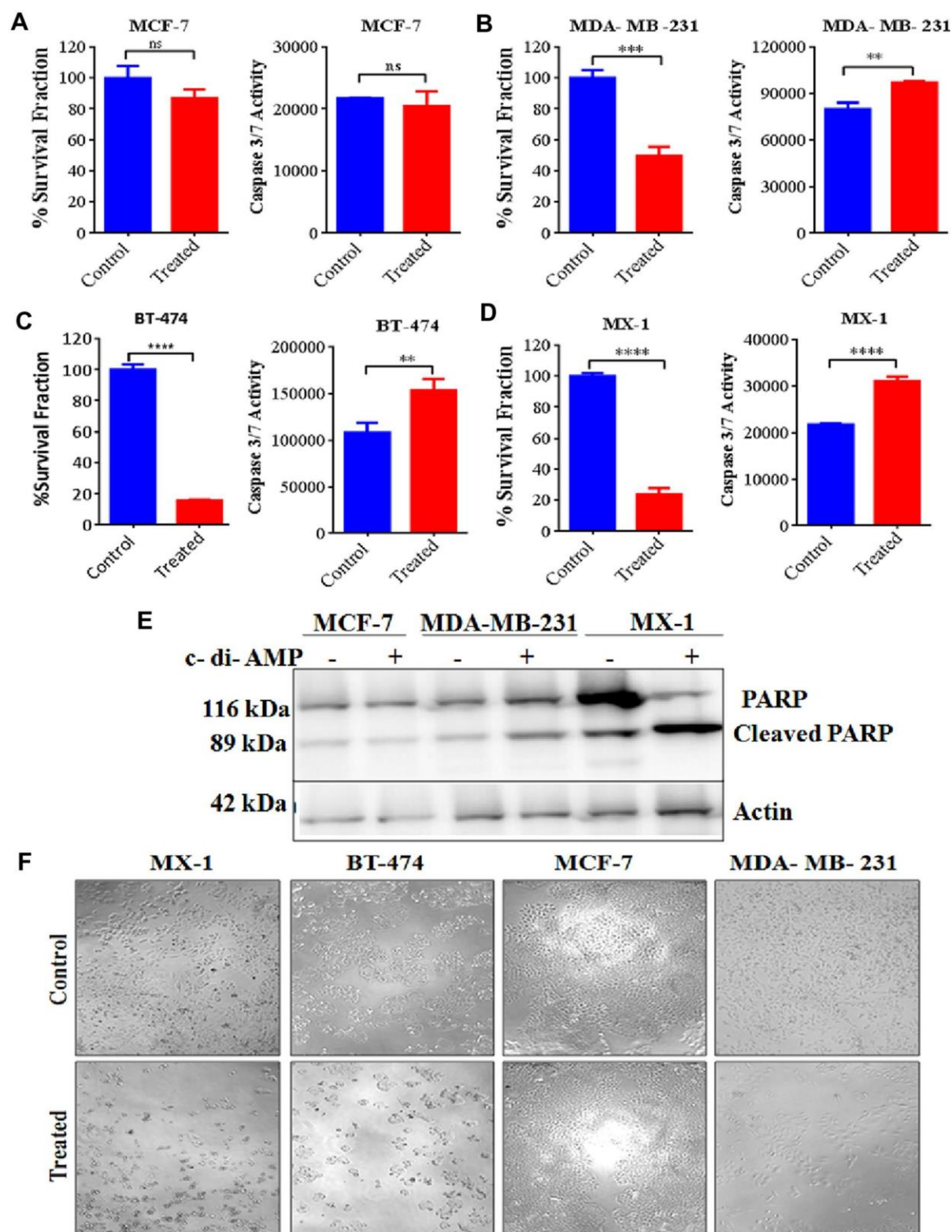
Fig. 1

Fig. 2

	Structure	Docking Score
E	c-di-AMP	−4.720
	c-di-GMP	−4.825
	c-di-Nucleotide	−4.288
	Co crystal ligand	−5.551

Fig. 3

Fig. 4

Fig. 5

Fig. 6