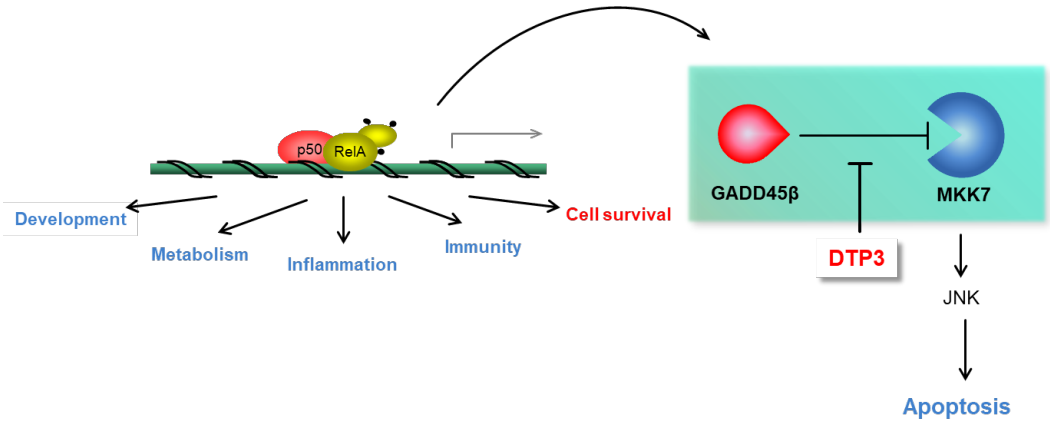
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXV CYCLE

Dr. Paola Arboretto

Interfering with GADD45β-MKK7 survival complex in anaplastic thyroid carcinoma



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Interfering with GADD45β-MKK7 survival complex in anaplastic thyroid carcinoma

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LIST OF ABBREVIATIONS

NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
IκB	Inhibitor of NF-κB
RHD	REL homology domain
TRAF	TNF Receptor Associated Factor
TNFα	Tumor necrosis factor α
IL-1β	Interleukin-1 beta
PAMP	Pathogen Associated Molecular Patterns
DAMP	Damage-associated molecular patterns
TAK	Transforming growth factor-β-activated kinase 1
IKK	IkappaB kinase
NIK	NF-κB-inducing kinase
NEMO	Nuclear factor-κB essential modulator
RANK	Receptor Activator of Nuclear Factor κ B
CD40	Cluster of Differentiation 40
IAP	Cellular inhibitor of apoptosis
TC	Thyroid cancer
PTC	Papillary thyroid cancer
FTC	Follicular thyroid cancer
PDTC	Poorly differentiated thyroid cancer
ATC	Anaplastic thyroid cancer
RET	Receptor tyrosine kinase
MAPK	Mitogen-activated protein kinase
PI3K	Phosphatidyl Inositol 3-Kinase
PDC	Programmed cell death
IRAK	Interleukin-1 receptor-associated kinase
TLR	Toll-Like Receptor
BCR	B-Cell Receptor

BTK	Bruton's tyrosine kinase
UPS	Ubiquitin–proteasome system
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
VEGF	Vascular endothelial growth factor
TAM	Tumor-associated macrophages
JNK	c-Jun N-terminal kinase
MKK7	Dual specificity mitogen-activated protein kinase kinase 7
ERK	Extracellular signal-regulated kinase
BCL-2	B-cell lymphoma 2
GADD45-β	Growth Arrest and DNA Damage 45 beta
MM	Multiple Myeloma
FITC	Fluorescein isothiocyanate
SCR	Scrambled
DEGs	Differentially expressed genes
ER	Endoplasmic reticulum

ABSTRACT

The alteration of the apoptotic machinery is an essential hallmark of cancer. Transcription factors belonging to NF-κB family play a crucial role orchestrating the balance of many apoptotic genes. Among the anti-apoptotic NF-κB effectors, GADD45β exerts a pivotal function hampering apoptosis and promoting cell survival. Alterations in GADD45β expression correlate with negative prognosis and worse outcome in several human malignancies. Therefore, GADD45β represented an attractive therapeutic target leading to the development of the first-in-class inhibitor DTP3 in Multiple Myeloma. Indeed, DTP3 showed to interfere with GADD45β aberrant activity with high efficacy and cancer-specificity.

The purpose of this study was to investigate DTP3 biological efficacy in anaplastic thyroid carcinoma (ATC) with high GADD45β expression levels.

For this purpose, ATC cell lines were screened for GADD45β expression levels and characterized for DTP3 dose-response assays. The results showed DTP3 effective permeability and biological activity in ATC cell lines. Accordingly, IC_{50} values revealed the correlation between GADD45β expression levels and DTP3 efficacy. Moreover, proliferation and apoptosis assays on DTP3-responsive cell lines demonstrated that DTP3 triggers cell cycle arrest followed by delayed apoptosis. Thus, the results revealed that DTP3 affects MAPK/ERK pathway followed by JNK signaling activation. Strikingly, RNA-seq data analysis unveiled that DTP3 treatment prompts reticulum endoplasmic (ER) stress response altering ER genes expression levels.

Overall, these findings demonstrate DTP3 specificity and efficacy in ATC cell lines expressing high Gadd45β levels via a novel mechanism that involves ER stress response and eventually the activation of apoptotic pathway.

BACKGROUND

The NF- κB transcription factors family

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) represents a family of inducible and ubiquitous transcription factors that coordinates the expression of a plethora of genes involved in key cellular processes (T. Liu et al., 2017). Significant research efforts over three decades have established the pivotal role of NF-κB proteins in innate and adaptive immunity, stress response, apoptosis, and cellular growth and differentiation (Hayden & Ghosh, 2011; Oeckinghaus et al., 2011).

In mammals, the NF-κB transcription factor family consists of five members known as p65 (RelA), RelB, c-Rel, p50/NF-κB1 (p105), and p52/NF-κB2 (p100), which associate by forming homodimeric and heterodimeric complexes (Hayden & Ghosh, 2012; T. Liu et al., 2017). These related subunits share a conservative N-terminal REL homology domain (RHD) responsible for the dimerization, the nuclear translocation, and the binding to the DNA sequences as well as the interaction with regulatory proteins (S. C. Sun & Ley, 2008). Unlike the other family members, p100 and p105 C-terminal regions lack the transactivation domain and need proteasomal partial degradation to produce the mature forms, p50 and p52 respectively, which act either as transcriptional repressors or activators. Therefore, NF-κB shows different transcriptional activity depending on the subunit composition of dimer complexes (Smith et al., 2019).

In resting cells, NF-κB is retained inactive in the cytoplasm by IκB proteins, including IκBα, IκBβ, IκBε, and IκBζ, and related protein members, which interact with the RHD domains masking the nuclear localization sequence (NLS). Interestingly, p100 and p105 precursors are themselves considered IκB-like proteins as they interact and inhibit NF-κB dimers via their RHD domains (Oeckinghaus & Ghosh, 2009). Following the proteasomal-dependent degradation of IκBs, NF-κB complexes are free to enter the nucleus, where they bind consensus DNA sequences, known as κB sites, and regulate the activation of tissues- and stimuli-specific transcriptional programs (Wan & Lenardo, 2009) Indeed, the expression pattern of the regulatory molecules, including surface receptors, along with the heterogeneous IKK and IκB distribution in each cell lineage, mirrors specific responses to the external stimuli (Tak & Firestein, 2001).

The NF-κB signaling pathway

Molecular agonists and different stimuli trigger the activation of NF-κB signaling through the interaction with several membrane receptors. The signal transduction is mediated by the recruitment of a variety of interacting and scaffold proteins, the nuclear translocation of active protein dimers and the interaction with κB sites culminating eventually in the transcription of target genes (Mitchell et al., 2016).

NF-κB signal cascade has been broadly studied and classified into three different pathways: the canonical, the non-canonical, and the recently characterized atypical pathway.

The canonical pathway, also known as classical, is rapidly activated by inflammatory cytokines, such as Tumor necrosis factor α (TNFα) and Interleukin 1β (IL-1β), pathogen-associated molecular patterns (PAMPs), and some danger-associated molecular patterns (DAMPs) (Taniguchi & Karin, 2018). Upon stimulation of their cognate receptors, these ligands trigger a signaling cascade that culminates in the activation of the IKβ kinase (IKK) complex, a large multicomponent protein kinases hub, that consists of the two homologous serine/threonine kinases IKK1 (IKKα) and IKK2 (IKKβ) and the regulatory subunit NFκB essential modulator (NEMO; also called IKKγ) (Israël, 2010).

The signal transduction cascade involves the recruitment of E3 ubiquitin ligases, adaptors, and protein kinases which in turn assemble proximal multiprotein complexes on polyubiquitin scaffolds modulating localization and site-specific phosphorylation signals. The functional assembly of this oligomeric protein complex is driven by polyubiquitin chain signals that are involved in a proteasomal degradation-independent mechanism acting as pivotal docking sites (Courtois & Fauvarque, 2018). Moreover, IKKγ/NEMO plays a fundamental role in this context as target of ubiquitination as well as a ubiquitin binding protein. Indeed, it has been demonstrated that NEMO binding to polyubiquitin chains promotes the recruitment of IκBβ proteins converging in NF- kB full activation (Wertz & Dixit, 2010). Structural studies have indicated that NEMO recognizes M1-linked di-ubiquitin and K63-linked polyubiquitin through the coiled-coil and zinc-finger (CC-LZ) domains serving as integration platform coupling upstream stimuli to IKK complex activation (Hadian et al., 2011; Rahighi et al., 2009). The clustering of the recruited proteins induces proximity of IKK multicomplex and IKK kinases, such as TAK1 or MEKK3 allowing the *trans*-autophosphorylation of Ser 177 and 181, for IKKβ, and Ser 176 and 180, for IKKα, located in their activation T loop and the subsequent full activation (Hinz & Scheidereit, 2014). The active IKK complex catalyzes the phosphorylation of IκB proteins (on Ser32 and Ser36 in IκBα) triggering their K48-linked polyubiquitination by Skp1–Cullin–F-box ($SCF^{\beta TrCP}$) ubiquitin

ligase complex at conserved lysine residues, K21 and K22, thus targeting them to 26S proteasome degradation (Collins et al., 2016; Xia et al., 2018). This results in release and translocation of p50, RELA and c-Rel dimers into the nucleus, where the inducible transcriptional program starts (Oeckinghaus & Ghosh, 2009)(Fig.1).

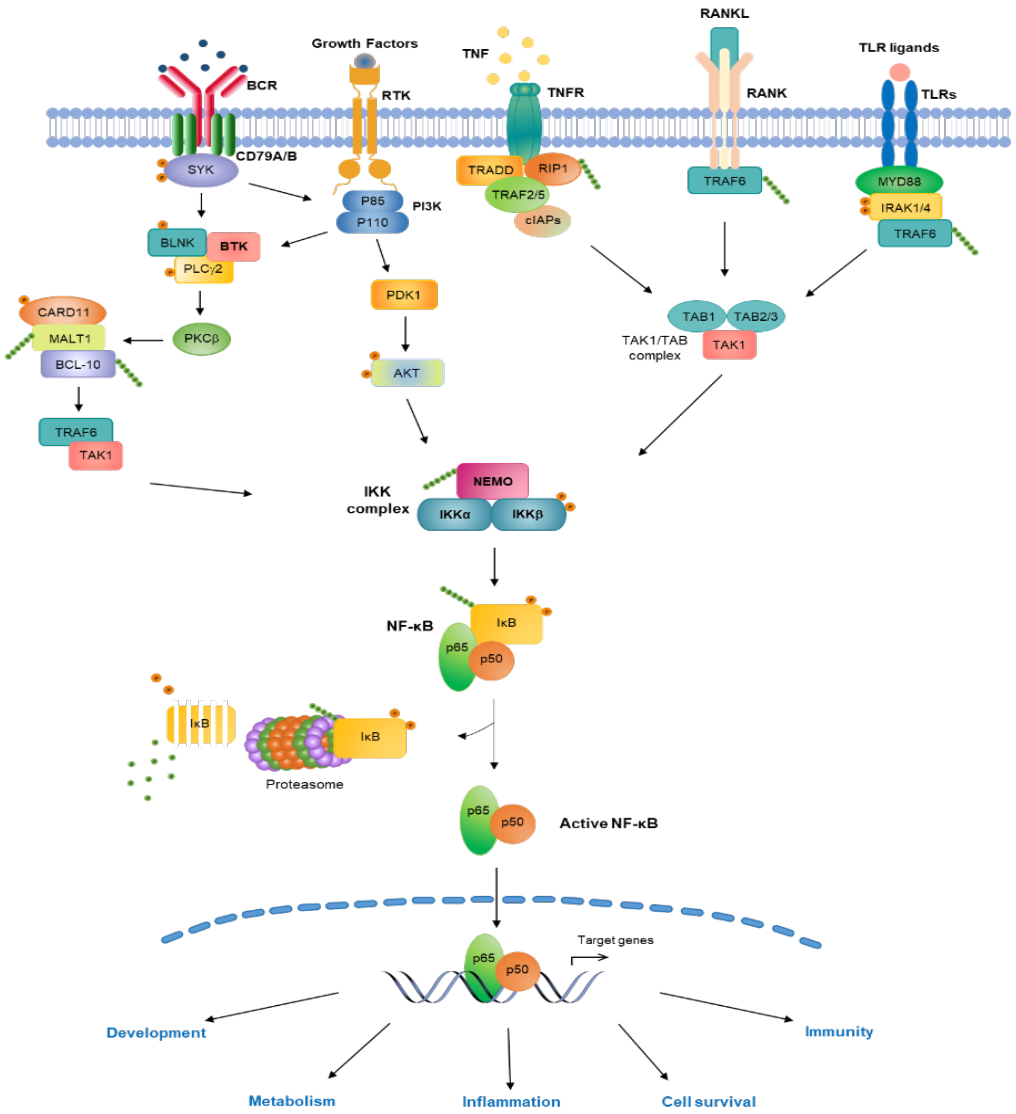


Figure 1. Schematic representation of the canonical NF-κB pathway.
TNF: tumor necrosis factor α; TNFR: tumor necrosis factor receptor; TLRs: toll-like receptors; IKK complex: IκB kinase enzyme complex to upregulate the NF-κB signaling; IκBα: IκB kinase 1 (IKK1); IκBβ: IκB kinase 2 (IKK2); NEMO: NF-kappa-B essential modulator; p50: NFKB1; p65: RelA; TRAF6: TNF receptor-associated factor 6. (Verzella et al., 2022)

Conversely, the alternative pathway of NF-κB activation, also designated as non-canonical, is induced by a different group of receptors including B-cell activation factor (BAFFR), lymphotoxin β-receptor (LTβR), CD40, receptor activator for nuclear factor kappa B (RANK), TNFR2 and Fn14 (S.-C. Sun, 2017).

In contrast to canonical NF-κB signaling, which is subject to a quick and transient activation, the non-canonical NF-κB pathway is characterized by a slower and persistent kinetic (S.-C. Sun, 2017; Yu et al., 2020). Additionally, it relies on NF-κB inducing kinase (NIK) proteins which phosphorylate and activate predominantly IKKα and does require neither IKKβ nor NEMO (Dejardin et al., 2002; Hoesel & Schmid, 2013).

In unstimulated cells a ubiquitin ligase complex, comprising TRAF3, TRAF2 and cIAP1/2, is responsible for NIK K-48-linked polyubiquitination and subsequent constitutive proteasome-mediated degradation thus controlling its expression levels (Cildir et al., 2016). Receptor engagement by specific ligands triggers NIK stabilization, which phosphorylates the downstream target IKKα on T-loop serine residues, S176 and S180 leading to its activation. Activated IKKα then phosphorylates p100 on C-terminal serine residues, S866 and S870, thereby creating a docking site for $SCF^{\beta TrCP}$ complex and Ubc4/5 ubiquitin E2 ligase (Begalli et al., 2017; Cildir et al., 2016).Therefore, the K48-linked polyubiquitination of p100 C-terminal domain enables its partial degradation by proteasome, thereby generating the mature p52 subunit. Hence, p52 together with RelB form RelB/p52 heterodimers, which translocate to the nucleus to regulate the transcriptional program governed by non-canonical NF-B signaling (Begalli et al., 2017; Cildir et al., 2016; Razani et al., 2011; S.-C. Sun, 2017; S. C. Sun, 2010)(Fig.2).

Finally, the third so called ‘atypical pathway’ refers to NF- κB activation in response to DNA damage or oxygen stress (Janssens & Tschopp, 2006). Unlike the canonical and non-canonical pathways, the atypical signaling cascade mirrors a retrograde nucleus-cytoplasm pathway induced by genotoxic agents, such as ionizing radiation, radiomimetic chemicals, topoisomerase I and II poisons, and oxygen radicals (Z. H. Wu & Miyamoto, 2007).
Huang et al. demonstrated that genotoxic stress prompts NEMO SUMOylation allowing its nuclear translocation (T. T. Huang et al., 2003). Then, sumo-NEMO is recognized by the DNA damage-responsive kinase ataxia telangiectasia mutated (ATM) which primes its mono-ubiquitination with subsequent cytoplasmatic translocation and IKK activation (T. T. Huang et al., 2003; Janssens & Tschopp, 2006; Miyamoto, 2010).
After NF-κB activation, a multi-layered network of negative feedback loops enables the termination of response. This negative feedback system ensures a temporally and spatially organized NF-kB signaling deactivation. Indeed, since

NF-κB signaling is crucial for many cellular processes, all negative feedback mechanisms are essential to restore the homeostasis and prevent excessive inflammation, tissue damage and the onset of several diseases (Begalli et al., 2017; Renner & Lienhard Schmitz, 2009)

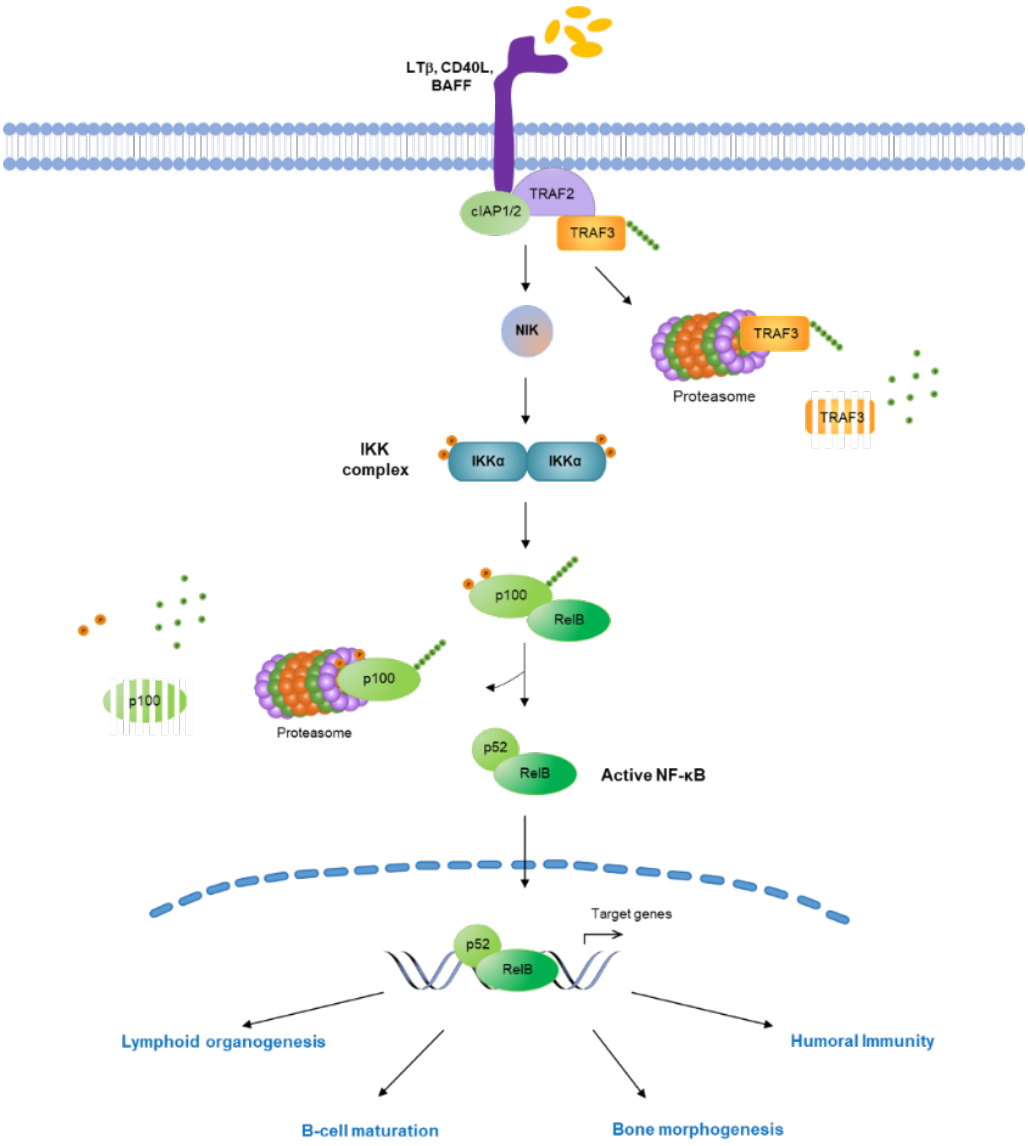


Figure 2. Schematic representation of the non-canonical NF-B pathway.
LTβ: lymphotoxin β; CD40L: cluster of differentiation 40 ligand, BAFF: B-cell activating factor; NIK: NF-κB inducing kinase; IKKα: Inhibitor of NFκ-B kinase subunit alpha P: phosphorylated; p100: NF-κB2 (p52) precursor protein; RelB: transcription factor RelB; cIAP1/2: Inhibitor of apoptosis protein 1and 2, TRAF: TNF receptor-associated factor. (Verzella et al., 2022)

NF-κB: an active player in thyroid cancer physiopathology

Thyroid cancer (TC) is the most common malignancy of the endocrine system arising from various cell types within the thyroid gland (Guenter et al., 2021). This endocrine malignancy comprises four histological subtypes: papillary thyroid carcinoma (PTC 80–85%), follicular thyroid carcinoma (FTC 10–15%), poorly-differentiated thyroid carcinoma (PDTC, <2%), and anaplastic thyroid carcinoma (ATC, <2%) (Laha et al., 2020).

PTC and FTC carcinomas are classified as well-differentiated thyroid cancers (DTC) and generally have good prognosis after appropriate treatment. However, about 10% of DTC eventually experience resistance to radioactive iodine therapy leading to cancer-specific death (Shin & Koo, 2022). Conversely, PDTC and ATC show aggressive features and are associated with lymph node and distant metastasis (D. Wen et al., 2019). Although PDTC and ATC are relatively rare, these cancers represent an unmet clinical question because of their poor survival and the inefficacy of classical therapies (Prete et al., 2021).

An emerging body of literature shows that NF-κB signaling pathway plays a major role in the initiation and progression of thyroid carcinoma (Giuliani et al., 2018). Constitutive NF-κB activation was reported in a panel of thyroid carcinoma cell lines which showed a significant increase of p65 activity in the nuclei compared to normal thyroid cells (Visconti et al., 1997). Subsequent studies confirmed the aberrant NF-κB nuclear activity on tissue specimens from papillary, follicular, and anaplastic thyroid cancers by immunohistochemical staining (Le et al., 2018; Pacifico et al., 2004; Pacifico & Leonardi, 2010) (Fig.3).

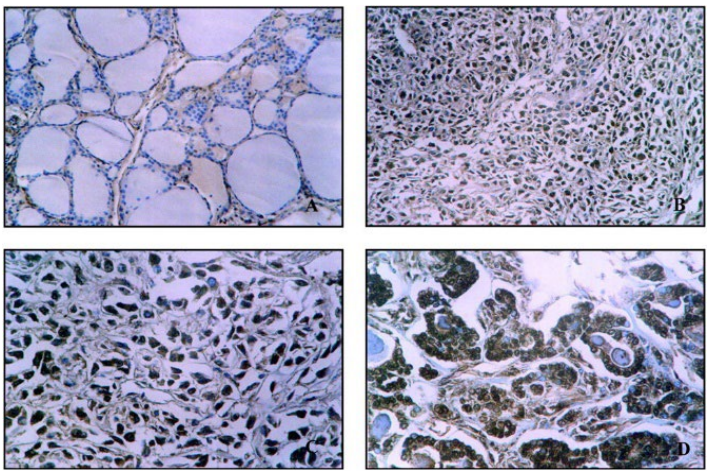


Figure 3. NF-κB is constitutively activated in anaplastic thyroid carcinoma. NF-κB constitutive activity in primary human anaplastic thyroid carcinomas. NF-κB staining of primary human anaplastic thyroid carcinomas (B–D) and normal thyroid tissue (A).(Pacifico & Leonardi, 2010)

The genetic landscape underpinning thyroid cancer development has been extensively studied (Hlozek et al., 2022; Prete et al., 2020). Thyroid cancer signature has shown frequent molecular lesions in crucial proteins involved in mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) cascades, such as RET, RAS, BRAF as well as PTEN (Younis, 2017). The alterations in the MAPK and PI3K signaling pathways in turn elicit NF-κB activation promoting a more aggressive phenotype (X. Li et al., 2013).

Furthermore, the crosstalk between NF-κB and MAPK signaling pathways has a crucial role in the control of the programmed cell death (PCD) (S. Papa et al., 2006). Indeed, it has been shown that NF-κB exerts a protective mechanism against PCD regulating c-Jun N-terminal kinases (JNKs) activities (Salvatore Papa, Zazzeroni, Pham, et al., 2004). These evidence demonstrated that the balance between JNK and NF-κB activity determines cell fate in response to external stimuli (Salvatore Papa, Zazzeroni, Pham, et al., 2004). JNKs are multifunctional kinases, also known as stress-activated protein kinases, that are involved in many physiological and pathological processes (Cui et al., 2007). JNKs were initially identified as UV and stress responsive protein kinases regulating downstream c-Jun transcription factor phosphorylation (Cui et al., 2007). Interestingly, Bulgin et al. demonstrated that JNKs play a crucial role in ATC cell lines survival diminishing ionizing radiation (IR) sensitivity (Bulgin et al., 2006).

It has been shown that NF-κB acts as oncogenic driver enhancing the expression of anti-apoptotic genes, promoting cell proliferation through mitogenic effectors, like c-MYC and Cyclin D1 and influencing tumor microenvironment via pro-inflammatory cytokines, chemokines, and growth factors (Giuliani et al., 2018). Moreover, recent studies revealed that NF-κB activation upregulates the expression of matrix metalloproteinases promoting invasive and metastatic features (Bauerle et al., 2010; Palona et al., 2006).

Additional findings showed NF-κB role in thyroid cancer chemotherapy and radiotherapy resistance (Pacifico & Leonardi, 2010). Indeed, several molecular approaches aimed to inhibit NF-κB signaling showed to be effective in reducing thyroid cancer cell growth, enhancing chemo-sensitivity, and increasing drug-induced apoptosis (Bauerle et al., 2010; Meng et al., 2012; Starenki, Namba, Saenko, Ohtsuru, Maeda, et al., 2004). Therefore, NF-κB pathway represents a promising candidate in the targeted therapy for thyroid cancer.

NF-kB pathway modulation in targeted cancer therapy

NF-κB signaling pathway regulates the transcription of a variety of genes involved in cell proliferation, survival, angiogenesis, metabolism, and differentiation (Xia et al., 2018)(Fig.4).

Molecular imbalance of the signaling pathways results in the development of several diseases and the pathogenesis of many disorders (Hoesel & Schmid, 2013; Yu et al., 2020). Constitutive NF-κB activation can be underpinned by intrinsic or extrinsic factors depending on the molecular lesions or the stimuli that fuel the signal cascade (Colombo et al., 2018). Among the intrinsic factors, there are strong evidence about mutations or epigenetic alterations involving regulator and effector proteins playing an active role in the signal transduction (Chaturvedi et al., 2011). However, many other signal networks as well as tumor microenvironment converge in NF-κB activation representing oncogenic extrinsic factors (Staudt, 2010).

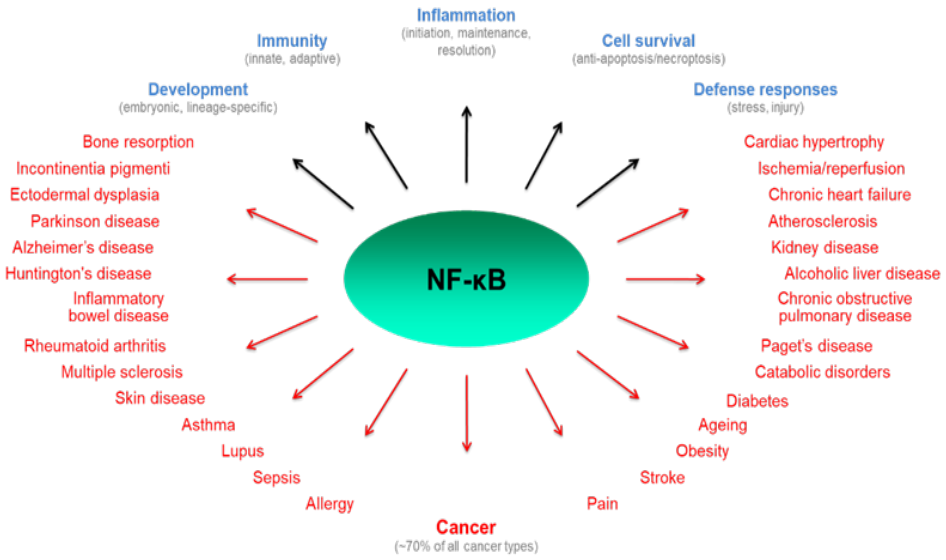


Fig.4. NF-κB: a pleiotropic pathway. NF-κB transcription factor family regulates many physiological processes (blue). Alterations in the NF-κB pathway are involved in human disorders (red).

Since deregulated NF-κB activation is a hallmark of most human cancers, over the last three decades lot of efforts have been addressed to therapeutically target this signal cascade (Begalli et al., 2017).

The available therapeutic approaches can be conventionally classified based on the hierarchical position of targeted-proteins in the NF-κB signal transduction network:

- *Agents Acting Upstream of IKK*

The variety of stimuli that trigger IKK activation provide a clear opportunity for therapeutic interventions (Fig.5).

Member of TNF-α receptor (TNF-α-R) superfamily and their ligands are strong inducers of NF-κB signaling as they are involved in self-enhancing loops. Several antibodies, such as Infliximab, as well as other TNF-α inhibitors are currently approved for the treatment of ulcerative colitis, rheumatoid arthritis, ankylosing spondylitis, psoriasis, and psoriatic arthritis (Gerriets et al., 2022). Likewise, brentuximab vedotin (Adcetris) directed against the TNF-R member CD30, is approved for the treatment of Hodgkin Lymphoma and anaplastic large-cell lymphoma (ALCL) which express high levels of CD30 (Gotlib et al., 2019; O'Connor et al., 2018). Moreover, antagonists of the RANK-RANKL signaling axis, which plays an essential role in osteoclast development, are being used to treat bone-associated malignant pathologies (Ming et al., 2020). However, no small molecule inhibitor of either RANK or RANKL is currently in use in a clinical setting (Ramadass et al., 2020).

Signaling intermediates of activated TLRs have been shown to be attractive therapeutic targets in cancer patients. Toll-like receptors (TLRs) sense pathogen-associated molecular patterns (PAMPs) to initiate innate immune responses (Kawasaki & Kawai, 2014). Except for TLR3, NF-κB activation by TLRs depends on the adaptor protein, MYD88, which is frequently targeted by gain-of-function mutations in hematological malignancies (J. Q. Wang et al., 2014). To date, trials evaluating synthetic antisense oligonucleotides or small-molecule antagonists targeting TLR7/TLR8/TLR9 have been completed, but the results have not been reported yet. However, additional promising therapeutic targets involved in TLR signaling are Interleukin-1 receptor-associated kinase (IRAK)1 and IRAK4 (Bennett & Starczynowski, 2022). The inhibition of IRAK proteins has demonstrated a clear efficacy against non-Hodgkin's lymphomas (NHLs), myelodysplastic syndrome (MDS), T-cell acute lymphoblastic leukemia (T-ALL), and melanoma in preclinical settings (H. Z. Lee et al., 2015; Srivastava et al., 2012).

An important role in the regulation of both canonical and non-canonical NF-κB signaling is played by cellular Inhibitor of Apoptosis Proteins (c-IAP). c-IAP genes are targeted by both gain-of-function and loss-of-function mutations in cancer (Gyrd-Hansen & Meier, 2010). Interestingly, non-peptidomimetic small-

molecule inhibitors and antagonists of c-IAP1/2 are currently in phase I/II trials in patients with advanced-stage solid tumors (Begalli et al., 2017).

Furthermore, somatic mutations in PI3K pathway trigger NF-κB in many human cancers (Hoesel & Schmid, 2013). Thus, several classes of PI3K inhibitors have been developed to block oncogenic signaling and the downstream NF-κB activation (Hoxhaj & Manning, 2020). In 2014, the orally available PI3K inhibitor, CAL-101 (idelalisib), became the first PI3K-targeting agent to receive FDA approval for the treatment of relapsed or refractory chronic lymphocytic leukemia (CLL) in combination with the anti-CD20 antibody, rituximab (Zirlik & Veelken, 2018). Numerous PI3K signaling inhibitors, are currently in several phase II and phase III trials, generally in combination with other agents. Nevertheless, the clinical utility of PI3K inhibitors in cancer treatment is still limited due to recurrence of adverse effects, such as infections and inflammation, as well as drug resistance (J. Yang et al., 2019).

In many lymphoid malignancies, NF-κB is frequently activated by genetic mutations targeting the B Cell Receptor (BCR) complex (Young et al., 2019). Bruton's tyrosine kinase (BTK) plays an essential role in driving NF-κB activation and B-cell survival BCR-mediated (S. Q. Zhang et al., 2015). The irreversible oral BTK inhibitor, ibrutinib, is the first agent in this class to be approved for medicinal use. Moreover, due to the onset of ibrutinib-resistance mechanisms, second-generation BTK inhibitors have been approved by the EMA and the FDA as monotherapies or in combination with other agents (T. Wen et al., 2020).

Despite the clinical results obtained by targeting upstream NF-κB signaling mechanisms, the clinical benefits have been limited by dose-limiting toxicities, primary resistance of certain cancer types, and eventually the onset of secondary drug resistance.

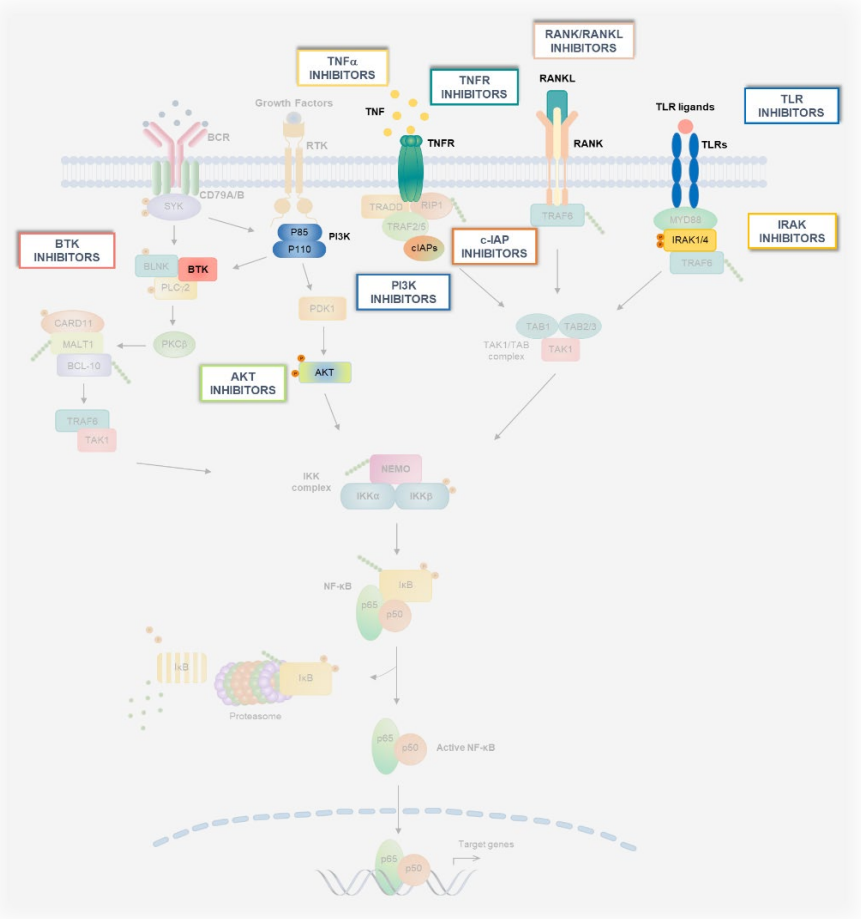


Figure 5. Schematic representation of agents acting upstream of IKK. NF-κB inhibitors acting by blocking activators ustream of IKK complex in the canonical NF-κB pathway. (Verzella et. al., 2022)

- *Agents Targeting Core Components of NF-kB Pathway*

The IKK family regulates NF-κB pathway during inflammation, immune cell activation, and tumorigenesis (Hayden & Ghosh, 2012). Aberrant activation of IKK family kinases is involved in different pathologic conditions including cancer (Antonia et al., 2021). Accordingly, IKK inhibitors were proven to be effective at inhibiting the NF-κB pathway. Nevertheless, they were found to be associated to severe toxicities due to the blockade of NF-κB ubiquitous functions (Dyer & Zhang, 2014). For instance, the deregulated activity of the IKKε/TANK-binding kinase 1 (TBK1) has been associated with a wide range of human diseases including autoimmune, inflammatory, and malignant disorders

(Barbie et al., 2014; Hasan & Yan, 2016; J. Y. Kim et al., 2013). Although several small molecules showed efficacy in inhibiting IKKε and TBK1, no specific molecules targeting these kinases have been evaluated in clinical trials (Thomson & Bergamini, 2021).

Since the non-canonical signaling is also frequently activated in several cancers by genetic alterations, NIK has emerged as an attractive target for therapeutic interventions (Verzella et al., 2022). Over the past decade, many NIK inhibitors have been investigated and showed good efficacy in treating inflammatory, metabolic, and immune diseases (Brightbill et al., 2018; Z. Li et al., 2020; Ren et al., 2017). Although encouraging preclinical results, no clear clinical candidates have emerged so far.

NF-κB pathway regulation is strongly related to the ubiquitin-proteasome system (UPS) and relevant partner enzymes (Laplante & Zhang, 2021). Therefore, several compound and small molecules have been introduced targeting different components such as the ubiquitin activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), ubiquitin ligases (E3s), the 20S proteasome catalytic core (20S), and the 19S proteasome regulatory particles (19S) as well as ubiquitin (Ub), ubiquitin-like proteins (Ubl), and Deubiquitinating enzymes (DUBs) (Komander et al., 2009; X. Zhang et al., 2020). For instance, Bortezomib and second-generation proteasome inhibitors have been approved by FDA for the treatment of several malignancies (Luttwak et al., 2020; Sivaraj et al., 2017). However, further optimization studies are needed due to the onset of numerous side effects and drug resistance (Suzuki et al., 2011).

In addition to ubiquitination, NF-κB transcriptional activity is regulated by other post-translational modifications such as acetylation, methylation, SUMOylation, and phosphorylation (Baud & Collares, 2016; B. Huang et al., 2010). Thus, the acetylation and methylation processes have been widely explored for therapeutic applications. Several HAT and HDAC, as well as DNA methyltransferase (DNTM) inhibitors (i.e., decitabine, vorinostat, romidepsin, belinostat, Panobinostat, tucidinostat and azacytidine), have been investigated in combination therapy in clinical settings, and most of them have been approved by FDA for the treatment of various pathologies (Kaminskas et al., 2005; N. Kim et al., 2022; H. Z. Lee et al., 2015; D. Wu et al., 2020). To date, two HDAC inhibitors, vorinostat and depsipetide, have been approved by the FDA to treat several hematological malignancies (Mann et al., 2007; Vandermolen et al., 2011). Additional studies are needed to evaluate the use of these inhibitors as monotherapies for the treatment of solid cancers.

Several other approaches aimed to interfere with NF-κB transcriptional program are still under investigation.

Despite the clinical improvements observed following treatment with core pathway inhibitors in specific tumors, patients often experience severe toxic effects. In addition, drug resistance remains the major unsolved question to the successful treatment of cancer. These findings underscore that the development of disease-specific, clinically useful anti-NF-κB agents is still an unmet medical need (Fig.6).

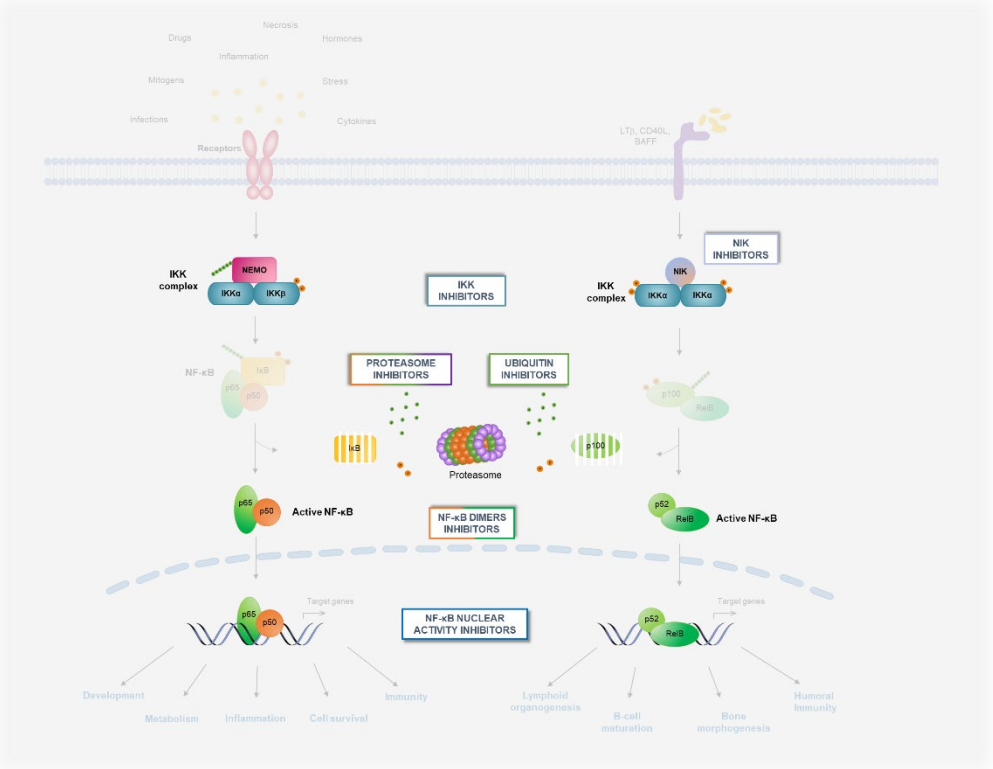


Figure 6. Schematic representation of agents targeting core components of NF-kB pathway. NF-κB-targeted therapeutics acting by inhibiting the canonical and non-canonical NF-κ core pathway. NEMO, Nuclear factor-κB (NF-κB) essential modulator (NEMO); IKK, IκB kinase. (Verzella et al., 2022)

- *Inhibitors of NF-kB Downstream Effectors*

NF-κB regulates the transcription of many anti-apoptotic target genes belonging to Bcl-2 family proteins, such as Bcl-XL and BCL-2 (D'Aguanno & Del Bufalo, 2020). Since Bcl-2 proteins result overexpressed in several cancer types, the pharmacological targeting has been extensively addressed for

therapeutical purposes. Recently, “BH3-mimetics” drugs—that mimic the pro-apoptotic functions of the BH3 domains of Bcl-2 proteins—have been the principal approach to blocking the anti-apoptotic function of Bcl-2 family members (Townsend et al., 2021). Although several agents have been tested in preclinical and clinical studies, only ABT-199 (Venetoclax) has been approved by the FDA for the treatment of patients with CLL; SLL patients; and newly diagnosed AML-aged 75 years or older (Juárez-Salcedo et al., 2019; Suvarna et al., 2019; Townsend et al., 2021). In addition, Venetoclax is currently being tested in combination therapies for the treatment of acute lymphoblastic leukemia (ALL) and DLBCL (Juárez-Salcedo et al., 2019). Moreover, several other BCL-2, BCL-XL and inhibitors are under investigation as single agents or in combination therapies in hematological and solid cancers to improve the selectivity for a specific Bcl-2 member and the safety profile (D’Aguanno & Del Bufalo, 2020; Montero & Letai, 2017).

Moreover, the principal effectors of NF-κB pro-angiogenetic function belong to the vascular endothelial growth factor (VEGF) family, which comprises five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PIGF). These factors interact with several tyrosine-kinase receptors such as VEGFR-1, VEGFR-2, and VEGFR-3 to promote tumor-associated angiogenesis, tissue infiltration, and metastasis formation (Ceci et al., 2020; Haibe et al., 2020) Bevacizumab (BVZ) is a humanized anti-VEGF-A monoclonal antibody which blocks the interaction between VEGF-A and its receptor by binding circulating and soluble VEGFA (Haibe et al., 2020). BVZ is the first anti-angiogenic drug approved by FDA and EMEA for the treatment, in combination with chemotherapy, of several solid tumors (Cheng et al., 2022; M. S. Lee et al., 2020). Additional VEGFR inhibitors have been clinically investigated and showed good efficacy. Collectively, these data support the use of angiogenetic inhibitors in combination with standard-of care chemotherapy for the treatment of several incurable advanced tumors.

Among NF-κB target genes, proinflammatory cytokines play crucial role in tumor-promotion and inflammation (Verzella et al., 2022). One of the key proinflammatory cytokines mediating oncogenic functions is interleukin-6 (IL-6). Effective anti-IL-6 agents have been developed and, to date, the monoclonal antibodies (mAb) tocilizumab (anti-IL-6R) and siltuximab (anti-IL-6) are approved by the FDA for the treatment of many cancers (i.e., lung, prostate, B-cell, renal cell carcinoma) (Kang et al., 2019). For instance, mAbs 1339 (OP-R003), ALD518/BMS-945429 and siltuximab have exhibited potential anti-tumor activity in vivo, inducing significant clinical response in patients with different disorders (Kang et al., 2019). Recently, Hailemichael and al. showed that IL-6R blockade abrogates immunotherapy-associated toxicity, thus reinforcing anti-tumor immunity in vivo (Hailemichael et al., 2022).

Accordingly, the combination therapy of tocilizumab and immune checkpoint inhibitors (anti-PD-1 and anti-CTLA-4) is currently in phase II trial for patients with melanoma, NSCLC, and urothelial carcinoma (NCT04940299) (Abdel-Wahab et al., 2022; Diab et al., 2021) Collectively, this bulk of evidence demonstrates that the safe and cancer-selective inhibition of the NF-κB pathway is clinically achievable and promises profound benefit to patients with NF-κB-driven cancers (Fig.7).

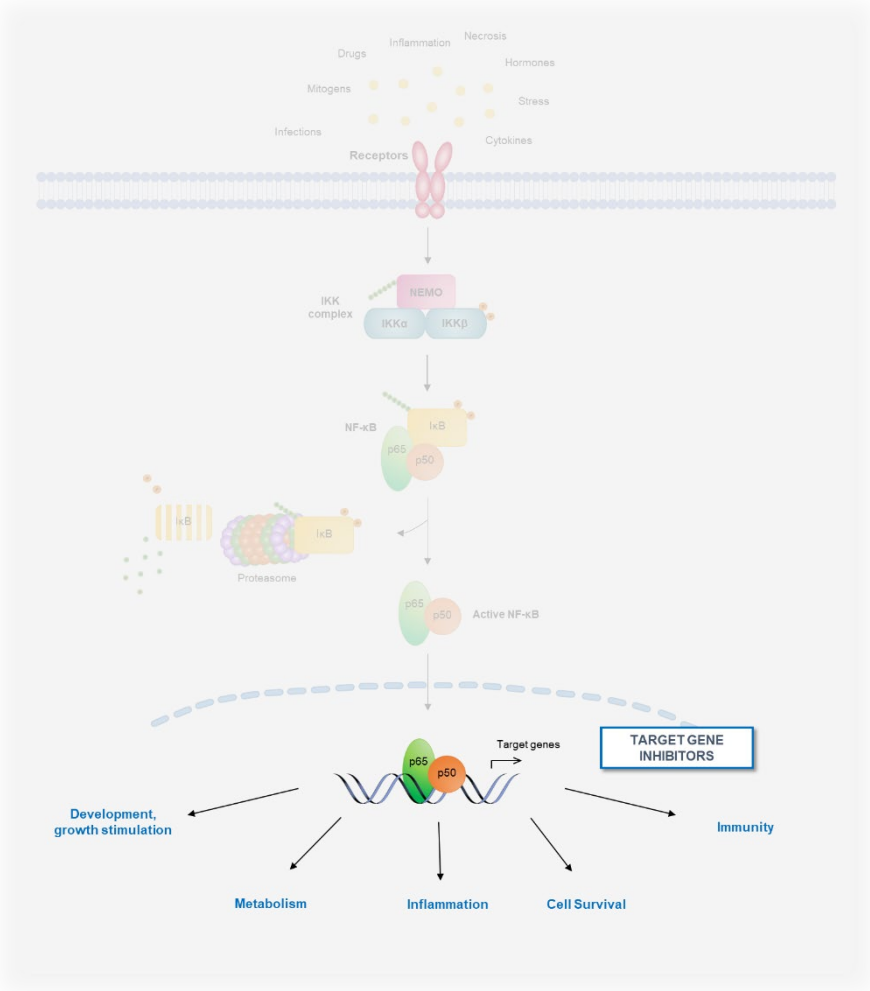


Figure 7. Schematic representation of inhibitors of NF-kB downstream effectors. NF-κB inhibitors acting by blocking downstream effectors of canonical NF-κB pathogenetic function. (Verzella et al., 2022)

The NF-κB downstream gene Gadd45β exerts a key role in the apoptotic pathway

NF-κB role in host defense and inflammatory responses has been extensively characterized. However, several studies on NF-κB–deficient mice and cells led to the identification of another biological function: the inhibition of apoptosis (Beg & Baltimore, 1996; Karin & Lin, 2002; Van Antwerp et al., 1996). Hence, NF-κB transcription factors regulate the balance of crucial genes involved in the inhibition or promotion of apoptosis, and in the cell cycle (Barkett & Gilmore, 1999).

Among the anti-apoptotic genes under NF-κB transcriptional control, it was identified *gadd45b* as a physiological regulator of the apoptotic response to TNF-α (De Smaele et al., 2001; Jin et al., 2002; Zazzeroni et al., 2003).

Growth Arrest and DNA Damage β (GADD45β), also known as MYD118, is a protein member of the GADD45 family. GADD45 members are involved in many physiological processes, including DNA repair, cell cycle arrest, cellular senescence, and epigenetic regulation (Moskalev et al., 2012). Moreover, GADD45 proteins are differentially expressed in multiple types of mammalian tissues and exert their biological function by forming homo- and hetero-dimers, as well as oligomers, with other family members (Kovalsky et al., 2001; Moskalev et al., 2012).

De Smaele et al. demonstrated that GADD45β antagonizes TNF-α-induced cell death following NF-κB activation (De Smaele et al., 2001). Indeed, *gadd45β* is rapidly induced by cytokine and stress through a mechanism that requires NF-κB. It has been shown that *gadd45b* promoter contains several putative κB elements, thus Gadd45b mRNA levels rely on nuclear NF-kB activity (Jin et al., 2002). Moreover, experiments in NF-κB-null cells revealed a virtually complete rescue from the TNF-α-mediated apoptosis following Gadd45β overexpression, indicating GADD45β as a NF-kB downstream regulator of the apoptotic response. Further evidence unveiled that GADD45β upregulation induces JNK apoptotic cascade suppression (De Smaele et al., 2001). Indeed, TNF-α-induced apoptosis involves the activation of JNK MAP kinases, which in turn trigger the apoptotic process by upregulating pro-apoptotic genes and modulating the activity of mitochondrial pro- and anti-apoptotic proteins (Deng et al., 2003; Dhanasekaran & Reddy, 2008; J. Liu & Lin, 2005).

Taken together, these findings underscore the NF-κB-dependent protective mechanism through the effector GADD45β against JNK activation in the response to TNF-α (De Smaele et al., 2001).

Subsequent studies identified the mechanism by which GADD45β exerts its protective function modulating JNK signaling pathway. Although GADD45

family proteins have not enzymatic activity, they are emerging as a new class of kinases modulators, including MAPK pathway kinases such as MEKK4, MKK7, and p38 (Humayun & Fornace, 2022; Liebermann & Hoffman, 2008; Mita et al., 2002; Ueda et al., 2017). In fact, GADD45β fulfils its anti-apoptotic function by associating with MKK7 in proximity of critical residues in the catalytic domain, thus inhibiting the enzymatic activity (Salvatore Papa, Zazzeroni, Bubici, et al., 2004). Essentially, site-directed mutagenesis data and molecular docking analyses suggest that MKK7-GADD45β complex formation prevents the access of ATP in the kinase catalytic pocket causing a conformational change which impairs the enzymatic function (Salvatore Papa et al., 2007).

Therefore, these findings unveil an intriguing model whereby NF-κB activation upregulates GADD45β expression, which interferes with MKK7 function, resulting in the suppression of JNK signaling and eventually in TNF-α-induced apoptosis inhibition (De Smaele et al., 2001; Salvatore Papa et al., 2007; Salvatore Papa, Zazzeroni, Bubici, et al., 2004) (Fig.8).

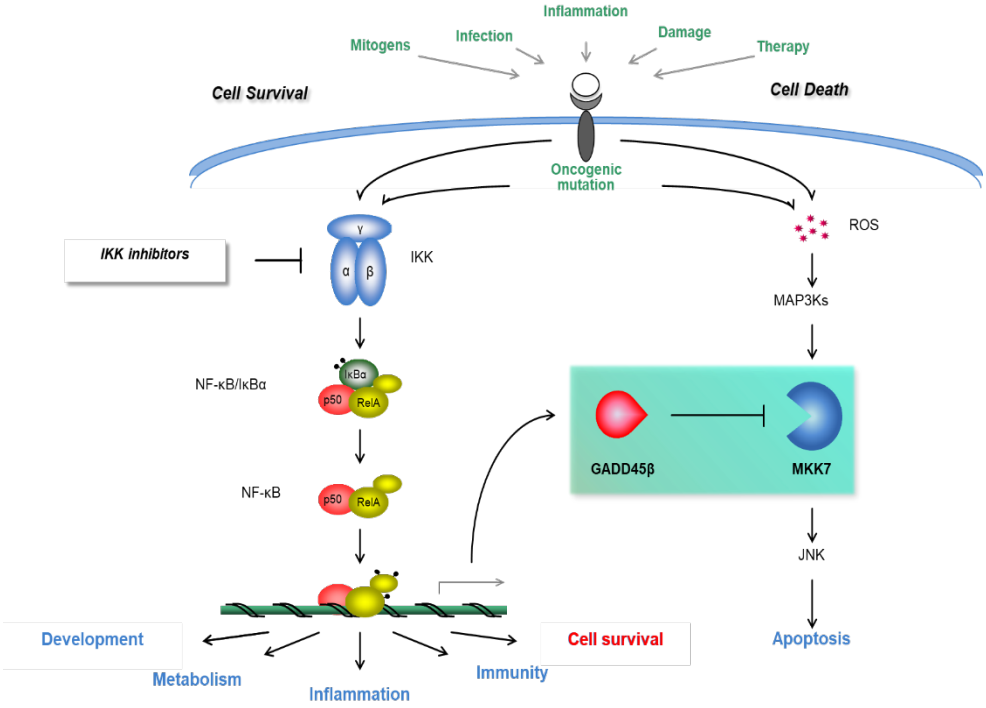


Figure 8. Schematic representation of the GADD45β/MKK7 survival complex signaling. Depicted is the crosstalk between the NF-κB and JNK pathways promoting cancer-cell survival and apoptosis, respectively. Also shown is the GADD45β/MKK7-dependent mechanism suppressing proapoptotic JNK signalling. (Tornatore et al., 2014)

Similarly, it has been found that GADD45β exerts an anti-apoptotic role in B lymphocyte acting as an important NF-κB effector during cytoprotective CD40 stimulation. The signaling cascade induced by CD40, another TNF-R family member, plays a critical role in controlling B-cell growth, differentiation, and fate in germinal center (Elgueta et al., 2009). Indeed, the CD40 signaling counterattack Fas-induced killing participating in selective rescue of B-cell clones with high-affinity antigen receptors (Koncz & Hueber, 2012). Therefore, following CD40 receptor engagement, NF-κB activation promotes the induction of anti-apoptotic genes, such as Bcl-xL, c-FLIPL and Gadd45β hampering Fas-mediated apoptosis (Schram & Rothstein, 2003; Zazzeroni et al., 2003).

Subsequent studies revealed that GADD45β is a prognostic and predictive biomarker of poor prognosis in several malignancies. Indeed, Gadd45β expression levels correlate with poor prognosis in II stage colorectal cancer, papillary thyroid carcinoma and pediatric acute myeloid leukemia patients (Barros-Filho et al., 2020; Depreter et al., 2020; Zhao et al., 2018). Accordingly, it has been found that Gadd45β expression levels are associated significantly with metastasis and poor survival in epithelial ovarian cancer (EOC) (Gong et al., 2021).

Interestingly, Verzella et al. identified an additional biological function of Gadd45β as innate immune "checkpoint". The research group showed that myeloid-associated Gadd45b ablation restored proinflammatory tumor associated macrophages (TAM) activation and intratumoral $CD8^+$ T-cell infiltration resulting in a reduction of tumor growth (Verzella et al., 2018).

Altogether, these findings uncover the role of Gadd45β as crucial NF-κB downstream effector in the regulation of apoptotic pathway and its relationship with cancer onset and development.

Cancer-selective targeting of NF-kB downstream MKK7-GADD45β survival complex.

Compelling evidence has established the pivotal role of aberrant NF-κB signal cascade in sustaining several human malignant diseases. Thus, there is a strong rationale for interfering with NF-κB pathway. However, despite the pharmaceutical industry's effort, no specific NF-κB inhibitor has been clinically approved, due to on-target toxicities of systemic NF-κB blockade (Didonato et al., 2012).

Since pathological NF-κB signaling deregulates physiological processes, an attractive therapeutic strategy would be to target essential oncogenic downstream effectors rather than NF- κB itself. In this perspective, agents targeting effectors restricted to cancer context could provide a safer and more selective therapeutic tool circumventing the dose-limiting toxicities of global NF- κB suppression (Begalli et al., 2017; Verzella et al., 2022).

Recently, this controversial question has been solved obtaining a proof-of concept in multiple myeloma (MM), the paradigmatic NF-κB-driven malignant disease.

MM is a blood cancer characterized by the clonal proliferation of plasma cells in the bone marrow (van de Donk et al., 2021). Several pioneering studies demonstrated that MM is underpinned by high level of NF-κB activity caused by intrinsic mutations in the components of the pathway as well as extrinsic stimuli from the microenvironment (Roy et al., 2018). To date, the medical treatments for MM include chemotherapy, steroids, immunomodulatory (IMiD) agents, proteasome inhibitors, and other active approved agents (i.e., monoclonal antibodies and CAR-T immunotherapy), and autologous-cell transplantation for selected eligible patients. However, despite the numerous therapeutic strategies available almost all patients with MM eventually relapse and/or develop a refractory disease (Rajkumar, 2022; Rajkumar & Kumar, 2020).

Therefore, given the oncogenic role of Gadd45β as downstream effector of NF-κB, our group investigated its potential involvement in MM pathogenesis. It has been found a strong correlation between Gadd45β mRNA expression levels in $CD138^+$ cells and disease progression in MM. Indeed, MM patients stratification based on Gadd45β mRNA expression levels in $CD138^+$ cells showed that high Gadd45β expression correlate with shorter progression-free survival and shorter overall survival (OS) (Tornatore et al., 2014)(Fig.9).

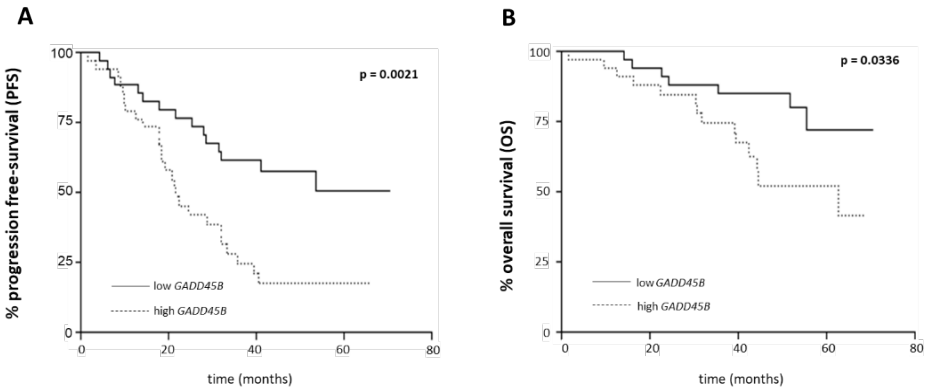


Figure 9. *GADD45B* is highly expressed in most MM cells and correlates with worse patient outcomes. PFS (**A**) and OS (**B**) in MM patients with low or high *GADD45B* expression. Patients were from the velcade/melphalan/prednisone arm of the trial and stratified at diagnosis, using the median *GADD45B* expression value as cutoff. (Tornatore et al.,2014)

Since Gadd45β fulfils its anti-apoptotic function by blocking MKK7 via direct physical interaction, our research group developed a selective inhibitor that disrupts the protein-protein complex thus restoring JNK cytotoxic signaling. Therefore, by screening a combinatorial library of 20,736 peptides, it was identified DTP3, a D-tripeptide with a molecular weight of 525 Da, which blocks GADD45β/MKK7 complex in vitro (Fig.10).

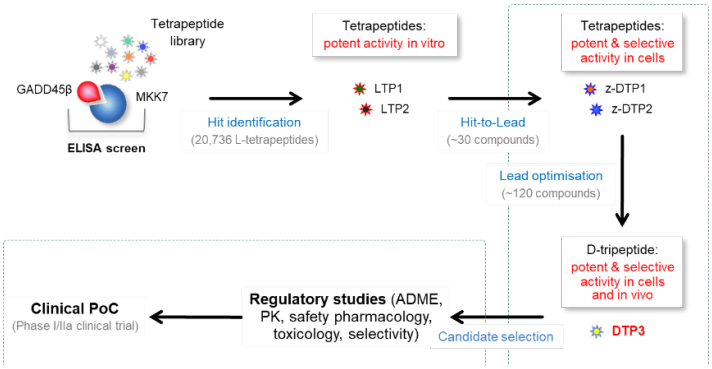


Figure 10. GADD45β/MKK7: the drug discovery pathway. Summary of the drug discovery strategy that led to the development of DTP3. (Tornatore et al.,2014)

Tornatore et al. showed that the stoichiometry and KD value of the DTP3/MKK7 interaction were 1:1 and 64.81 ± 6.22 nM, respectively (Fig.11) (Tornatore et al., 2014). Furthermore, co-immunoprecipitation analysis showed

the efficacy and the specificity of DTP3 compound to disrupt GADD45β/ MKK7 complex compared to a scrambled (SCR) control D-tripeptide (Fig.11C-B).

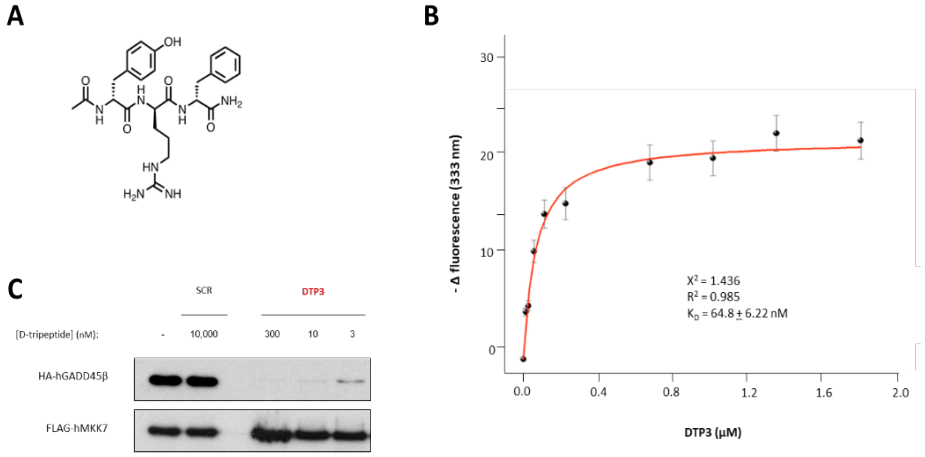


Figure 11. DTP3 efficacy and specificity in GADD45β/MKK7 complex interference. (A) The structure of DTP3. (B) Tryptophan fluorescence quenching analysis showing the dose-response curve of the D fluorescence values of GST-hMKK7 at 333 nm plotted against the concentration values of DTP3. (C) Co-immunoprecipitation assays showing the disruption of the GADD45β/MMK7 complex by DTP3, but not by the SCR control D-peptide, at the indicated concentrations. (Tornatore et al.,2014)

The DTP3 binding to GADD45β/MKK7 complex has been further characterized by Mass spectrometry (MS)-based footprinting and chemical cross-linking (CX-MS) analysis and modelling studies. Strikingly, the structural investigation showed that DTP3 interacts with two spatially adjacent N-terminal MKK7 regions inducing a conformational rearrangement eventually resulting in the dissociation of GADD45β from MKK7. These data also suggested that the interactions of GADD45β and DTP3 with MKK7 are mutually exclusive (Rega et al., 2018; Sandomenico et al., 2020).

The potential efficacy has been demonstrated in MM cell lines, where DTP3 induces a dose-dependent cytotoxicity in correlation to GADD45β expression levels demonstrating the addiction of MM cell lines to this survival pathway (Tornatore et al., 2014) (Fig.12-A,B). The selective cytotoxic effect of DTP3 has been showed on MM plasma cells isolated from patients at nanomolar concentration demonstrating the suitability of this potential tool on primary cell lines (Tornatore et al., 2014)(Fig. 12 - C-E). Moreover, in plasmacytoma mouse models, treatment with DTP3 at the dose of 14.5 mg/kg/day virtually eradicated subcutaneous myeloma xenografts in mice, with no apparent side effects, demonstrating the potent therapeutic efficacy as well as the excellent in vivo tolerability (Tornatore et al., 2014)(Fig.12- F,G).

Therefore, regulatory studies confirmed the DTP3 on-target-selective pharmacology, favorable pharmacokinetic (PK) and absorption, distribution, metabolism and excretion (ADME) profiles, leading to MHRA approval for a first-in-human study in relapsed or refractory MM patients (Tornatore et al., 2019). The results from this study (EudraCT: 2015-003459-23) confirmed the ability of DTP3 to trigger JNK activation followed by apoptosis in MM, but not normal cells, demonstrating clinical benefit and no significant adverse events (Tornatore et al., 2019). Notably, DTP3 produced these pharmacodynamic (PD) and initial efficacy signals in refractory MM patients, warranting further investigation of its novel mechanism of action in a larger follow-on trial (EudraCT number: 2021-004028-13).

Collectively these findings identify DTP3 as an attractive pharmacological tool targeting the GADD45β/MKK7 survival complex and confirm its therapeutic application in MM malignancy which rely on NF- κB down-stream survival module GADD45β/MKK7.

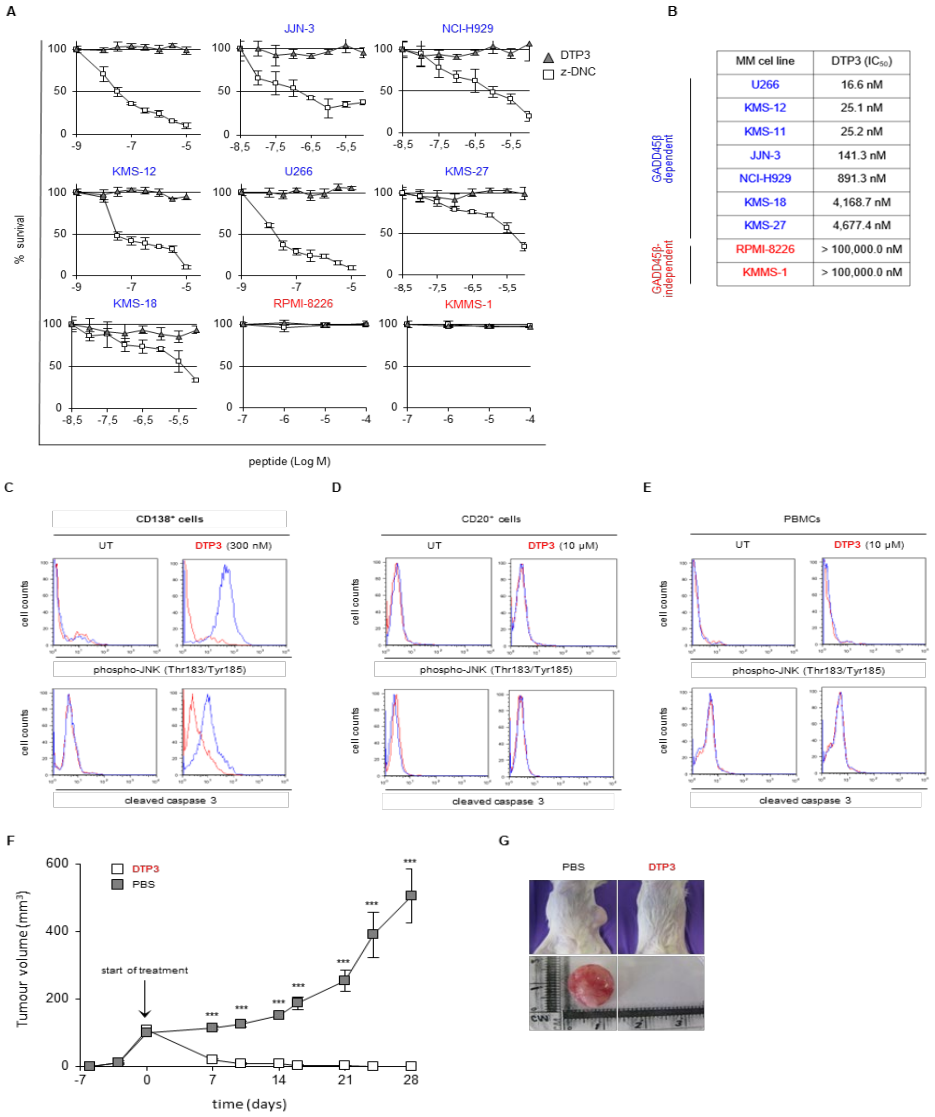


Figure 12. Cancer-selective target engagement by DTP3 in MM. (A) [3H]Thymidine incorporation assays showing the survival of GADD45β-dependent (blue) and GADD45β-independent (red) MM cell lines after a 6-day treatment with the indicated concentrations of DTP3 or a negative control D-peptide (z-DNC).(B) Shown are IC_{50} values of DTP3 at 144 hr for the experiment reported in (A).(C-E) FACS analysis showing the levels of phosphorylated (phospho) JNK and active (cleaved) caspase 3 in malignant CD138+ cells (C), and normal CD20+ cells (D) and PBMCs (E) isolated from the same representative MM patient and treated with DTP3, ex vivo, at the indicated concentrations or left untreated (UT) for 24 hr. Blue lines, phospho-JNK-specific or active caspase 3-specific antibodies; red lines, appropriate immunoglobulin (Ig) isotype controls. (F) Shown are the volumes of s.c. U266 MM xenografts in NOD/SCID mice treated by continual infusion with DTP3 at the dose of 14.5 mg/kg/day or PBS for the times indicated. (G) Images of representative U266 MM-bearing mice (top) and isolated tumours (bottom) from (F) at day 28. (Tornatore et al. 2014)

AIM

Anaplastic thyroid carcinoma (ATC) accounts for 2-3% of all thyroid gland neoplasms (Limaiem et al., 2022). Although ATC is a very rare malignant tumor, it represents one of the deadliest diseases worldwide and carries a very poor prognosis. Due to the undifferentiated and aggressive behavior, ATC is still an unmet clinical problem.

NF-κB showed to drive ATC development and progression, thus representing an attractive therapeutic target to improve medical treatment (Pacifico & Leonardi, 2010; Starenki, Namba, Saenko, Ohtsuru, & Yamashita, 2004). However, NF-κB inhibitors currently available revealed side effects and severe toxicities due to NF-κB systemic blockade.

The discovery of Gadd45β as an anti-apoptotic downstream NF-κB effector prompted to the development of DTP3 that represents the first-in-class downstream NF-κB inhibitor. Interestingly, DTP3 showed to be an effective and specific tool in multiple myeloma malignancy suggesting its potential therapeutic applicability (Tornatore et al., 2014, 2019).

The aim of this study is to determine Gadd45β biological role in ATC cell lines thus to virtually extend the DTP3 potential therapeutic effects on those malignancies addicted to Gadd45β-dependent survival mechanism.

MATERIALS AND METHODS

Cell culture and thyroid patient specimens

BHT101, CAL-62, 8505c, a kind gift of Prof. M. Santoro, Federico II University of Naples, and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO USA) supplemented with 10% fetal bovine serum (Invitrogen). RNA patient samples were obtained processing thyroid patient formalin-fixed paraffin-embedded (FFPE) tissue specimens.

Tripeptide compounds synthesis

DTP3 (Ac-D-Tyr-D-Arg-D-Phe-NH2), the related scramble (SCRB) peptide (Ac-D-Arg-D-Phe-D-Tyr-NH2) and FITC-labeled variants (FITC-βAla2-DTP3 and FITC-βAla2-SCRB) were provided by Dr. Ruvo Menotti from Dipartimento delle Scienze Biologiche, Istituto di Biostrutture e Bioimmagini (IBB), CNR. Tripeptide compounds were synthetized as previously reported (Tornatore et al., 2008; Sandomenico et al., 2020).

Human thyroid cancer dataset analysis

The human dataset of thyroid cancer was part of The Cancer Genome Atlas (TCGA) program and downloaded from the UCSC Cancer Genomic Browser. Gene expression profiling was performed on fresh or frozen tissue biopsies using the Illumina HiSeq 2000 RNA Sequencing platform. The estimates of GADD45B expression levels were derived from the normalized values in the UCSC Cancer Genomic Browser. Patients were stratified into two groups based on the median value of GADD45B mRNA expression levels.

RNA extraction, cDNA synthesis, and quantitative PCR

Total RNA was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription reactions were performed on at least 500 ng of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosistems). qRT-PCR was carried out using cDNAs reverse transcribed from total RNA by using SensiFAST SYBR® NoROX Master Mix (Bioline), in LightCycler480 system (Roche). Experimental $2^{-\Delta\Delta Ct}$ values were normalized to Cyp-A housekeeping gene.

qRT-PCR primers used were:

Gadd45β Forward: 5'-GGGAAGGTTTTGGGCTCTC-3'

Gadd45β Reverse: 5'-GGTCACCGTCTGCATCTTC-3'

gRNA Forward: 5'-AGAGCTAAGCTGGAAACAGCA-3'

gRNA Reverse: 5'-CGACTCGGTGCCACTTTTTC-3'

Cyp-A Forward: 5'-GCCGAGGAAAACCGTGTACT-3'

Cyp-A Reverse: 5'-TGTCTGCAAACAGCTCAAAGG-3'

XBP1 Forward: 5'-TGCTGAGTCCGCAGCAGGTG-3'

XBP1 Reverse: 5'-GCTGGCAGGCTCTGGGGAAG-3'

ATF4 Reverse: 5'-CTATCTGGAGGTGGCCAAGC-3'

ATF4 Forward: 5'-TCTGTCCCGGAAAAGGCATC-3'

HSPA5 Forward: 5′-TTGACTCCGACCTTCACCTTCC-3'

HSPA5 Reverse: 5′-TTTCACAGTGGCCAAGAGTC-3'

DDIT3 Reverse: 5′-GCACCTCCCAGAGCCCTCACTCTCC-3'

DDIT3 Forward: 5′-GTCTACTCCAAGCCTTCCCCCTGCG-3'

Confocal microscopy

Cells on coverslips were fixed with 4% PFA for 10' at RT, washed three times with PBS and stained with DAPI [150 ng/ml] at RT. Cells were again washed three times with PBS and coverslips were mounted on standard glass slides using 1:1 PBS/Glycerol Mounting Medium. All the procedures were performed in the dark. Images were acquired with the same settings using Zeiss LSM-710 confocal microscope and analysed with FIJI software.

Plasmid vectors, lentiviral particles production and cells transduction

Lenti-EF1a-dCas9-KRAB-Puro was purchased by Addgene (plasmid # 99372). EZ-Tet-pLKO-Puro (from Addgene, plasmid # 85966) was modified cloning the gRNA scaffold containing AjuI recognition sequence by NheI and EcoRI restriction enzymes. Lentiviral vectors were produced and concentrated as described by Kutner et al. (2009), with minor modifications. Briefly, a total of 7×10^6 HEK293T cells were seeded in 150mm-dishes 24 hours before transfection in 20 ml complete medium. A total of 42 µg of plasmid DNA was transfected in each dish: 7 µg of the envelope plasmid pMD2G, 14 µg of packaging plasmid pR8.91, and 21 µg of transfer vector plasmid. The medium was harvested after 24, 48 and 72 hours after the transfection, pooled and filtered using 0.45 µm PES filtration units. Viral particles were then subjected to PEG concentration.

Western blot

Total protein extracts were obtained lysing cells in RIPA lysis buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with Complete Protease Inhibitor (Roche) and Phosphatase-Inhibitor-Mix II solution (Serva). Total proteins from cell lysates (30 µg) were analysed by SDS-PAGE. After electroblotting of gels onto polyvinylidene difluoride sheets (Millipore, Bedford, MA), filters were blocked for 1 h at room temperature with 5% non-fat dry milk in TBST buffer (10 mm Tris-HCl (pH 8.0), 0.1% Tween 20, 150 mm NaCl). The filters were then incubated in the same buffer for 14-16 h at 4 °C with primary antibody according to the manufacturer's instruction. After TBST washing, blots were incubated for 1 h at room temperature with secondary horseradish peroxidase-conjugated antibodies diluted according to manufacturer's instructions in TBST buffer and then revealed by ECL (Amersham Biosciences).

The antibodies used were: anti-Gadd45β (Invitrogen, #PA5-43160, 1:1000), anti-JNK (Cell Signaling, 9258S 1:1,000), anti-P-JNK (Cell Signaling, 9251S 1:1,000), anti-ERK1/2 (CellSignaling, 4695S; 1:1,000), anti-P-ERK1/2 (CellSignaling, 4377S;1:1,000), anti-β-actin (Sigma, A11978, 1:5000), Cyclin D1 (SantaCruz, sc-246, 1:1000).

Cell viability assays

Crystal Violet staining

Cells were seeded in a 96-well plate at a density of 1×10^3 cells/cm^2. Cells were treated with appropriate DTP3 concentration or DMSO as vehicle. After 96 hours, media were removed and cells were washed with PBS, the plates were inverted and tapped gently to remove excess liquid. A total of 50 µL of a 0.1% crystal violet (Sigma-Aldrich) staining solution in 25% methanol was added to each well and incubated at room temperature for 30 minutes on a bench rocker with a frequency of 20 oscillations per minute. The cells in the wells were briefly washed with deionized H_2O, and the plates were left to air dry without a lid for 10 minutes. For crystal violet extraction, 200 µL of methanol was added to each well, and the plate was covered with a lid and incubated at room temperature for 20 minutes on a bench rocker set at 20 oscillations per minute. The absorbance of the plates was measured at 570 nm.

MTS assay

Cells were seeded in a 96-well plate at a density of 1×10^3 cells/cm^2. Cells were treated with appropriate DTP3 concentration or DMSO as vehicle. After 96 hours, 20 µl MTS solution (Promega) containing PES was added to each well and incubated 2 hours at 37°C. Then, the absorbance was measured at 490 nm.

CellTiter-Glo® Luminescent assay

Cells were seeded in a 96-well plate at a density of 1 × 103 cells/cm2. Cells were treated with appropriate DTP3 concentration or DMSO as vehicle. After 96 hours, 100 µl CellTiter-Glo® reagent (Promega) was added to each well. Then, plates were placed on a shaker for 5 minutes. After 30 mins incubation at 37°C, luminescence signals were recorded by luminometer.

FACS analysis

BrdU staining protocol

Before harvesting cells were exposed to BrdU (BD Bioscience) at the final concentration of 10 µM for 30 minutes in the CO_2 incubator at 37°C. Then, cells were harvested and washed in 1% BSA/PBS and centrifuged at 500g for 5 minutes at room temperature. The pellet was fixed using cold EtOH 70% drop by drop on vortex and centriguge at 500g at 4°C for 10 minutes. Supernatant was discarded. Cells were resuspended in 500 µL 2N HCl, 0.5% Tween-20 for 30 minutes. Then, cells were pelleted at 500g at 4°C for 10 minutes. Supernatant was discarded. Then samples were resuspended in 500 µL of 0.1M $Na_2B_4O_7$ • 10 H_2O (Borax) pH 8.5 to neutralize the acid. Cells were centrifuged again as described above. Pellets were resuspended in a variable volume of PBS 1%BSA 0.5% Tween-20 depending on the pellet volume. Cells were counted using Trypan-Blue. A volume containing $5x10^5$ cells was placed in a clean 1.5 mL tube and stained with 10 µL of anti-BrdU-FITC (BD Bioscience) for 30 minutes at room temperature in the dark. Eventually, cells were centrifuged and resuspended in 200 µl PBS containing 5 µg/mL of PI (BD Bioscience) and analysed using FACSCanto II BD and FlowJo software.

AnnexinV/Propidium Iodine double staining

Cells were harvested, washed cells twice with cold PBS and then resuspended cells in 1X Binding Buffer at a concentration of ~1 x 10^6 cells/ml. Apoptosis was detected by flow cytometry using FITC Annexin V Apoptosis Detection Kit II (BD Biosciences). Briefly, cells were double-stained with Annexin V-FITC and PI following the manufacturer's instructions. Early apoptosis is defined by annexin V-positive/PI negative staining, and late apoptosis/necrosis is defined by annexin V-positive/PI positive staining. Samples were analysed using FACSCanto II BD and FlowJo software.

Rna-Seq data and identification of DEGs

ATC cell lines were treated with 200 µM DTP3 or DMSO as control for 24 hours. Reverse transcription reactions were performed on using High-Capacity cDNA Reverse Transcription Kit (Applied Biosistems). RNA was extracted

using the Direct-zol RNA Miniprep (Zymo Research) and RNA-Seq analysis was performed at Imperial BRC Genomics Facility.

The raw data used for the analysis were processed using the Limma package(Law et al., 2014) in R language. Overexpressed and underexpressed genes in treated samples will have positive or negative base 2 logarithmic fold change (log fold change, logFC) values, respectively.

Statistical analysis

Data are presented as means of at least three independent experiments with Standard Deviation (SD), or representative data are shown. Statistical analysis was performed using GraphPad Prism software version 9.0 (GraphPad Software, San Diego, CA, USA) using two-tailed paired Student t test or two-way analysis of variance (ANOVA) according to experimental design.

RESULTS

High GADD45β expression levels are detected in human thyroid cancer biopsies and correlate with a reduced Overall Survival in patients

Several studies have demonstrated that NF-κB is constitutively activated in many human thyroid carcinomas (TC)(Giuliani et al., 2018; Pacifico et al., 2004, 2010). Furthermore, high levels of NF-κB target gene Gadd45β correlate with poor clinical outcome (Barros-Filho et al., 2020; Gong et al., 2021; Zhao et al., 2018).

Based on these findings, we hypothesized that Gadd45β may be involved in thyroid cancer development and progression.

Thus, to investigate Gadd45β potential biological role in thyroid cancer, we performed qRT-PCR experiments on mRNA samples isolated from human biopsies of normal thyroid (N), papillary (P), and anaplastic thyroid carcinomas(A). The qRT-PCR analysis showed elevated Gadd45β mRNA expression levels in several papillary (PTC) and anaplastic carcinoma thyroid (ATC) samples. Notably, A1 and A3 ATC specimens exhibited higher Gadd45β expression among analysed samples. Conversely, Gadd45β mRNA is almost undetectable in healthy thyroid samples (Fig.13).

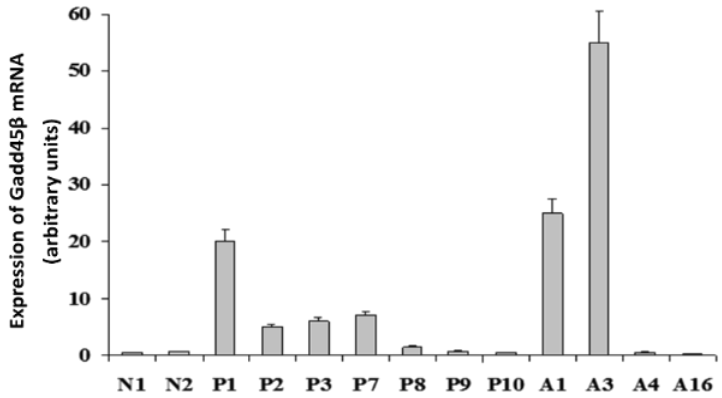


Figure 13. Gene expression levels of Gadd45β in TC biopsies. qRT-PCR analysis showing Gadd45β mRNA expression levels in in healthy controls (N), papillary (P) and anaplastic carcinoma thyroid (A) biopsies. Levels of mRNA are expressed in arbitrary units relative to expression of GAPDH mRNA.

Considering this result, we decided to evaluate the correlation of Gadd45β gene expression with clinical outcome among patients affected by TC. Thus, we processed RNA-seq dataset of TC patients available in The Cancer Genome Atlas (TCGA) to calculate overall survival (OS) by Kaplan-Meier statistical method.

The cohort of patients were stratified based on Gadd45β gene expression in two groups: high Gadd45β (red) and low Gadd45β (black).

The results showed that Gadd45β gene is associated with a shorter OS in patients with TC (Fig.14). The association reached statistical significance (P=0.02924).

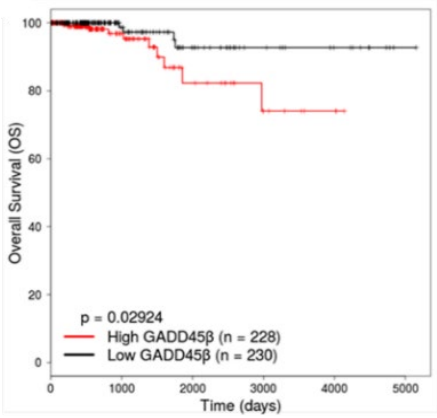


Figure 14. Correlation between GADD45B mRNA levels and poor clinical outcome of TC patients. Kaplan–Meier plot showing a shorter overall survival in cases presenting higher Gadd45β expression in TC patients from the TCGA database (RNA sequencing). The X and Y axes represent follow-up time in days and the percentage of survival, respectively. Low and high Gadd45β mRNA levels were stratified based on the median of Gadd45β expression levels. Comparisons between groups were analyses by using the log-rank test.

GADD45β is differentially expressed among human thyroid cancer cell lines

Gadd45β differential expression levels in TC biopsies and its potential role as prognostic marker in ATC patients prompted us to translate our studies on human TC cell line models.

Therefore, we examined Gadd45β mRNA expression levels by qRT-PCR analysis in a panel of human thyroid cancer cell lines. We analysed human PTC

(TPC-1 and B-CPAP) and ATC (8505c, ACT-1, BHT-101, C-643 and CAL-62) cell lines.

Since Gadd45β is an inducible stress sensor and its expression levels are almost undetectable in normal tissues (Cretu et al., 2009), human embryonic kidney cell line (HEK293) was used as control for gene expression relative quantification. The results showed that Gadd45β mRNA levels were differently expressed among TC cell lines (Fig. 15).

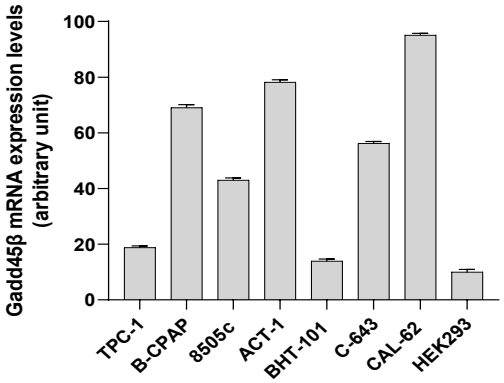


Figure 15. Gene expression levels of Gadd45β in TC cell lines. qRT-PCR analysis showing Gadd45β mRNA expression levels in TC cell lines. Levels of mRNA are expressed in arbitrary units relative to HEK293 cell line.

To further characterize Gadd45β biological function, we selected three representative ATC cell lines showing low (BHT-101), medium (8505c) and high (CAL-62) mRNA expression levels respectively.

Then, we analysed Gadd45β protein expression by Western Blot. Our data correlated with results observed in qRT-PCR analysis (Fig. 15). Indeed, Gadd45β protein expression is almost undetectable in BHT-101 cell line, while showed intermediate and high levels in 8505c and CAL-62 respectively (Fig.16, first panel).

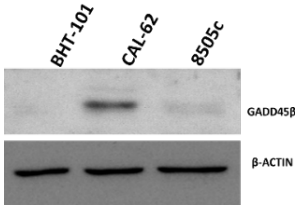


Figure 16. Gadd45β protein expression levels in ATC cell lines. Western Blot analysis of Gadd45β protein expression. β-actin was used as the loading control.

The DTP3 and the related scrambled peptides are permeable to cellular plasma membranes.

Pharmacological inhibition of NF-κB downstream effectors is a challenging and potentially safe therapeutic strategy.

Gadd45β modulation via the novel first-in-class inhibitor DTP3 represents a starting point to extend this therapeutic tool to other NF-κB-addicted malignancies. Therefore, we wondered if DTP3 compound exerted its biological function in anaplastic thyroid carcinoma cell lines.

To validate our hypothesis, we first investigated the cellular permeability of DTP3 in ATC cell lines. Therefore, we synthetized and labelled DTP3 tripeptides with fluoresceine isothiocyanate (FITC-DTP3) to allow compounds detection by flow cytometry and confocal microscopy experiments. We used FITC-scramble tripeptides (FITC-SCR) as control.

We treated ATC cell lines BHT-101, 8505c and CAL-62 with SCR-FITC and DTP3-FITC and processed the samples at three different time points (1h, 3h and 6h) to study the labelled tripeptides uptake kinetic (Fig.17).

Flow cytometric analysis indicated high fluorescence intensity in ATC cell lines treated with SCR-FITC (green) and DTP3-FITC (red) compared with untreated cells (blue) (Fig.17).

We observed that FITC positive cells in treated samples showed similar distribution over the time points. indicating that tripeptides entry was effective after 1 hour of treatment and detectable until 6 hours.

Notably, the fluorescence intensity showed no significant differences among the ATC cell lines and the tripeptide compounds, suggesting that neither cell-specific context nor tripeptide-sequence influence the entry mechanism.

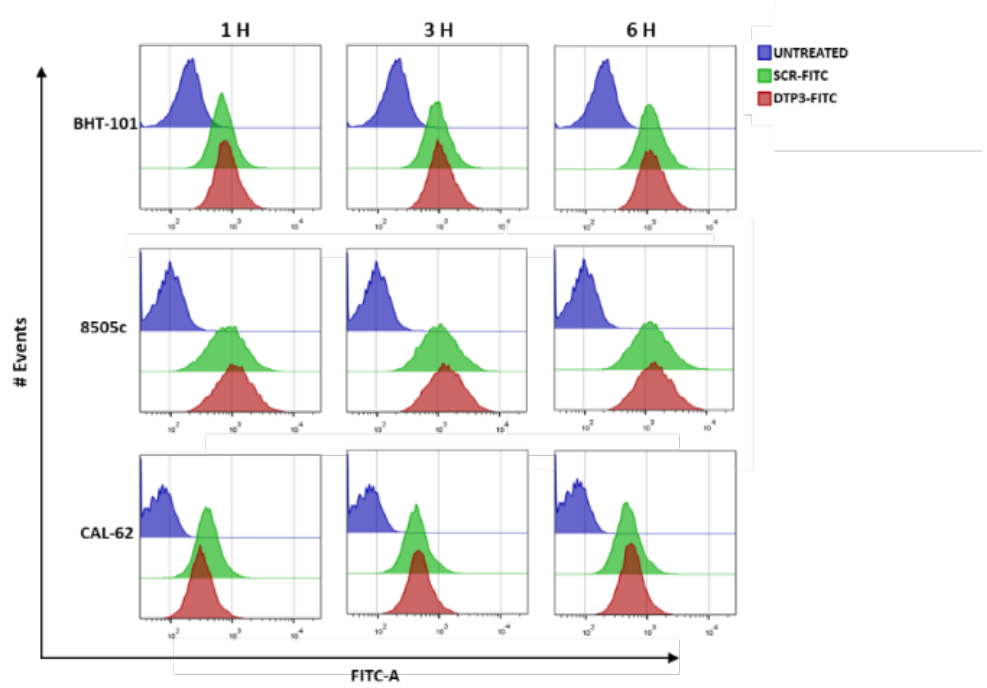


Figure 17. Flow cytometric analysis of FITC-labelled tripeptides on ATC cell lines. Representative flow cytometric analysis of ATC cell lines treated with 50µM SCR-FITC and DTP3-FITC. Untreated represent the negative control. The histograms show a comparison of the distribution of FITC negative (blue) and positive (green and red) cells. The X-axis shows linear fluorescence intensity, and the Y-axis shows the number of events.

Since flow cytometry analysis does not provide information about the cellular localization of fluorescence signals, we questioned if labelled tripeptides crossed cellular plasma membranes. Thus, we performed confocal microscopy experiments at the same experimental conditions. We exposed ATC cell lines to 50µM of both labelled tripeptide compounds and processed the samples after 1, 3 and 6 hours.

The confocal microscopy analysis was consistent with flow cytometry results as FITC fluorescence signals (green) were detectable in BHT-101 (A), 8505c (B) and CAL-62 (C) cell lines with a comparable trend (Fig.18). Notably, we observed a different pattern in fluorescence signal localization among ATC cell lines comparing DTP3-FITC and SCR-FITC tripeptides. Indeed, fluorescence signal showed a diffuse distribution throughout the cells, with no significant differences among ATC cell lines. Conversely, only DTP3-FITC showed a punctate structures pattern restricted to CAL-62 cell line which showed high Gadd45β expression levels, 8505c and BHT-101 These data might mirror the

DTP3-FITC direct localization in MKK7-Gadd45β complex which correlates with Gadd45β expression levels.

These data demonstrated that both DTP3 and tripeptides compound are efficiently permeable to cellular plasma membranes.

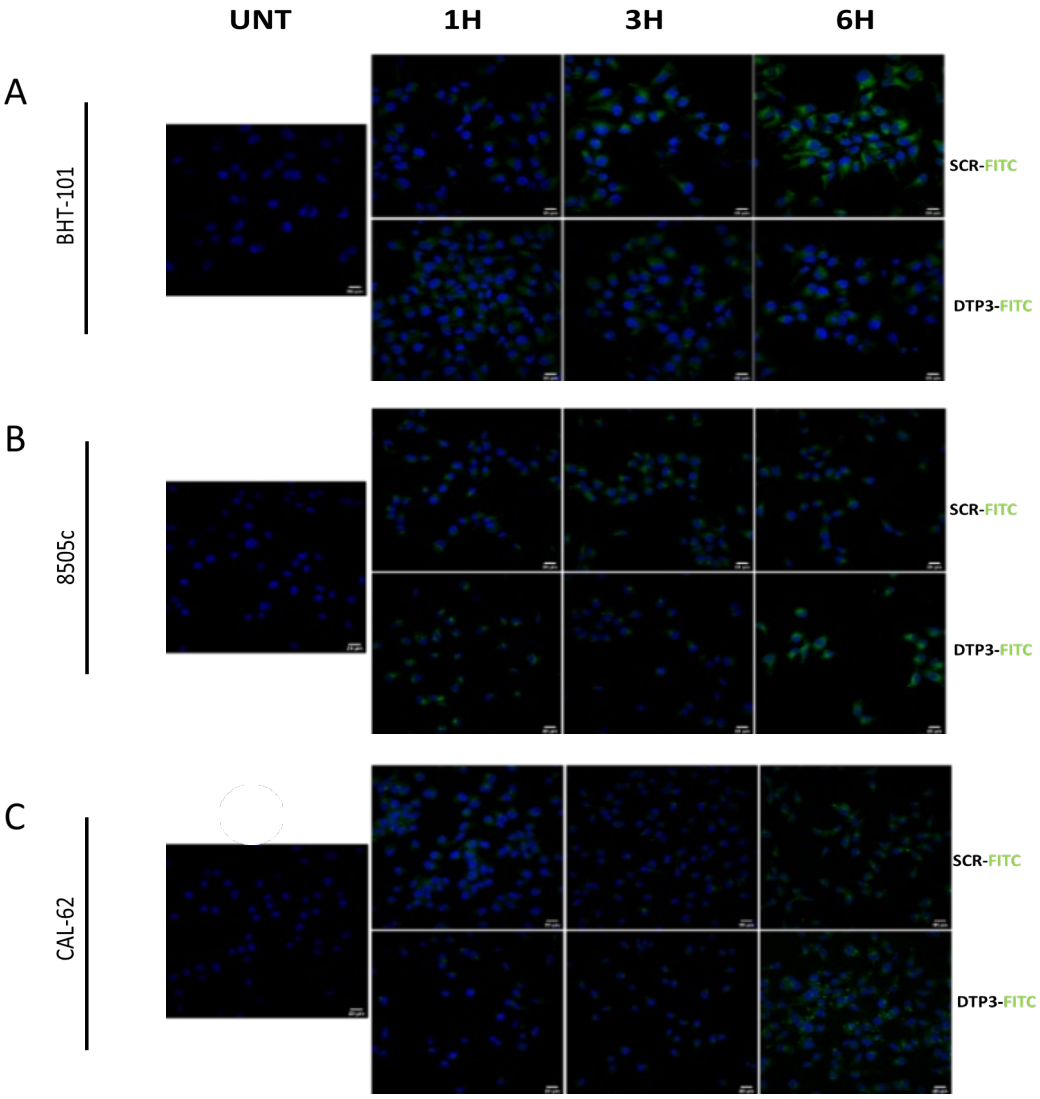


Figure 18. Confocal microscopy images of FITC-tripeptides in ATC cell lines. Representative confocal images (merged) of fixed control (UNT) BHT-101 (A), 8505c (B) and CAL-62 cells or cells treated with 50μM SCR-FITC and DTP3-FITC (green color) at indicated time points. Nuclei were stained with DAPI (blue color). Scale bar, 20 μm.

DTP3 biological effects specifically correlate with GADD45β expression levels in ATC cell lines

Gadd45β acts as an anti-apoptotic NF-κB effector impairing the physiological MKK7 kinase function (Salvatore Papa, Zazzeroni, Bubici, et al., 2004).

It has been demonstrated that DTP3 rescues MKK7 catalytical activity by interfering with the GADD45β/MKK7 complex. Moreover, DTP3 biological effects are tight related to Gadd45β expression levels as demonstrated in previous studies conducted in MM cell lines (Tornatore et al., 2014).

Therefore, we explored the potential DTP3 biological activity in ATC cell lines which showed differently Gadd45β expression levels. Accordingly, we treated BHT-101, 8505c and CAL-62 cell lines at different concentration of DTP3 and performed Crystal Violet cell viability assays after 96 hours.

Surprisingly, 8505c and CAL-62 cells showed a statistically significant survival reduction upon DTP3 treatment (Fig. 19). Indeed, 8505c and CAL-62 viability decreased at higher (300µM) and lower (100 and 200µM) DTP3 concentrations respectively, while BHT-101 cells were not affected by DTP3 treatment even at maximum dosage.

The data showed a dose-response trend that correlate with Gadd45β expression levels (Fig.15-16). Indeed, BHT-101 cells were not susceptible to DTP3 treatment in contrast to 8505c and CAL-62 which showed proportional drug sensitivity.

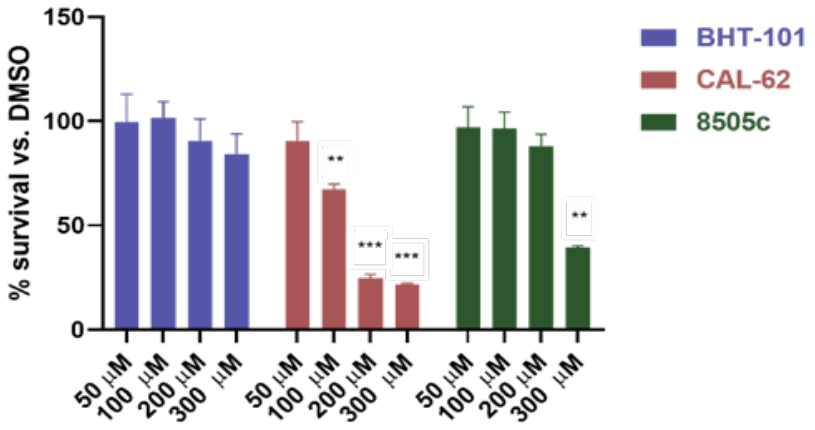


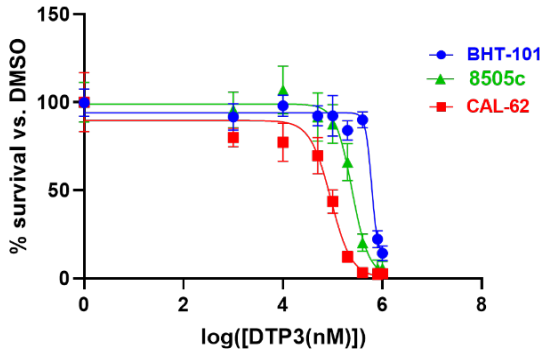
Figure 19. Crystal violet cell viability assay on ATC cell lines following DTP3 treatment. Crystal violet staining was conducted on BHT-101, 8505c and CAL-62 cell lines exposed to indicated DTP3 dosages for 96 hours. Results are expressed as a percentage of vehicle (DMSO). Data were shown as mean ± SD from three independent experiments. Two-way ANOVA analysis: **P < 0.01; ***P < 0.001.

Further, to determine DTP3 effectiveness and specificity we reasoned to quantify the half-maximal inhibitory concentration (IC_{50}). Thus, we measured IC_{50} values by Crystal Violet Staining both for DTP3 and SCR tripeptides (Fig 20).

The Crystal Violet staining data indicated a dose-response pattern in ATC cell lines consistent with our previous experiments. Surprisingly, IC_{50} values showed to correlate with Gadd45β levels (Fig.15-16, Fig.20, A-B), suggesting that DTP3 effectiveness is strictly Gadd45β-dependent. Notably, IC_{50} for SCR tripeptide is not calculable as no significant cell viability reduction was observed (Fig.20, C).

Overall, the results show a linear correlation between Gadd45β expression levels and DTP3 dose-response suggesting that the biological effects are Gadd45β and peptide-sequence specific.

A



B

	BHT-101	8505c	CAL-62
IC_{50}	>500 µM	245 µM	92 µM
R^2	0,945	0,946	0,928

C

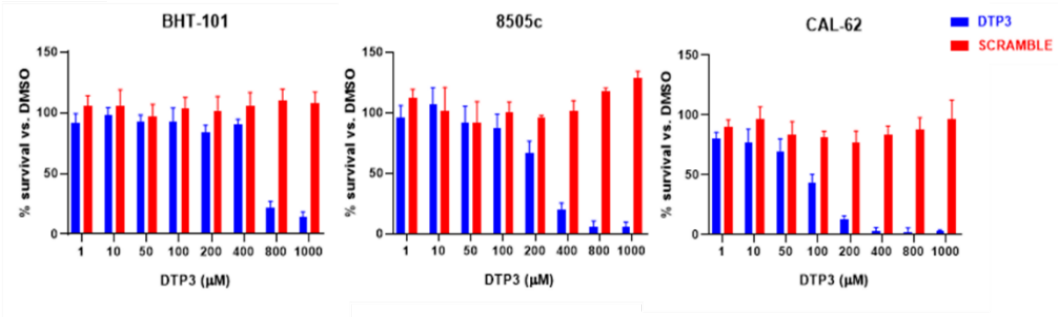


Figure 20. IC_{50} estimation by Crystal Violet cell viability assay on ATC cell lines. (A) Indicated cell lines were treated with a range of DTP3 concentrations indicated in log scale for 96 hours. All values are averages of replicates expressed relative to cell viability values in cells treated with vehicle (DMSO) normalized to 100%. Cytotoxicity curves represent n=3 experiments with 3 replicates per drug concentration for each experiment. Dose-response curves were calculated nonlinear regression analysis. (B) Table showing IC_{50} and R^2 values of cell in (A). (C) Histograms presenting percentage of cell survival on cell in Fig.20 (A). Bars represent averages of replicates expressed relative to cell viability values in cells treated with vehicle (DMSO) normalized to 100%. X axes show DTP3 concentration (µM).

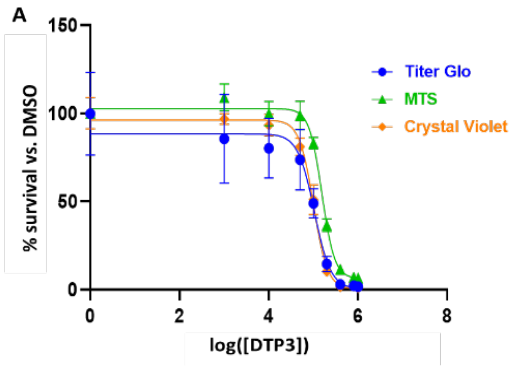
DTP3 IC_{50} values show high reproducibility in CAL-62 cell line using different biochemical assays

Drug-dose response assays are commonly used to evaluate drug efficacy and potency. However, biological and technical factors may affect data replicability and reproducibility. Ideally, a robust assay should be able to provide precise information about drug efficacy allowing statistically significant results(Larsson et al., 2020). Furthermore, the assay protocols to assess drug anticancer effects may be significantly influenced by chemical and biological properties of the compounds of interest.

Therefore, to verify DTP3 IC_{50} estimation accuracy we compared different technique protocols performing cell viability assays. So, we focused our attention on CAL-62 cell line which showed significant DTP3 sensitivity.

Briefly, we treated CAL-62 cell line with a range of DTP3 concentrations and assessed Crystal Violet, MTS and CellTiter-Glo® assays.

Intriguingly, the relevant dose-response curves showed a similar trend (Fig. 21, A) and IC_{50} values were comparable between experimental protocols (Fig.21, B). Interestingly, CellTiter-Glo® and Crystal Violet assays showed a tight correlation demonstrating the consistency of DTP3 IC_{50} values. These results underline that IC_{50} estimation is reproducible and DTP3 chemical properties do not influence experimental procedures.



B

	TiterGlo	MTS	Crystal Violet
IC_{50}	105 µM	155 µM	100 µM
R^2	0,875	0,987	0,986

Figure 21. Comparative IC_{50} estimation using TiterGlo, MTS and Crystal Viole viability assays on CAL-62 cell line. (A) Cal-62 cell line was treated with a range of DTP3 concentrations indicated in log scale for 96 hours. All values are averages of replicates expressed relative to cell viability values in cells treated with vehicle (DMSO) normalized to 100%. Cytotoxicity curves represent n=3 experiments with 3 replicates per drug concentration for each experiment. Dose-response curves were calculated nonlinear regression analysis. (B) Table showing IC_{50} and R^2 values of cell in (A).

DTP3 induces G0/G1 cell cycle arrest followed by delayed apoptotic cell death

It is well known that cell death by apoptosis is a natural barrier against neoplasia development. Indeed, cell death resistance represents a hallmark of cancer(Hanahan & Weinberg, 2011). GADD45β interacts with MKK7 kinase leading to an impairment of the apoptotic machinery(De Smaele et al., 2001; Salvatore Papa, Zazzeroni, Bubici, et al., 2004).

To better clarify DTP3 biological effects on ATC cell lines, we assessed cell cycle analysis by BrdU incorporation assays.

Basically, we treated BHT-101, 8505c and CAL-62 with 200 µM DTP3 for 48 hours and determined alterations in cell cycle distribution and phases by flow cytometer analysis (Fig. 22). We observed that in CAL-62 cell line DTP3 treatment triggers remarkable decrease of S cell phase (DMSO: 58,8%, DTP3: 23,6%) and increase of G0/G1 phases (DMSO:26,9%, DTP3:57%). Similarly, the results showed that 8505c cells distribution is slightly affected by DTP3 treatment as suggested by the percentage of G0/G1 phase in vehicle DMSO (42%) compared with DTP3 (46,2%). Conversely, BHT-101 showed no significant changes (Fig. N).

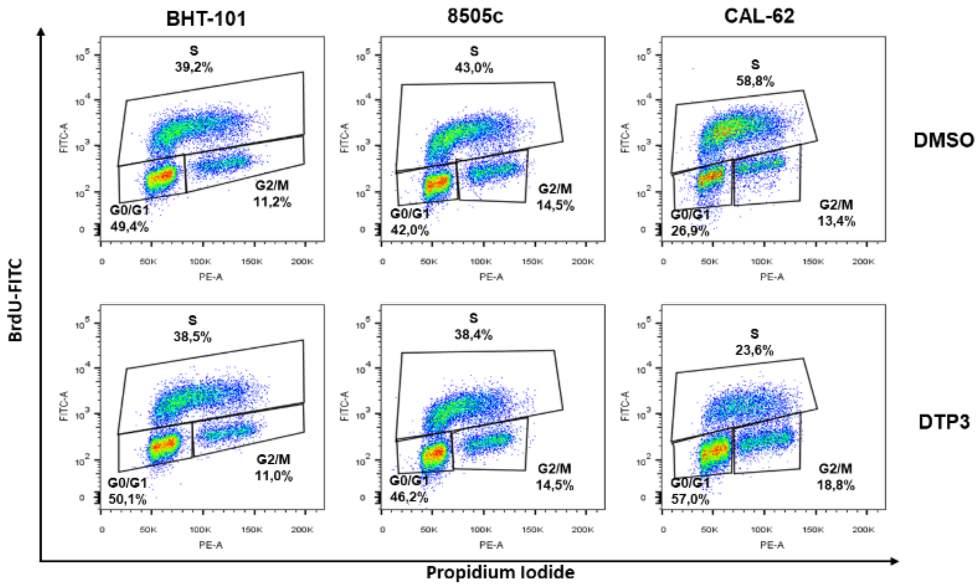


Figure 22. **Flow cytometry analysis of cell cycle distribution following DTP3 treatment.** BrdU incorporation assay was used to detect the cell proliferation status by flow cytometry. In this figure, biparametric dot plots of control or 200 µM DTP3-treated ATC cells after 48 hours. BrdU incorporation and DNA content of nuclei were visualized using anti-BrdU Alexa 488 conjugate and propidium iodide, respectively. Three boxes are shown, representing nuclei in G0/G1, S and G2/M, respectively.

It has been established that prolonged cell cycle arrest culminates to divergent outcomes: the cell-division restoration, the commitment to senescence and the activation of the apoptosis (Schmitt et al., 2007).

We have previously demonstrated DTP3 ability to activates apoptosis in MM cell lines (Tornatore et al., 2014).

Based on our data, we decided to examine ATC cells fate upon DTP3 treatment at late time points.

To address this point, we performed flow cytometry apoptosis assays by Annexin V/Propidium Iodide (PI) double staining. We decided to detect apoptosis in CAL-62 cell line which showed remarkable G0/G1 accumulation upon DTP3 treatment. So, CAL-62 were exposed to DTP3 at different concentration (50-100-200µM) for 3 and 9 days, labelled with Annexin V FITC-conjugated and PI and then analysed by flow cytometer.

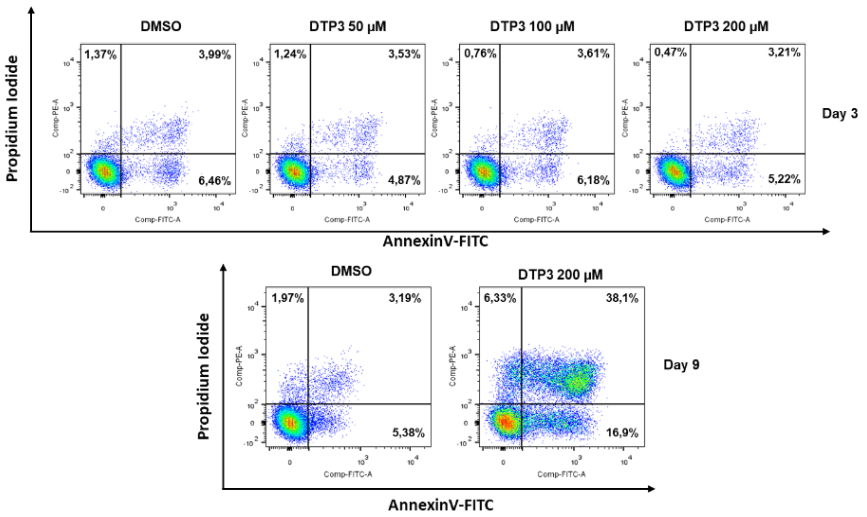


Figure 23. Flow cytometry apoptosis analysis of CAL-62 cell line treated with DTP3. CAL-62 cells were treated at indicated DTP3 concentrations or vehicle (DMSO). Cells were stained with Annexin V and Propidium Iodide (PI) and analysed by flow cytometry after 3 and 7 days. Apoptotic cells positive for annexin V can be seen in the bottom right quadrant and dead cells positive for both annexin and PI in the top right quadrant. Top left quadrant shows necrotic PI positive cells. Healthy cells are negative for both stains.

The panel in Fig. 23 indicate that DTP3 showed no remarkable biological effects after 3 days. Indeed, neither apoptotic nor necrotic positive cells showed significant differences at early time points (top). Strikingly, the analysis revealed higher levels of Annexin V/PI positive cells (bottom) than DMSO control at dosage of 200µM of DTP3 after 9 days.

These data suggest that DTP3 triggers G0/G1 cell cycle arrest at early time points followed by delayed apoptotic pathway activation.

DTP3 induces G0/G1 cell cycle arrest modulating phospho-ERK and Cyclin D1 levels

Cell-division cycle in regulated by a tight balance between 'driving' and 'braking' forces. Numerous studies have shown that the ERK 1/2 MAP kinase induces the transcriptional regulation of Cyclin D1, thereby promoting cell cycle progression (Villanueva et al., 2007; Z. Wang, 2021).

So, we questioned if DTP3-mediated cell cycle arrest was related to ERK1/2 signaling pathway alteration.

To test our hypothesis, we evaluated ERK 1/2 phosphorylation (pERK 1/2) and Cyclin D1 expression levels by Western blot analysis. Hence, we treated CAL-62 cell line at different DTP3 dosages and analysed protein extracts following 48 hours of treatment.

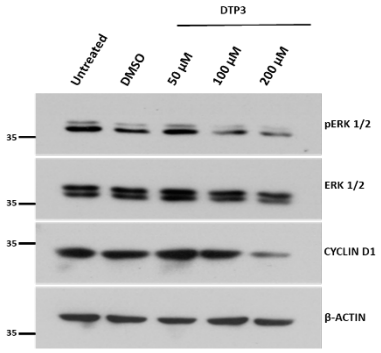


Figure 24. ERK 1/2 and Cyclin D1 Western Blot analysis. Representative Western blots showing phospho-ERK1/2, total ERK1/2 (first and second panels) and Cyclin D1 protein levels (third panel). β-actin was used as the loading control.

Remarkably, ERK phosphorylation showed a reverse correlation with DTP3 concentration. In fact, pERK 1/2 levels decreased according to DTP3 dosages (Fig.24 - first panel). A similar phenomenon was observed in Cyclin D1 protein levels confirming that pERK 1/2 downregulation prevents Cyclin D1 expression (Fig 24 – third panel).

These preliminary data are consistent with our flow cytometry results demonstrating that DTP3 impairs cell cycle progression via ERK1/2 causing a G0/G1 phase arrest.

DTP3 triggers a delayed and sustained activation of JNK kinase

It has been established that many scaffold and kinases proteins play fundamental role in JNK phosphorylation and subsequent activation. Moreover, the coordination of the so called 'signalosome' relies on the physical proximity of JNK upstream MAP kinases MKK7(Yarza et al., 2016).

Our previous studies demonstrated that DTP3 interacts with MKK7 kinase displacing Gadd45β thus restoring JNK phosphorylation(Tornatore et al., 2014).

To further confirm DTP3 mode of action in our cellular models, we designed time-course experiments to evaluate phospho-JNK (pJNK) expression levels.

Representative Western Blot results are shown in Fig.25.

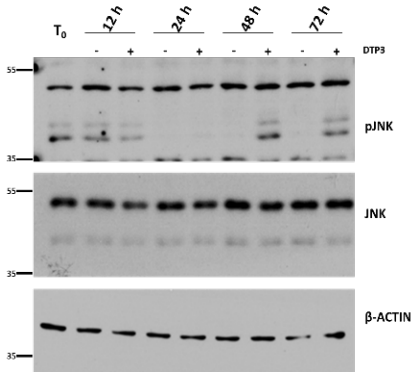


Figure 25. pJNK Western blot analysis. Representative pJNK Western Blot analysis showing pJNK (first panel) and total JNK (second panel) expression levels. CAL-62 cells were treated with 200μM DTP3, harvested and processed at indicated time point. β-actin was used as the loading control.

We observed an increase in pJNK levels from T_0 up to 12 hours in 200 μM DTP3- and vehicle- treated samples. Conversely, JNK phosphorylation levels appeared to decrease after 24h with no significant differences between treated and control cells. Surprisingly, we detected higher pJNK levels in DTP3 treated cells than relevant controls at late time points (48h and 72h). These data demonstrate that DTP3 induces a persistent and delayed pJNK activation in CAL-62 cell line.

Overall, the results confirmed that GADD45β plays an important role in the regulation of ATC cell lines survival. Moreover, as demonstrated in the hematological malignancy MM, the interference with NF-κB signaling via its pro-survival effectors represents a promising and effective therapeutic strategy in solid tumors, as such anaplastic thyroid cancer. However, experiments in ATC

xenograft mouse models are ongoing to further confirm DTP3 cancer-cell efficacy and cancer-cell specificity.

Ongoing research and preliminary data:

Gadd45β gene expression control by the CRISPR interference (CRISPRi) system

Our results showed that DTP3 biological function correlates with Gadd45β expression levels. Moreover, GADD45β-MKK7 complex disruption leads to growth arrest followed by apoptotic cell death in ATC cell lines. Thus, we decided to investigate GADD45β-MKK7 complex ATC cells addiction. Firstly, we assessed RNA interference (RNAi) experiments using specific shRNAs directed against Gadd45β mRNA. However, RNAi approach showed to be not effective in producing Gadd45β knockdown cells (data not shown).

Thus, to achieve our purpose we selected *S. pyogenes* dCas9 with a C-terminal KRAB fusion, which has been used previously for endogenous gene repression (Thakore et al., 2015). This repurposed CRISPR system consists of catalytically inactive version of Cas9 (dCas9) that lacks endonucleolytic activity because of two point mutations in both its RuvC-like (D10A) and HNH nuclease (H840A) domain171. The catalytically inactive version of Cas9 (dCas9) when coexpressed with a gene-specific gRNA induces the repression of transcription initiation and the silencing is inducible and reversiblecas9 (Larson et al., 2013).

We designed four gRNAs targeting the proximity of *Homo sapiens Gadd45b* transcription starting site (TSS). gRNA target sites were selected for minimal predicted off-target activity and maximal on-target activity according to CHOP CHOP tool algorithms (Montague et al., 2014)(Fig.28).

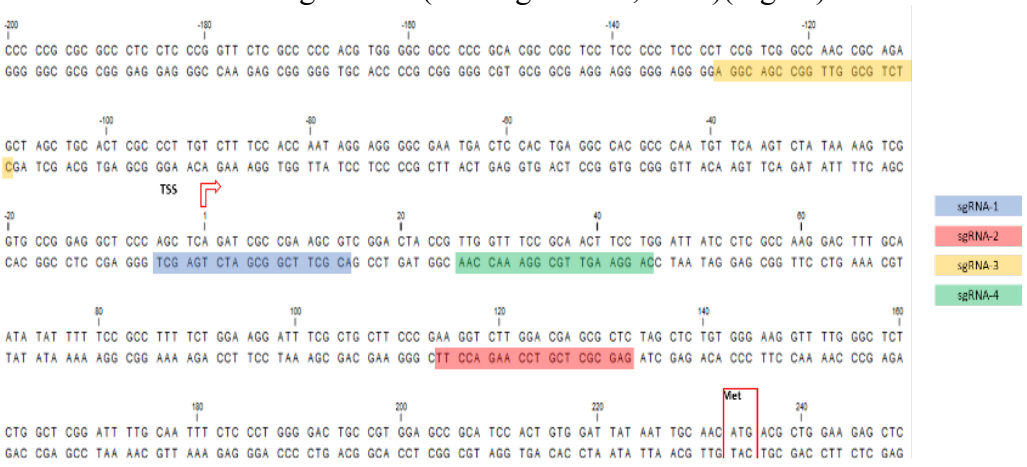


Figure 28. Schematic representation of gRNAs target sequences. The figure shows Gadd45 genomic sequence (-200bp, +250bp respect to TSS). The gRNAs target sequences are highlighted by different colours. TSS, transcription starting site, Met, starting Methionine.

We delivered the d C a s 9 -KRAB system by lentiviral particles encoding Blasticidin resistance gene, which allowed antibiotic cells selection. Accordingly, we transduced CAL-62 and dCas9- CAL-62 cells with lentiviral vectors encoding doxycycline (Dox)-inducible gRNAs (gRNA1-2-3-4) and Puromycin resistance gene.

To validate CRISPRi system, we performed qRT-PCR analysis on CAL-62 transduced cells using wild type cells as control of relative quantification. Thus, we isolated RNA samples from the relevant cell populations following Dox induction.

First, we analysed the efficiency of gRNAs induction in transduced cell populations upon 1µg/mL Dox induction after 48 hours. As a result, transduced population exhibited almost undetectable gRNAs expression levels in absence of doxycycline induction. Notably, gRNAs-cells exhibited effective induction of gRNAs expression (Fig. 29, A).

Also, Gadd45β mRNA expression levels decreased according to gRNAs induction. Moreover, Dox induction does not affect Gadd45β expression levels in CAL-62 relevant controls (CAL-62 and no gRNA) indicating that the mRNA reduction is gRNA-related (Fig.29, B).

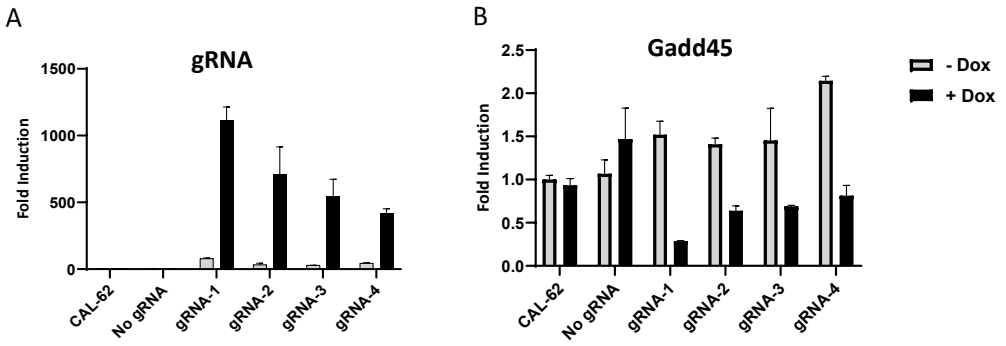


Figure 29. Validation of gRNAs efficacy by qRT-PCR analysis. qRT-PCR analysis testing the induction and specificity of gRNAs designed against Gadd45β genome sequence. (A) qRT-PCR analysis of gRNAs expression levels upon Dox treatment 1µg/mL after 48h. (B) qRT-PCR analysis of Gadd45β expression levels upon Dox treatment 1µg/mL after 48h. CAL-62, wild type. No gRNA, dCas9-CAL62.

To further validate functional efficacy of gRNAs, we evaluated Gadd45β protein expression levels in Dox-induced CAL-62 cell lines by Western Blot analysis (Fig.30).

We confirmed that Gadd45β protein expression levels decreased in gRNA-transduced cells according to qRT-PCR analysis (Fig.29).

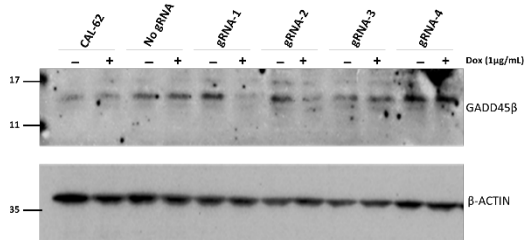


Figure 30. Validation of Gadd45β gRNAs by Western blot analysis Western Blot analysis showing Gadd45β protein expression levels treated with 1µg/mL of Dox for 48h. β-actin was used as the loading control.

Since the CRISPRi allows reversible effects on gene expression, we questioned how long the interference mechanism affects Gadd45β protein expression. Thus, we selected gRNA1 transduced CAL-62 cells, which showed high downregulation efficacy, and performed time course experiments by Western Blot.

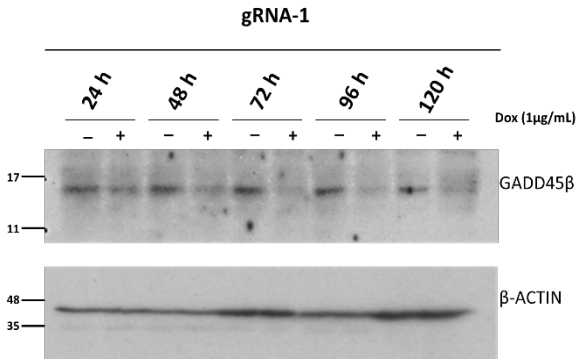


Figure 31. gRNA1 time course Western Blot analysis. Western Blot analysis showing Gadd45β protein expression levels treated with 1µg/mL of Dox and processed at indicated time point. β-actin was used as the loading control.

As a result, gRNA1 induction revealed to be efficient in Gadd45β protein downregulation up to 120 hours (Fig.31).

Therefore, CRISPRi system is a functional and efficient tool to interfere with protein expression. Moreover, designed gRNAs induced a strong reduction of both mRNA and protein Gadd45β expression levels.

Overall, these results are a promising starting point to further demonstrate Gadd45β biological role in ATC cell lines interfering with Gadd45β/MKK7 complex formation.

DTP3 triggers reticulum endoplasmic (ER) stress in responsive ATC cell lines

Our data demonstrated that DTP3 selectively induces cell growth arrest and activation of delayed apoptosis in ATC Gadd45β expressing cell lines. Thus, to investigate gene expression variations eliciting DTP3 biological activity we performed RNA-seq analysis in ATC cell lines upon the tripeptide treatment.

Transcriptome analysis is a reliable and high-throughput technique which provides a snapshot of gene expression profiling under various conditions. The study of gene signatures through RNA-seq approach gives information about temporal and spatial expression of specific genes at the resolution of individual transcript (X. Yang et al., 2020).

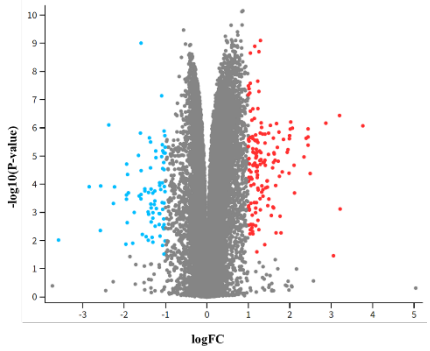
Our results showed that DTP3 mechanism of action in ATC cell lines hinges on cellular growth arrest followed by delayed apoptosis.

ATC cell lines were exposed to DTP3 treatment for 6 hours and we compared RNA-seq profile of DTP3 treated cells with vehicle controls. We performed differential expression (DEGs) gene analysis in the treated samples versus the controls. We assessed this analysis using the voom function of limma, employing an FDR cut-off of 0.05 to identify differentially expressed genes (DEGs). We focused our attention on CAL-62 cell line transcriptome analysis as they showed lower DTP3 IC_{50} value and higher Gadd45β expression levels. Surprisingly, we found significant deregulation of genes involved in endoplasmic reticulum (ER) stress as shown in Volcano plot (Fig. 26, E-F). BHT-101 and 8505c did not showed significant changes in genes involved in ER stress. These results correlate with our findings as BHT-101 showed to be not sensitive to DTP3 treatment (Fig.26, A-B). Conversely, in 8505c cells expressing medium Gadd45β levels, DTP3 concentration is not sufficient to appreciate changes or biological effects at the addressed time point (Fig.26, C-D).

Our results suggest that Gadd45β-MKK7 complex disruption triggers ER stress in ATC cell line context.

BHT-101

A

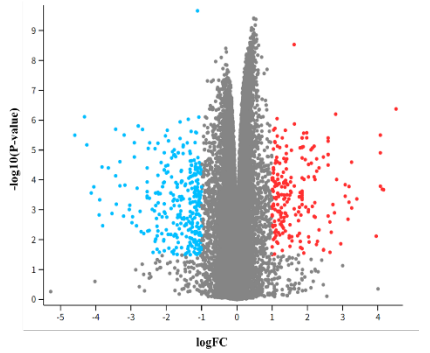


B

Ensemble	Symbol	Gene name	logFC	Log10(P-value)
ENSG00000100219	XBP1	X-box binding protein 1	-0.08377	4.291
ENSG00000175197	DDIT3	DNA damage inducible transcript 3	-0.5673	4.617
ENSG00000162772	ATF3	activating transcription factor 3	-0.1604	1.927
ENSG00000128965	CHAC1	ChaC glutathione specific gamma-glutamylcyclotransferase 1	0.3753	2.956
ENSG00000105327	BBC3	BCL2 binding component 3	0.5841	3.178
ENSG00000087074	PPP1R15A	protein phosphatase 1 regulatory subunit 15A	0.3719	6.628
ENSG00000101255	TRIB3	tribbles pseudokinase 3	0.284	5.774
ENSG00000130766	SESN2	sestrin 2	0.434	5.153

8505c

C

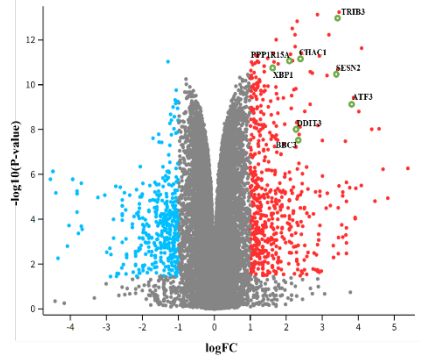


D

Ensemble	Symbol	Gene name	logFC	Log10(P-value)
ENSG00000100219	XBP1	X-box binding protein 1	0.3844	7.815
ENSG00000175197	DDIT3	DNA damage inducible transcript 3	0.2169	2.598
ENSG00000162772	ATF3	activating transcription factor 3	-0.2404	4.425
ENSG00000128965	CHAC1	ChaC glutathione specific gamma-glutamylcyclotransferase 1	0.5752	5.841
ENSG00000105327	BBC3	BCL2 binding component 3	0.265	1.282
ENSG00000087074	PPP1R15A	protein phosphatase 1 regulatory subunit 15A	0.05138	3.002
ENSG00000101255	TRIB3	tribbles pseudokinase 3	0.1578	4.838
ENSG00000130766	SESN2	sestrin 2	0.401	5.321

CAL-62

E



F

Ensemble	Symbol	Gene name	logFC	Log10(P-value)
ENSG00000100219	XBP1	X-box binding protein 1	1.658	11.02
ENSG00000175197	DDIT3	DNA damage inducible transcript 3	2.287	8.311
ENSG00000162772	ATF3	activating transcription factor 3	3.868	9.42
ENSG00000128965	CHAC1	ChaC glutathione specific gamma-glutamylcyclotransferase 1	2.422	11.41
ENSG00000105327	BBC3	BCL2 binding component 3	2.355	8.144
ENSG00000087074	PPP1R15A	protein phosphatase 1 regulatory subunit 15A	2.117	11.36
ENSG00000101255	TRIB3	tribbles pseudokinase 3	3.466	13.24
ENSG00000130766	SESN2	sestrin 2	3.438	10.71

Figure 28. RNA-seq analysis of the upregulated and downregulated DEGs. The volcano plots show the upregulated and downregulated differentially expressed genes (DEGs) of the BHT-101 (A), 8505c (C) and CAL-62 (D) cell lines. For each plot, the x-axis represents the log 2-fold change (FC), and the y-axis represents-log 10 (p values). The blue and red represent downregulated and upregulated genes respectively. ER stress marker in CAL-62 Volcano plot are depicted (green). Tables show ER stress deregulated genes in BHT-101 (B), 8505c (D) and CAL-62 (F) emerged from Volcano plot analysis in CAL-62 (E). Total RNA extracts were processed after 6 h of DTP3 treatment.

Therefore, X-box binding protein 1 (XBP1) , DNA Damage Inducible Transcript 3 (DDIT3), Activating transcription factor 4 (ATF4) and heat shock 70 kDa protein 5 (HSPA5) are known to be ER stress markers (Fusakio et al., 2016; Han et al., 2013; Oyadomari & Mori, 2003; Park et al., 2021).

Thus, we confirmed our RNA-seq results by qRT-PCR experiments. We measured ER stress markers expression levels in ATC cell lines upon either DTP3 or SCR treatment. Remarkably, qRT-PCR data showed that DTP3 treatment significantly upregulate ER stress markers expression levels in CAL-62 cell line suggesting that DTP3 biological activity relates to this pathway. Moreover, ER markers showed no significant changes in BHT-101 used as negative control. Notably, SCR compound showed no significant effect on ER stress markers mRNA induction neither in BHT-101 nor in CAL-62 cell lines. The data suggest that the ER stress response is specifically related to DTP3 biological function and its aminoacidic sequence.

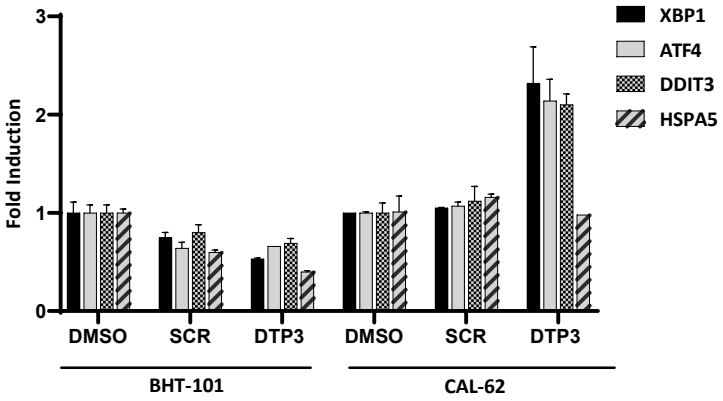


Figure 27. qRT-PCR analysis showing ER stress markers. Gene expression visualized as fold induction of mRNA levels relative to control sample (DMSO). BHT-101 and CAL-62 RNA extracts were processed upon 6h of DTP3 or SCR treatment.

DISCUSSION

In this work, we investigated the biological function of the downstream NF-κB target gene Gadd45β in anaplastic thyroid carcinoma (ATC) cellular models.

ATC is a rare malignant tumor with undifferentiated phenotype and poor prognosis. Available treatments are still poor effective thus ATC represents a controversial clinical challenge.

Cancer development is a multi-layered process sustained by genetic lesions which alter the cellular homeostasis leading to neoplastic transformation. Multiple studies showed the involvement of NF-κB signaling pathway in the pathogenesis of most human malignancies. In addition to its role in immune response, NF-κB is a pleiotropic factor regulating cellular proliferation, apoptosis, angiogenesis, and metabolism. In the last three decades multiple studies have shown that alterations in the tight regulated NF-κB cascade promote oncogenesis.

Interestingly, it has been demonstrated the correlation between NF-κB oncogenic function and ATC physiopathology. Given the driving role in cancer development and progression, NF-κB has been considered a potential therapeutic target for those malignancies addicted to its signal cascade. Indeed, molecular biology approaches aimed to interfere with NF-κB aberrant activity have showed promising results. However, the process "from the bench to bedside" revealed NF-κB as a challenging druggable target. Indeed, despite the aggressive efforts by the pharmaceutical industry to develop specific NF-κB inhibitors, few drugs have been clinically approved, due to the dose-limiting toxicities associated with the global suppression of NF-κB.

To circumvent this limitation, research studies focused their attention targeting downstream NF-κB genes, rather NF-κB itself. Among NF-κB downstream target genes, Gadd45β has been identified as crucial anti-apoptotic effector. Indeed, it has been demonstrated that Gadd45β exerts a cytoprotective function impairing apoptotic pathway. Accordingly, several studies demonstrated that Gadd45β expression levels correlate with poor prognosis in several human malignancies. Subsequent studies revealed that GADD45β impairs JNK phosphorylation by interacting with MKK7 kinases. JNK signaling activation involves the transcription of many pro-apoptotic genes and the balance of pro-survival proteins. Thus, JNK cascade alterations result in apoptosis evasion that is a known cancer hallmark.

With the purpose to interfere with Gadd45β-MKK7 survival complex it has been developed the first-in-class inhibitor DTP3. DTP3 is a tripeptide compound

able to disrupt GADD45β-MKK7 survival complex restoring JNK signal cascade. Regulatory studies confirmed DTP3's on-target pharmacology, anti-cancer efficacy, favorable PK/ADME, and tolerability leading to the ongoing phase-I/II trial (EudraCT/2021-004028-13) in relapsed/refractory Multiple Myeloma patients.

Virtually, DTP3 is an intriguing tool suitable for malignancies sustained by high Gadd45β signature. Our results showed heterogeneous Gadd45β expression levels in ATC patient specimens as well as cancer cell lines suggesting that Gadd45β may be involved in thyroid cancer apoptosis resistance and cell proliferation. Although its expression levels are associated with worse prognosis in TC patients, very little is known about Gadd45β biological role in TC development and progression. Therefore, we validated our hypothesis investigating Gadd45β biological function in BHT-101, 8505c and CAL-62 cell lines which showed low, medium, and high Gadd45β mRNA and protein expression levels, respectively. Thus, we investigated DTP3 biological activity in ATC cell lines expressing differential Gadd45β levels. Interestingly, the linear correlation between Gadd45β expression levels and DTP3 sensitivity suggests a potential addiction to GADD45β-MKK7 survival complex. Further, our data showed that ATC cell lines are permeable both to FITC-labelled DTP3 and SCR tripeptides. The fluorescence intensity detected by flow cytometry and confocal microscopy analysis demonstrate the efficient uptake of tripeptide compounds. However, the specific mechanism of tripeptides entrance is still unknown, indeed further studies are needed to better clarify if culture conditions (e.g., pH, nutrients, O_2/CO2 ratio) may facilitate the transport across plasma membranes. Yet, the fluorescence signals were detectable at early time points suggesting that the entrance mechanism may involve facilitated diffusion rather active transport. Remarkably, we observed a punctate structure pattern in fluorescence signal distribution only in CAL-62 cell lines treated with DTP3-FITC. Conversely, BHT-101 and 8505c cell lines showed a diffuse localization fluorescence signal both with SCR-FITC and DTP3-FITC. Since CAL-62 showed higher Gadd45β expression levels and DTP3 sensitivity, we speculated that DTP3 fluorescence peculiar pattern distribution might indicate Gadd45β signalosome localization. However, we are planning to perform further colocalization analysis to validate these preliminary insights.

IC_{50} data confirmed DTP3 specific mechanism of action in reducing cell viability is tightly related to its aminoacidic sequence. Indeed, SCR tripeptides, having the same aminoacidic composition as the input sequence, showed no biological effects on ATC cell lines. Moreover, Sandomenico et al. demonstrated that at biologically relevant concentrations DTP3, but not SCRB was capable of binding to MKK7. These findings have been supported by further analysis showing the stronger interaction of MKK7 with DTP3 than with SCR compounds (Sandomenico et al., 2020).

Although DTP3 showed both in vitro KD (64.81 ± 6.22 nM) and IC_{50} in MM cells in the nanomolar range, our data showed higher IC_{50} values in our cellular model system. Several studies showed that NF-κB constitutes the central pathway driving MM disease, representing a paradigmatic model to study NF-κB cancer addiction. Thus, it is not surprising that the perturbation of NF-κB signal cascade results in a potent cancer cell killing efficacy. However, ATC genetic background is characterized by mutations of multiple genes and combinations of genes which interplay in aggressive disease behavior. The alteration of cooperating signaling cascades might sustain ATC cell survival thus explaining the higher dosage needed to detect biological effects. Moreover, cell viability results revealed that DTP3 efficacy is tight related to Gadd45β expression levels suggesting its target specificity. Indeed, IC_{50} calculated upon DTP3 treatment showed strong consistency with Gadd45β mRNA and protein expression levels detected by qRT-PCR and Western Blot analysis. These results confirm that DTP3 safe and effective activity is restrained to GADD45β-MKK7 complex oncogenic signaling.

Interestingly, we demonstrated that DTP3 triggers cell cycle arrest in ATC sensitive cell lines followed by the activation of the apoptotic program. Cell cycle arrest is a cellular response to environmental stimuli and stress conditions. Many proteins operate as cell cycle checkpoints to monitor the completion of upstream events prior to proceeding to the next phase. We found that the disruption of GADD45β/MKK7 survival complex results in a perturbation of cell cycle progression. Indeed, CAL-62 cells showed a remarked accumulation in G0/G1 phase with a concomitant reduction of cells in S phase upon 48 hours of treatment. Further investigations confirmed that DTP3-induced cell cycle arrest is due to alteration in p-ERK 1/2 and CyclinD1 protein expression levels. Numerous studies confirmed that ERK1/2 sustained phosphorylation during G0/G1 phase determines cell cycle entry. Indeed, ERK activation is implicated in the induction of cyclin D1 expression and in the inhibition of CDKIs, which are necessary pre-requisite for G1/S phase transition (Chambard et al., 2007). Furthermore, cyclin D1 transcriptional induction is a rate-limiting event regulating cell cycle fate and G1/S phase transition(Montalto & De Amicis, 2020). Thus, these findings suggest that DTP3 exerts anti-proliferative effects on CAL-62 by inducing G0/G1 phase arrest through the inhibition of the MAPK/ERK pathway. On the other hand, our flow cytometry data indicate that treatment of CAL-62 cells with 200 μM of DTP3 resulted in delayed apoptosis activation. Thus, our data demonstrated that DTP3 exerts delayed cytotoxic effects on sensitive ATC cell line showing a new mechanism of action. Indeed, accumulating evidence showed that prolonged cell cycle arrest may eventually result in apoptotic cell death suggesting a link between cell cycle progression and apoptosis. Strikingly, it has been shown that cyclins D unbalanced

expression levels are involved in the regulation of the apoptosis in response to signals of cell cycle arrest (Pucci et al., 2000). These findings support our data showing that DTP3 treatment reduces Cyclin D1 expression levels, thus determining an impairment of cellular proliferation at early time point followed by the activation of apoptotic pathway.

GADD45β-MKK7 survival complex impairs JNK phosphorylation. It has been already demonstrated that DTP3 efficacy relies on its ability to rescue MKK7 kinase activity thus prompting JNK-mediated apoptosis in MM. We confirmed DTP3 mechanism of action in our cellular model detecting p-JNK expression levels by Western Blot analysis. However, our data showed that JNK activation is detectable at early time point as in control as in DTP3-treated CAL-62 cells. Then, at late time points (48h and 72h) p-JNK levels increased in cells exposed to DTP3 treatment compared to controls. Several studies showed the controversial nature of JNK signaling pathway in response to mitogenic stimuli, pro-inflammatory cytokines, and a multitude of environmental stresses. Since JNKs are stress sensors, culture conditions may affect their activation status. Moreover, it has been reported that JNK proteins promote either survival or apoptosis depending on the timing of the downstream signaling cascade. Indeed, sustained JNK activation triggers apoptotic pathway, while JNK pulse signals are compatible with cell survival. Our results suggest that JNK activation at early time point depends on seeding density and/or culture conditions that is compatible with cell survival (data not shown). Conversely, DTP3 enhances JNK signaling over time resulting in a sustained and prolonged activation status triggered by MKK7 activity reconstitution.

RNA-seq analysis reveals the transcriptional state of cells providing information about the temporal and spatial expression of specific genes. To characterize DTP3-induced transcriptional landscape, we performed RNA profiling of ATC cell lines upon DTP3 treatment. Our preliminary data showed that in CAL-62 cell line DTP3 treatment deregulates many genes altering cells transcriptional profile. Indeed, differential expression analysis showed that DTP3 significantly affects genes related to reticulum endoplasmic (ER) stress response. Moreover, these results were strengthened by qRT-PCR analysis of ER stress markers which showed a significant increase in XBP1, ATF4, DDTI3 and HSPA5 mRNA expression levels. ER is the eukaryotic cells 'protein factory' that is involved in protein synthesis, protein folding, and transporting of synthesized proteins. Its functional perturbation activates ER stress pathway followed by the unfolded protein response (UPR) to restore cellular homeostasis.

Remarkably, we demonstrated that ER stress is a specific response related to DTP3 molecular mechanism and its specific aminoacidic sequence. DTP3 tripeptide is composed of amino acids in D configuration, a strategy

to make small peptides resistant to proteolysis. It has been reported that UPR is triggered by accumulation of misfolded protein or inefficiencies in protein degradation (Adams et al., 2019). Notably, we demonstrated that DTP3-mediated ER stress response is tightly related to its biological mechanism of action and Gadd45β expression levels. Thus, ER stress response is a consequence neither of D tripeptide accumulation nor impairment in compound degradation. As a proof, our IC_{50} results showed that SCR tripeptide exerted no biological effects on ATC cell lines regardless Gadd45β expression levels. We confirmed these insights analyzing ER stress markers expression levels where no significant changes were observed in ATC cell lines upon SCR treatment.

Although UPR aims to restore cellular function, prolonged ER stress response results in apoptotic pathway activation, leading to cell death(Bhattarai et al., 2020). Thus, this pathway allows the elimination of damaged cells by apoptosis, indicating the existence of mechanisms that integrate information about the duration and intensity of ER stress stimuli. Indeed, persistent ER stress leads to BAX- and/or BAK-dependent apoptosis as the transcription of the key UPR players, termed DNA Damage Inducible Transcript 3 (DDIT3, also known as CHOP), promotes the activation of BCL-2 apoptotic proteins, contributing to the induction of apoptosis (Hetz, 2012). Surprisingly, latest evidence coupled ER stress to activation of JNK protein kinases through stress sensors inositol-requiring protein 1α (IRE1α) (Urano et al., 2000). IRE1α is the key UPR pathway activator propagating the UPR signal from the ER to the cytosol. Moreover, IRE1α interacts with the tumor necrosis factor receptor-associated factor 2 (TRAF2) resulting in the activation of apoptosis signal-regulating kinase (ASK-1) and downstream target JNK and p38 MAPK. Eventually, JNK activation stimulates pro-apoptotic factors BID and BiM, whilst inhibiting anti-apoptotic factors BCL-2, BCL-XL and MCL-1 (Adams et al., 2019). Further, it has been shown that ER stress triggers activation of NF-κB pro-survival transcriptional program via the adapter molecule TRAF2 (Leonardi et al., 2002). However, we hypothesize that NF-κB anti-apoptotic function is impaired by DTP3 which interferes with the downstream target Gadd45β allowing delayed JNK-mediated apoptosis activation.

Thus, these findings raise an important question about the sequence of events that converges in JNK activation. Previous data showed that DTP3 mechanism of action converges in JNK activation as latest consequence of GADD45β-MKK7 complex disruption. However, our results suggest that in ATC cellular context DTP3 enhance ER stress response which in turn activates JNK signaling leading to apoptotic pathway. Yet very little is known about the crosstalk between Gadd45β-MKK7 complex and ER stress. Further studies are needed to better clarify this new signaling interplay and its potential implications.

Therefore, our data showed that DTP3 biological activity is strictly related to the high Gadd45β signature, so we decided to further confirm Gadd45β cells addiction by interfering with its expression levels. However, RNAi approach showed to be not efficient in the generation of Gadd45β knockdown cell population (data not shown). Hence, we speculated that Gadd45β depletion might be lethal impairing cloning cell selection. However, we addressed this point by using CRISPR interference (CRISPRi) system which allows sequence-specific repression of gene expression. Since Gadd45β is a DNA damage stress sensor we decided to interfere with its expression levels avoiding canonical genome editing techniques (CRISPR-Cas9, TALEN). Indeed, lentiviral infections and genetic manipulation induce Gadd45β expression levels (data not shown). Thus, we circumvented this issue by using the enzymatically deficient Cas9 (dCas9) protein which contains mutations in two active endonuclease domains, losing the DNA cleavage. We cloned under doxycycline (dox)-inducible Tet-On system specific Gadd45β gRNAs for a controlled transcriptional regulation. Our data showed a reduction of Gadd45β gene expression demonstrating that CRISPRi system is suitable and efficient in interfering with target expression. However, further experiments are ongoing to evaluate the biological response upon Gadd45β knock-down.

CONCLUSION

The purpose of cancer targeted therapy is to find new drugs and molecules to overcome malignancies. Cancer is a complex disease characterized by the transformation of normal cells which acquire malignant features and gain the ability to proliferate aberrantly. The origin of cell transformation resides in genetic lesions which drive biological processes deregulation.

In this study we showed that NF-κB pathway deregulation is involved in human thyroid cancer especially in undifferentiated and aggressive ATC subtypes. We demonstrated that NF-κB aberrant activity sustains ATC cell proliferation upregulating the downstream target Gadd45β. Moreover, our data demonstrated that GADD45β-MKK7 complex represents a druggable target through the first-in-class inhibitor DTP3. Indeed, we revealed that DTP3 biological activity is strictly related to Gadd45β expression levels suggesting its specificity and efficacy in ATC cell lines. Strikingly, we unveiled a new mechanism of action of DTP3 in ATC cellular model related to ER stress response activation. Further experiments are ongoing to better clarify Gadd45β and ER stress crosstalk.

Overall, our findings reveal that GADD45β-MKK7 complex exerts a pivotal survival function in ATC cell lines characterized by high Gadd45β signature. Moreover, the discovery of ATC cells addiction to Gadd45β suggest DTP3 as new promising therapeutic strategy for this clinical unmet disease.

LIST OF PUBLICATION

The results obtained by Dr Paola Arboretto have been included in the following publications:

- **Arboretto, P**., Cillo, M., & Leonardi, A. (2021). New Insights into Cancer Targeted Therapy: Nodal and Cripto-1 as Attractive Candidates. International journal of molecular sciences, 22(15), 7838.

- Capece, D., Verzella, D., Flati, I., **Arboretto, P**., Cornice, J., & Franzoso, G. (2022). NF-κB: blending metabolism, immunity, and inflammation. Trends in immunology, 43(9), 757–775.

- Verzella, D., Cornice, J., **Arboretto, P**., Vecchiotti, D., Di Vito Nolfi, M., Capece, D., Zazzeroni, F., & Franzoso, G. (2022). The NF-κB Pharmacopeia: Novel Strategies to Subdue an Intractable Target. Biomedicines, 10(9), 2233.

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