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REVIEW

Proceedings of the discoveries on post-transcriptional *Bcl-2* deregulation in human leukemias/lymphomas

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The *Bcl-2* (B-cell lymphoma 2) antiapoptotic gene has been discovered in virtue of its over-expression occurring in B-cell leukemias/lymphomas carrying the 14;18 chromosomal translocation [t(14;18)], which places the *Bcl-2* gene next to the immunoglobulin heavy chain (*IgH*) locus. In this condition, the transcription of the *Bcl-2* moiety of the *Bcl-2/IgH* fusion gene is driven by the four enhancers located in 3' of the *IgH* moiety and is, therefore, excessive. This leads to overproduction of Bcl-2 protein, which confers a survival advantage that contributes to neoplastic transformation. Nevertheless, in most malignancies, comprising chronic lymphocytic leukemias, breast, prostate, colorectal and lung cancer, the over-expression of *Bcl-2* does not imply chromosomal rearrangements, suggesting that alterations at post-transcriptional level could be involved. Collaborating with the group of Angelo Nicolin (University of Milan, Italy), we first disclosed the existence of a *Bcl-2* post-transcriptional control based on interplay among an Adenine and uracil-Rich cis-acting Element (ARE) located in the 3'UTR of *Bcl-2* mRNA and several trans-acting ARE-Binding Proteins (AUBPs). We also demonstrated its deregulation in human leukemias/lymphomas. In particular, we have identified some *Bcl-2* AUBPs - such as AUF-1, TINO/hMex-3D, the Bcl-2 protein itself and ζ-Crystallin - and described their qualitative or quantitative alterations in cancer cells. Moreover, in the attempt to correct *Bcl-2* deregulation in the human diseases characterized by defects or excesses of apoptosis, we have modulated exogenously *Bcl-2* expression by means of different antisense strategies. In this research highlight, we briefly report our proceedings, in which a long non-coding *Bcl-2/IgH* antisense RNA (*Bcl-2/IgH* AS) we discovered in a serendipitous manner has played a key role.

Keywords: Bcl-2; Post-transcriptional control; AU-Rich Elements (AREs); ARE Binding Proteins; Antisense strategies

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The key role of apoptosis defects consequent to overexpression of *Bcl-2* (B-cell lymphoma 2) gene in cancer development and therapy has been widely recognized [1]. *Bcl-2* has been discovered in B-cell leukemias/lymphomas carrying the 14;18 chromosomal translocation t(14;18), which places the *Bcl-2* gene next to the immunoglobulin heavy chain (*IgH*) locus and is therefore over-transcribed by four enhancers located in 3' of the *IgH* moiety. This leads to the production of excessive amounts of the Bcl-2 antiapoptotic oncoprotein [2], responsible for a survival advantage leading to neoplastic transformation [3]. Nevertheless, in most malignancies, such as chronic lymphocytic leukemias, breast, prostate, colorectal and lung cancer, *Bcl-2* over-expression can occur in the absence of chromosomal rearrangements, which suggests that it could be caused by alterations at post-transcriptional level. A large amount of evidences indicates that up- and down-regulation of *Bcl-2* expression is modulated at transcriptional,

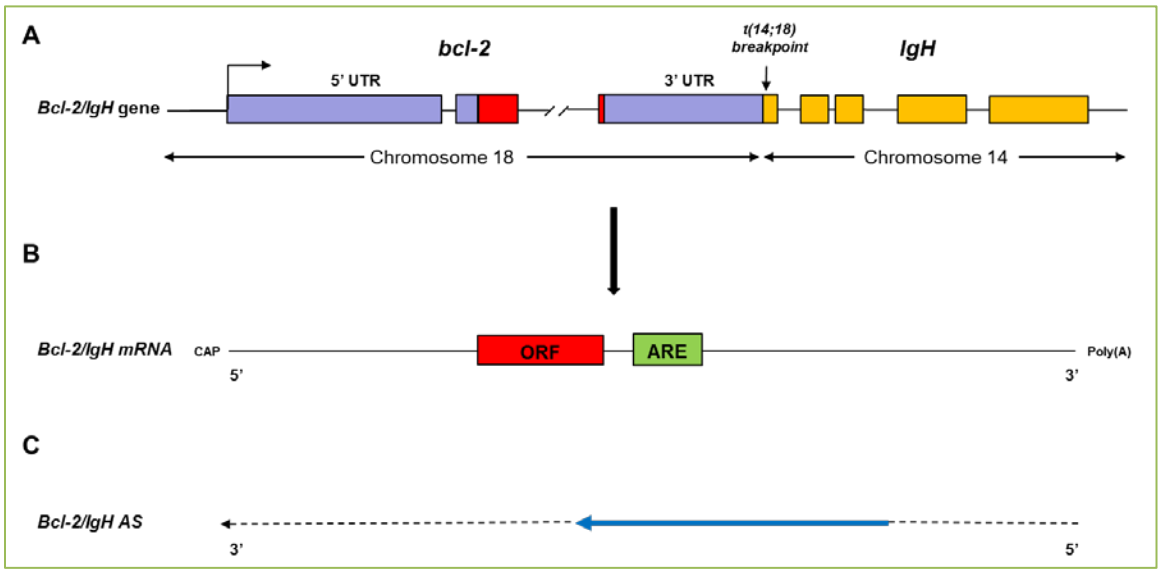

Figure 1. Schematic structure of t(14;18) translocation juxtaposing the *Bcl-2* gene to the *IgH* locus. (A) The hybrid *Bcl-2/IgH* gene showing UTRs of the *Bcl-2* moiety (purple hatched) flanking the coding region (red hatched) and the *IgH* moiety (orange hatched). Intronic sequences are reported as lines. (B) The hybrid *Bcl-2/IgH* mRNA showing its ORF (red hatched) and its ARE (green hatched). (C) The hybrid *Bcl-2/IgH* antisense RNA showing the extension of the portion revealed (blue line) by the strand specific RT-PCR, which includes the ARE overlapping stretch.

post-transcriptional, including mRNA stability and translational control, and post-translational levels. Expression of the *Bcl-2* gene was known to be regulated transcriptionally by a negative regulatory element [4] and by two estrogen-responsive elements identified within its coding region in a breast cancer cell line [5]. An 11 amino-acidic upstream Open Reading Frame (uORF) located within the 5'UTR of *Bcl-2* mRNA inhibits translation of Bcl-2 protein [6]. In addition, *Bcl-2* translation is controlled by the presence of an Internal Ribosome Entry Site (IRES) within the *Bcl-2* mRNA 5'UTR. *Bcl-2* IRES activity is induced upon cell stress, when cap-dependent translation is repressed, and enables to replenish levels of Bcl-2 protein preventing unwarranted apoptosis induction [7]. A mechanism of post-translational control of *Bcl-2 expression* has been described to be mediated by phosphorylation of Bcl-2 protein at different amino acid positions [8, 9]. Two decades ago, starting from the serendipitous identification of a long non coding *Bcl-2*/IgH antisense RNA in t(14;18) leukemic cells, in collaboration with the group of Angelo Nicolin (University of Milan, Italy) we identified a complex post-transcriptional mechanism of *Bcl-2* regulation, which proceedings are described below.

Aimed to reduce *Bcl-2* over-expression in t(14;18) cells carrying the *Bcl-2/IgH* fusion gene by antisense strategies we surprisingly noted that, whilst synthetic antisense oligodeoxyribonucleotides (aODNs) targeting *Bcl-2* or *IgH* RNA did not elicit any effect, the relevant oligodeoxyribonucleotides designed in sense orientation (sODNs) as controls induced a marked decrease of *Bcl-2* mRNA and protein [10]. The ability of sODNs in down-regulating *Bcl-2* expression suggested that *bona fide* they could target a natural *Bcl-2/IgH* antisense RNA that, since its inactivation by sODNs leaded to down-regulation of Bcl-2 expression, could overlap/mask a negative regulative element located in the hybrid *Bcl-2/IgH* mRNA. We obtained the direct evidence of the actual existence of a long non-coding antisense *Bcl-2/IgH* RNA (*Bcl-2/IgH* AS) by a strand-specific PCR analysis, followed by directly sequencing of PCR products. The *Bcl-2/IgH* AS was present in t(14;18) follicular lymphoma DOHH2 cells while was absent in untranslocated Burk1itt's lymphoma Raji and Acute Lymphatic Leukemia (ALL) Jurkat cells, which indicated that its existence was conditioned by the t(14;18) translocation generating the *Bcl-2/IgH* hybrid oncogene (**Figure 1**). Originating in the *IgH* locus, encompassing the t(14;18) fusion site and spanning at least the complete 3' UTR region of the *Bcl-2* mRNA, the hybrid *Bcl-2/IgH* AS has a certain relationship with another antisense transcript previously identified in Burkitt lymphomas starting in the mu-switch region of the *IgH* locus and spanning the c-*myc* gene [11]. The study of the pathophysiological role of the long non-coding RNAs is one of the most intriguing aspects of post-transcriptional control of gene expression [12].

For many years, the fusion sequences arising from chromosomal translocations have been recognized highly tumor-specific molecular targets for ODNs [13, 14]. In analogy, the *Bcl-2/IgH* AS has proven an optimal target for synthetic

ODNs, being of more general relevance respect to the single fusion points of each individual t(14;18) cell line we have tested [15]. Indeed, we have targeted the *Bcl-2/IgH* AS either within the *Bcl-2/IgH* fusion regions, which have a sequence specificity presumably limited to a single cell line, or within the ectopic *Bcl-2* region upstream from the major breakpoint region and the *IgH* segment, which sequence specificity is extended to all cells carrying the t(14;18). Although all sODNs complementary to the *Bcl-2/IgH* AS induced a fast reduction of proliferation and a late but massive apoptosis, while the effectiveness of ODNs targeting the *Bcl-2/IgH* fusion regions was limited to each cell line, the effectiveness of all ODNs targeting the *Bcl-2* or *IgH* regions was extended to all t(14;18) cell lines. The selectivity and efficacy of all sODNs tested provided support for the development of therapeutic ODNs targeting *Bcl-2/IgH* AS expressed in human follicular lymphomas.

Searching for the negative regulative element we supposed to be harbored in the *Bcl-2* mRNA moiety on *Bcl-2/IgH* RNA, we found that the 3' untranslated region (3'UTR) of *Bcl-2* contained a 107-nucleotide Adenine+uracyl Rich Element (ARE) provided with a series of AUUUA repeats similar to others elements endowed with mRNA negative regulative functions [16]. Besides its impressive evolutionary conservation (from *C. elegans* to humans), the *Bcl-2* ARE had all the features of a typical ARE, included a particular distribution of the AUUUA pentamers near an UUAUUUAUU nonamer, which let it ascribe to the class II AREs according to the classification proposed by Shyu *et al*. [17, 18]. The class II AREs usually impart a biphasic kinetic of degradation to their relevant mRNA, are sensitive to actinomycin D treatment, and do not necessarily act on translation. *Bona fide*, the *Bcl-2/IgH* AS could stabilize the *Bcl-2* mRNA in t(14;18) cells by overlapping its ARE.

AREs modulate the fate of relevant mRNAs (in terms of stability, localization and translation) by interacting with a series of trans-acting factors included RNA-binding proteins, namely ARE-binding proteins (AUBPs) and miRNAs. On this basis, the exhaustive clarification of the ARE dependent post-transcriptional control of *Bcl-2* expression required identification and functional analysis of the *Bcl-2* AUBPs. For this purpose, we firstly demonstrated in Jurkat cells that the ARE of *Bcl-2* ARE bound to several cytoplasmic proteins, which molecular weights were from 35 to 100 kDa, and whose pattern underwent modifications in response to apoptotic stimuli. We hypothesized that these proteins must be trans-acting regulators in the ARE mediated degradation of *Bcl-2* mRNA during apoptosis [19]. Considering the antiapoptotic activity of *Bcl-2*, our observations strongly suggested that possible alterations of *Bcl-2* AUBPs could contribute to carcinogenesis and neoplastic progression.

The first analyses of the *Bcl-2* AUBPs in *Bcl-2* over-expressing cell lines demonstrated significant alterations with respect to the normal counterpart. In particular, the observation that proteins ranging from 30–50 kDa underwent the most noticeable increase led us to hypothesize that AU-rich element RNA-binding protein 1 (AUF1) could be a *Bcl-2* ARE-binding protein. Indeed, AUF1, first identified as an RNA-binding protein with selective affinity for AREs located within mRNAs such as c-*myc*, c-*fos*, and *GM-CSF* [20, 21], is comprised of four isoforms of 37, 40, 42, and 45 kDa. We demonstrated that AUF1 bound to the *Bcl-2* mRNA both *in vitro* and *in vivo* and that potentially all its isoforms constituted complexes with the *Bcl-2* ARE in Jurkat cells [22]. At doses able to induce apoptosis, UVC irradiation induced an increase of cytoplasmic levels of the p45 AUF1 isoform, which paralleled an enhancement of a *Bcl-2* mRNA/AUF1 complex and subtended a mechanism requiring caspase activation. These results indicated that ARE-mediated *Bcl-2* mRNA down-regulation during apoptosis involved AUF1 and suggested different roles for its four isoforms.

By using a non-radioactive cell-free mRNA decay system we observed that the degradation of *Bcl-2* mRNA was related to the amount of Bcl-2 protein expressed by different cell types at steady state, was lost upon Bcl-2 depletion and was reconstituted by adding recombinant Bcl-2. This clearly indicated that Bcl-2 was necessary to activate the degradation complex on the relevant RNA target [23]. Successively, in the context of a AUBPs silencing approach, we demonstrated that Human antigen R (HuR) knockdown reduced the expression of endogenous *Bcl-2*, whereas increased significantly a *Bcl-2* ARE-reporter transcript, which suggested that HuR expression has opposite effects on endogenous and ectopic *Bcl-2* ARE [24]. Having also demonstrated that Bcl-2 protein had a specific and dose dependent role in regulating its own mRNA degradation and that its activity overcame the activity of HuR, we suggested that Bcl-2 was the main determinant of *Bcl-2* mRNA turnover [24]. Confirming our observations, Ishimaru D. *et al.* demonstrated that HuR plays a positive role in *Bcl-2* mRNA stability and translation regulation in HL60 leukemia and A431 epidermoid carcinoma cells [25]. We have also shown by UV cross-linking that KH-type splicing regulatory protein (KSRP) and Tristetraprolin (TTP) bound *in vitro* to the *Bcl-2* mRNA [26]. While the functional role of KSRP on *Bcl-2* remains to be disclosed, Park SB *et al.* have very recently demonstrated the ability of TTP to down-regulate *Bcl-2* expression in head and neck cancer cells in response to cisplatin [27].

In an attempt to search for other *Bcl-2* mRNA binding proteins, we used the yeast RNA three-hybrid system assay

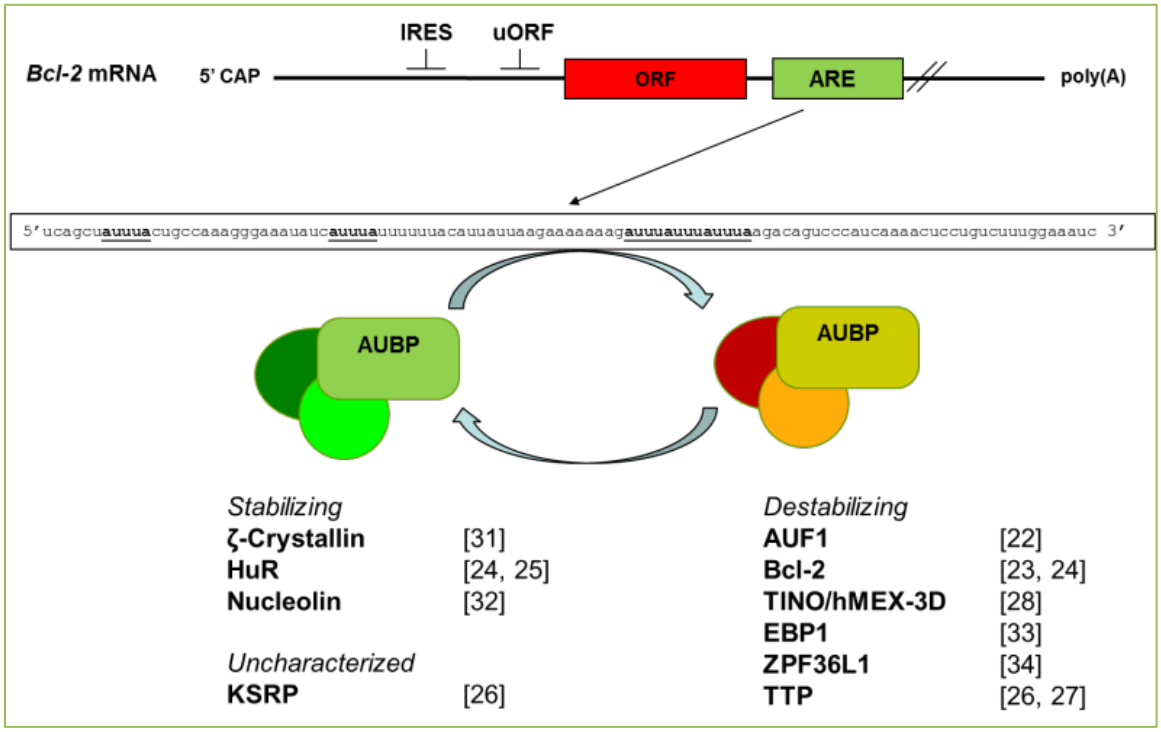

Figure 2. Scheme of the *Bcl-2* mRNA. The position and sequence of the *Bcl-2* mRNA AU-Rich Element (ARE) and its stabilizing or destabilizing ARE-Binding proteins (AUBPs) are shown. References are listed in brackets.

and identified a novel human protein we named TINO, which interacted with *Bcl-2* ARE [28]. Upon binding, TINO had a post-transcriptional negative activity on *Bcl-2* expression, demonstrated by its ability to destabilize a chimeric reporter construct containing the *Bcl-2* ARE. The predicted sequence of TINO had two heterogeneous nuclear ribonucleoprotein K homology (KH) amino-terminal motifs for the nucleic acid binding and a carboxy-terminal RING domain endowed with a putative protein E3 ubiquitin ligase activity. In addition, using the protein-protein BLAST analysis, we observed that the novel protein was evolutionarily conserved. In particular, we identified Posterior End Mark-3 (PEM-3) of *Ciona savignyi* and Muscle EXcess protein-3 (MEX-3) of *Caenorhabditis elegans* as TINO orthologous proteins. More recently, TINO has been recognized as a variant form of hMex-3D [29]. Compared to hMex-3D, TINO is truncated in its N-terminal region, beginning at the first KH domain. This form of hMex-3D also differs at its C-terminal end, with 19 amino acids, encoded by a potential alternative exon, replacing the four last terminal amino acids of hMex-3D. We did not succeed in identifying TINO protein in any experimental model and conditions we have explored. Although the function of TINO/hMex-3D remains to be clarified, some data reported in the literature combined with a series of our preliminary results strongly suggest that it could be involved in a conserved circuit comprising Quaking/GLD-1 and ζ-Crystallin, which alteration leads to stem cell polarity disruption and acquisition of cancer phenotype [30].

Five years ago, by means of a bidimensional SDS-PAGE carried out on *bcl-2* AUBPs from phytoemoagglutinin (PHA)-activated T lymphocytes or Jurkat T-cells followed by mass spectrometry analysis, we have identified ζ-crystallin as a new *Bcl-2* AUBP, which augmented binding to the *Bcl-2* mRNA in ALL T-cells and increased *Bcl-2* expression by enhancing the stability of its mRNA [31]. The specific association of ζ-crystallin to the *Bcl-2* ARE was significantly higher in T cells of ALL patients respect to normal T cells, which accounted for the higher stability of *Bcl-2* mRNA and suggested a possible contribution of ζ-crystallin to *Bcl-2* overexpression occurring in this leukemia. Surprisingly, we found that the cytoplasmic levels of ζ-crystallin did not differ in normal PHA-activated T-lymphocytes with respect to leukemia T-cells, indicating that the different binding of ζ-crystallin to the *Bcl-2* ARE in leukemia T-cells did not depend on its concentration and might be explained by other mechanisms, which remain to be disclosed. We propose two alternative scenarios: the first predicts that qualitative alterations of the ζ-crystallin protein in ALL T-cells allow increased *Bcl-2* ARE binding; the second proposes that modifications of *Bcl-2* AUBPs pattern in ALL T-cells could advantage ζ-crystallin interaction with the *Bcl-2* ARE.

Others have identified further Bcl-2 AUBPs. They are

Nucleolin, which overexpression and altered subcellular localization in Chronic Lymphatic Leukemia (CLLs) leads to excessive *Bcl-2* mRNA stability [32]; EBP1, endowed with destabilizing activity on a chimeric construct harboring the *Bcl-2* ARE in HL-60 leukemia cells [33]; ZFP36L1, which *Bcl-2* mRNA destabilizing activity has been demonstrated in leukemia, lymphoma and renal carcinoma cell lines [34] (**Figure 2**). Furthermore, some miRNA have also been demonstrated to be involved in *Bcl-2* post-transcriptional control and in its alterations in human leukemias [35-38]. Very recently, Díaz-Muñoz MD *et al.* have disclosed the ability of *Bcl-2* AUBP/ARE association to stabilize *in vivo Bcl-2* mRNA, contributing to Bcl-2 protein over-production and B cell survival [39].

The complex of the past and current literature clearly indicate that the pathogenesis of most human diseases underlies either defects or excesses of apoptosis and that *Bcl-2* deregulation plays a key role in apoptosis execution. On this basis, *Bcl-2* still represent a preferred target for innovative cancer therapies [40-42] and some clinical trials have also been recently described [43]. For more than two decades, we have used the antisense strategy as potential therapeutic tool, and (besides the *Bcl-2/IgH* AS in t(14;18) cells), we have chosen the *Bcl-2* ARE as rationally preferred oligonucleotide target to down or up regulate *Bcl-2* expression. Indeed, the simulated folding of *Bcl-2* ARE by the MUFOLD program [44] indicates that it forms a relatively wide loop and is therefore an optimal target both for natural endogenous molecules (the *Bcl-2* AUBPs) and for synthetic exogenous molecules (such as, antisense oligonucleotides and ribozymes).

To downregulate *Bcl-2* expression in apoptosis-defective *Bcl-2* overexpressing Raji cells, we targeted the *Bcl-2* ARE with a synthetic hammerhead ribozyme [45], designed relying on *in vitro* results obtained by probing RNA accessibility to antisense ODNs. The cellular uptake of this lipotransfected ribozyme resulted in a marked reduction of *Bcl-2* mRNA and Bcl-2 protein levels and a dramatic increase of cell death by apoptosis. Although the *Bcl-2* ARE is not a tumour specific target, we proposed to evaluate such ribozyme as potential therapeutic tool for the treatment of *Bcl-2* overexpressing tumors.

Symmetrically, we have attempted to prevent *Bcl-2* dowregulation, thereby inhibiting apoptosis in pathological conditions characterized by apoptosis excesses, by targeting the *Bcl-2* ARE with three 26-mer 2'-*O*-methyl oligoribonucleotides (ORNs) homologous to the core region of the *Bcl-2* ARE used as decoy-aptamers. Sense-oriented ORNs competed with the *Bcl-2* ARE for the interaction with both destabilizing and stabilizing AUBPs in cell-free systems and in cell lines [26]. Moreover, ORNs induced mRNA stabilization and therefore up regulated both *Bcl-2* mRNA and protein levels. Furthermore, *Bcl-2* ORNs stabilized other ARE containing transcripts and up regulated their expression. We also demonstrated that treatment of the SHSY-5Y neuronal cells with *Bcl-2* ORNs prevented *Bcl-2* down-regulation in response to apoptotic stimuli, such as glucose/growth factor starvation or oxygen deprivation, inhibited cell cycle entry and induced a markedly increase of cellular neurite number and length, a hallmark of neuronal differentiation resulting from *Bcl-2* up-regulation [46]. Enhancement of apoptotic threshold and induction neuronal differentiation by *Bcl-2* ORNs suggested evaluating their potential application to prevent pathological apoptosis and neuronal degenerations.

The previously described results confirmed that the destabilizing activity of *Bcl-2* mRNA ARE, we discovered thanks to the *Bcl-2/IgH* AS, underlies a new mechanism of post-transcriptional control of *Bcl-2* expression, whose disruption could contribute to the oncogenicity of this antiapoptotic gene. Furthermore, they demonstrated that the *Bcl-2* ARE represents an optimal target for antisense strategies aimed to down- or up-regulate *Bcl-2* expression, thereby modulating apoptosis in apoptosis deregulation-related diseases.

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