Chapter 7

Analysis of Apoptotic and Autophagic Pathways in Neuroblastoma by Treatment with Copper Compounds

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1. Introduction

Neuroblastoma (NB) is the most common extra-cranial tumor of childhood with more than 600 new cases per year in the United States. The clinical presentation is heterogeneous and dependent on age at diagnosis, staging, histology, and alterations such as *MYCN* amplification and chromosome 1p loss or 17q gain. High-risk patients with evidence of metastases have an overall survival rate of less than 40% despite intensive multimodality treatment. This highlights the urgent need for new therapeutic intervention strategies. However due to NB characteristic molecular features, in about 10% of patients with metastases, tumors disappear by apoptosis [1].

In the search of new anticancer agents with increased chemotherapeutic spectrum, and lower toxicity, new substances based on metals have shown initial promising results. Casiopeínas® (Cas) are a family of compounds with a central Cu^{2+} core atom and an amino acid acidates or α, β-diketonates to seem give them tumor specificity [2]. To date, their action mechanisms are still not completely understood. A possible mechanism may be envisaged however, since it has been described that metals such as Copper and some complexes of them, participate in redox reactions that may generate reactive oxygen species (ROS) including Hydrogen peroxide (H_2O_2), hydroxyl radical (HO●) and superoxide (O_2^-●) [3] that are probably the main factors, leading to apoptosis in cells exposed to these anti-tumor compounds [4-6]

Programmed cell death is an important process for the regulation of different pathways such as cellular homeostasis, embryonic development or regulation of the immune system [7-9].

Apoptosis, a form of programmed cell death, is a highly cotrolled process which includes several well-characterized morphological changes, like membrane blebbing and cell shrinkage, chromatic condensation and nuclear fragmentation [10].The induction of apoptosis may involve either extracellular triggering signals as tumor necrosis factor or endogenous signals such as a cytochrome c (cyt C) release [11], followed by caspase-and endonuclease-activation [12], resulting in the disassembly of nuclear chromatin and degradation of oligonucleosomal DNA.

Several studies have identified other cell death programs clearly distinct from apoptosis [13], and even if we know they are genetically regulated and often have morphological features resembling necrosis, their underlying molecular mechanisms remain unclear. Autophagy is a process that regulates the amount of cell death that occurs in response to specific stimuli like blocking apoptosis after growth factor depletion [14-15] or external insults as DNA damaging agents [16] protecting against caspase-independent death [17] and also by the action of a large variety of anticancer drugs [18].

2. Casiopeínas, a new compound against cancer

Copper is an essential transition metal involved in diverse biological functions, quite especially in redox balance processes [19]. Due to its properties, Copper and its complexes have the ability to catalyze the generation of ROS by means of the Fenton reaction [20]. This process might cause oxidative modification of cellular components like lipids, DNA and proteins, thus disturbing the redox balance and interfering with the redox-related cellular signaling pathways [21].

Casiopeínas is a family of new antineoplastic agents that have been synthesized, characterized and patented in base of chelated Copper (II) complexes. Their general formula is [Cu(N–N)(a-L-aminoacidate)]NO_3 and [Cu(N–N)(O-O)]NO_3, where the N–N donor is an aromatic substituted diimine (1,10-phenanthroline or 2,20-bipyridine) and the O-O donor is acac or salal (Figure 1). The underlying hypothesis is that nature, number and position of the substituents on the diimine ligands, and modification of a-L-amino acidate or O–O donor will have an effect either on the selectivity or on the degree of biological activity shown by the mixed ternary Copper (II) complexes. Chemical data for Casiopeínas are: Cas IIgly [elemental analysis data: calculated (%) for CuC16H16O5N4 2H20 (443.90 g/mol)]: C, 43.29; N, 12.62; H, 4.54. Found (%): C, 43.59; N, 12.61; H, 4.52); Cas IIIia [(elemental analysis data: calculated (%) for CuC17H19O5N3 2H20 (444.93 g/mol): C, 45.89; N, 9.44; H, 5.21. Found (%): C, 46.59; N, 9.80; H 4.93)] and Cas IIIEa [(elemental analysis data: calculated (%) for CuC19-H19O5N3 H20 (450.94 g/mol): C, 50.61; N, 9.32; H, 4.69. Found (%): C, 51.37; N, 9.40; H 4.46)] [22].

Cas have been tested, both *in vitro* and *in vivo*, and have shown cytotoxic, genotoxic [23] and antitumor activity [5, 24]. Cas have been shown good therapeutic indexes in human ovarian carcinoma (CH1), murine leukemia (L1210), AS-30D rat hepatoma, cervix-uterine (HeLa), breast, colon (HCT40) carcinomas, murine glioma C6, and human medulloblastoma (Daoy)

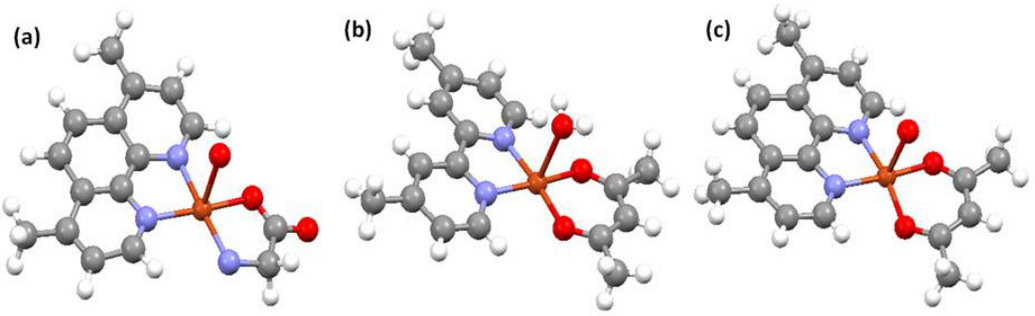

Figure 1. Structures of Casiopeínas.(a) Cas IIgly [Cu (4,7-dimethyl-1,10-phenantroline) (glycinato) (H_2O)] NO_3; **(b)** Cas IIIia [4,4′-dimethyl-2,2′-bipiridina) (acetylacetonato) (NO_3)]. **(c)** Cas IIIEa [Cu (4,7-dimetyl-1,10-phenantroline) (acetylacetonato) (H_2O)] NO_3

and neuroblastoma (CHP-212 and SK-N-SH) cells [25-29]. As well as in animal tumor models such isolated rat hearts [30], dogs [31-32], and nude mice models [5].

The precise mechanism of action for each Cas is still not completely understood and a detailed description of the events that lead to cell death remains unexplained. However, there is evidence that supports that these compounds are able to inhibit cell proliferation and produce cell death by apoptosis by means of mechanisms dependent of caspases activation and independent of caspases throughout ROS generation [24, 26, 29]. It has been shown that Cas are able to block oxidative phosphorylation and to bind DNA by adenine and thymine interactions [33-35], and computational modeling has been done in order to explain such an interaction [36-37]. This suggests that there is more of than one biochemical action mechanism for Cas.

3. Apoptosis by means of Casiopeínas in neuroblastoma

According to previous research results of our group, neuroblastoma cell lines CHP-212 and SK-N-SH have showed that Cas IIgly, IIIia and IIIEa, were active even at very low concentrations (8 μg/ml) when compared to cisplatin, their more efficient competing treatment [29]. These NB cells also showed a differential sensitivity for every Cas treatment and cell lineage at 24 h. This last finding may be due a selectivity given for the specific ligands in the Copper core. For instance, Cas IIgly and Cas IIIEa contain the same imine (4,7-dimethyl,1,10-phenanthroline) with a different charged ligand (glycine *vs* acetylacethonate). Then Cas IIIEa turned out to be more active for CHP-212, whereas Cas IIgly is more active for SK-N-SH cell line. Cellular origin of NB cell lines or even the lack of caspase-8 cleaved expression [38], are features that may define the behavior of NB towards Cas.

Since caspase-8 is commonly silenced in NB, mitochondrial apoptosis is the preferential route to apoptotic cell death, which may involve endogenous process such as cyt C release from mitochondria, resulting in disruption of the mitochondrial transmembrane potential ($\Delta\psi m$) [39]. This event generates a reduction in ATP levels with an influx of ions that leads to decreased mitochondrial activity, and opening of the mitochondrial permeability transition pores [40]. This is an essential component for caspase-3 activation [41].

Under stress conditions such as those produced by Cas, the cell could promote several survival *Stimuli*, one of them is expression of the Bcl-2 family of proteins. Even when Bcl-2 protein is found in the cytoplasmic fraction of treated cells, its expression does not result enough to stop the apoptotic process. In contrast, the expression of the well-known apoptosis promoter Bax is decreased in neuroblastoma cells treated with Cas. Casiopeínas then possess several mitochondrial targets in NB. The main effects of these is production of ROS that in turn provoke $\Delta\psi$m loss, with an augmented Bcl-2 and cyt C release and a massive entrance of Bax protein. By considering, all this body of evidence together we may support the position that the apoptotic pathway favored by Casiopeínas in NB is the intrinsic route [29].

4. Autophagy and Copper compounds in neuroblastoma

Autophagy is a dynamic process that involves the arrest of cytoplasmic portions and intracellular organelles in large double-membrane vesicles (autophagosomes). When these vesicles are fused with lysosomes they generate autophagolysosomes and mature lysosomes, where the arrested material is degraded, inducing cell death [42-43].

Constitutive autophagy enables the physiologic turnover of intracellular components, thus playing an important function in cell homeostasis. However, autophagy can be rapidly induced as a defensive stress response [44-45]. Although apoptosis and autophagic cell death present distinct morphological features among themselves, the two pathways overlap at the level of various signaling steps and may converge and be integrated at the level of the same organelles (i.e. the lysosome and the mitochondrion) [48].

Another molecule involved in the autophagic process is the cytoplasmic form of LC3-I, which during the formation of autophagosomes is cleaved and liquefied to give rise to the membranous form LC3-II. The expression levels of LC3-II can be used to estimate the abundance of autophagosomes before they are degraded by lysosomal hydrolases and the subcellular localization of LC3 redistributes from a cytosolic diffuse pattern to punctuate staining in vacuolar membranes when autophagy is induced. Increased autophagic activity is then reflected by the enhanced conversion of LC3-I to LC3-II.

In a model of rat glioma C6 treated with Cas IIIia were found augmented levels of LC3, particularly of LC3-II, leading to an increased ratio of LC3-II/LC3-I. These results indicate that Cas IIIia induced autophagy promoters such as LC3-II and Beclin-1 [47]. When autophagy was subsequently prevented with 3-methyladenine (3-MA), localization of LC3 at the autophagosomal membrane was inhibited and tumor cells were rescued from cell death [48]. Meanwhile, when neuroblastoma cells (CHP-212) were treated with Cas IIgly, Cas IIIia, Cas IIIEa and cisplatin, LC3-II increases protein at 2 h and 10 h were observed. However at 24 h this molecule was absent, indicating that there was another event different to cellular death [50].

Beclin-1 is a clue regulator of autophagy that directly interacts with Bcl-2, because when they are bound, Beclin-1 is incapable of activating autophagy. However, autophagy is induced by the release of Beclin-1 from Bcl-2 by pro-apoptotic BH3 proteins, Beclin-1 phosphorylation by

DAP kinase (DAPK), or Bcl-2 phosphorylation by JNK [50-51]. Conversely, over-expression of Bcl-2 or Bcl-X_L can inhibit autophagy [52-54]. Another Beclin-1-dependent mechanism by which apoptosis can inhibit autophagy is through caspase-3 cleavage of Beclin-1 to produce a truncated protein that is unable to promote autophagy, thus leading to the overall inhibition of autophagy [55].

Depending of the *status* of caspase-3, neuroblastoma cells may switch between autophagic and apoptotic cell death. It was found that a targeted toxin kills glioma cells *via* a caspase-independent mechanism, and when autophagy is inhibited, this increases (modestly) the amount of death, but changes dramatically the mode of death by allowing the toxin to activate caspases. These data show that autophagy can alter the way cells die, not just whether they die or not [56].

Autophagy in cells treated with Casiopeínas may be result of a diminished effect of this compound that on low doses may not be efficient enough to produce apoptosis. Thus, caspase-3 activity was found in neuroblastoma cells, yet at very early times (2 and 4 h); whilst at 24 h this protein was totally absent [29]. This event is probably seemed to point out that low doses of Cas treatments enable the physiologic turnover of the tumoral cells. In a model of C6 rat glioma cells treated with low doses (5-10 μg/ml) of Casiopeína IIIia, effects at 24 h were also compatible with autophagic features [32].

5. The role of ROS in apoptosis and autophagy in NB

Among several effector mechanisms are involved in the control and regulation of cell death pathways, including autophagy and apoptosis, it seems that the starting point is related with changes in the cellular redox *status*. In the cell, this stage is determined by the balance between rates of production and breakdown of ROS, including free radicals such as superoxide, hydroxyl radical and non-radicals capable of to generate free radicals (i.e., H_2O_2) [57].

A deeper understanding of the mechanisms linking the oxide-radicals-induced autophagy response to cell death pathways, may suggest new therapeutic strategies for the treatment of oxidative stress-associated diseases and phenotypic conditions. For instance, apoptosis observed with Cas treatments, might be the result of one or several events which lead to this final effect: these signals could be mediated by generation of ROS [23-24, 29], by mitochondrial toxicity [58], or both, and might play –either alone or cooperatively- an important role in the regulation of cell death induced by this type of complexes. Several studies have shown that inhibition of cell proliferation and DNA degradation [59-60]) in the presence of reducing agents is simultaneous to ROS generation, suggesting that DNA oxidation observed on cells might also be triggering cell death.

Exposure of C6 glioma cells to Cas IIIia resulted in cell death, with structural and biochemical features consistent with autophagy and apoptosis. Furthermore, the involvement of ROS generation and JNK activation, were showed to be the main features of the autophagic and apoptotic pathways [47]. Hydrogen peroxide reacts with the superoxide radical to constitute itself into a non-radical reactive species. Even toughH_2O_2 is less reactive than radical oxygen

it can produce higher levels of cellular damage. Since it hydrolyzes metals -specially iron Fe(II) and Copper Cu(I)- giving rise to Fenton reactions, a phenomenon already documented in Cas-therapy [6, 58].

On the other hand, it has been reported that low H_2O_2 concentrations (as low as 100μM), are able to induce several events including morphological cell changes, DNA fragmentation and caspase-activation in leukemia cell lines [61]. For this reason H_2O_2 increase by Cas treatment might be implied, either in apoptosis induction or participating as a substrate in Fenton's reaction, producing hydroxyl radical which is a highly reactive and affecting different biomolecules, which not only may cause an apoptotic process but also secondary necrosis. ROS increase could then promote p53-mediated increase of both Bax messenger and protein, implicating cyt C release and consequently promoting caspase-3 activation [62]. In neuroblastoma cells, Cas treatments can induce ROS expression that in turn is related with mitochondrial apoptosis [29].

Superoxide radical is produced when molecular oxygen accepts an electron which remains unpaired. Within the cell this process takes place mainly in the mitochondria, since as a consequence of metabolism, oxygen receive transported electrons [63]. Superoxide synthesis is increased by the influence of factors as radiation or chemicals like pharmaceutical drugs and narcotics. Treatment by Cas increase the concentrations of superoxide thus promoting an unusually high oxidizing environment. This highly oxidant environment cannot be regulated by means of the usual antioxidant defense mechanisms of the cell [6, 23]. For this reason oxidative stress appears, thus damages biomolecules, in particular mitochondrial DNA [58]. As a consequence, cells die *via* the intrinsic route to apoptosis [29, 49]

Previous findings suggest that Copper–phenanthroline complexes react in a redox-cycle with thiols and H_2O_2, resulting in ROS production [64]. On the other hand, glutathione (GSH) indexes haves been related directly to apoptosis due to the ROS expression. In order to maintain the basal concentrations of GSH in the cell, glutathione reductase (Grd) reduces GSS to GSH [65]. Intracellular GSH decrement is thus related to ROS production, because the sulphydril group of Cas is charged in order to trap reactive species that forms a coordinated covalent link with the metal, thus reducing GSH to GSS, by means of a nucleophilic substitution reaction. Platinum is, in the other hand, a soft metal which has affinity to soft donors like those in glutathione's cysteine group. This link may be the mechanism by which the cells eliminate cisplatin. Such processes are implied in producing the low levels of GSH detected in both cell lines after cisplatin treatment, since elimination of cisplatin related to GSS avoids Grd regeneration of the basal levels of GSH. This effect has been reported in several neoplasic lines after the treatment with cisplatin [6, 58].

In fact, Copper has been reported as reacting with GSH to form a stable complex [58]. Nevertheless, it is not clear if this is the elimination mechanism for Cas, or if this complex interferes with the process of GSH regeneration. Decrease in intracellular levels of GSH has been reported in murine melanoma and pulmonary cancer cells treated with Cas IIgly, since Cas catalyzes Fenton's reaction and GSH acts catching the produced ROS [6, 29, 58]. These results allow us to explain the decreased intracellular levels of GSH resulting after treatment with Cas. This comes as a result of a pro-oxidant atmosphere, which could be produced since Fenton's

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reaction is catalyzed through the active Copper core in Casiopeínas. Our results support the hypothesis that, under certain conditions, GSH can be a substrate for pro-oxidant reactions in the cell. CasII gly has been previously shown to interfere directly with the mitochondrial respiratory chain, another effect that could account for an increase in ROS [66]. However, both, delayed ROS burst and mitochondrial depolarization observed by flow cytometry, support Cas IIgly-mediated indirect effects, i.e. GSH depletion and disruption of the mitochondrial respiratory chain due to mitochondrial DNA damage. Given the fast drop in GSH levels, such delay may be due to the ability of the cells to initially control ROS levels, including O_2-● and HO●; however, after some time this ability results exhausted.

The activation of either the pro-survival or the pro-death pathways by oxidative stress depends on the type of ROS and the site of its generation [65, 67], the dose and length of exposure, as well as on the genetic and metabolic background of the target cell [68-73]. Understanding the mechanisms linking the oxide-radicals-induced autophagy response to cell death pathways could suggest new therapeutic strategies for the treatment of neuroblastoma.

6. Pathways and biological processes

As we have seen the action mechanisms of Cas-based chemotherapeutics involves system-level interactions of a number of biochemical processes in the cell, including apoptosis, autophagy, and response to oxidative stress as main motifs; but also involving (to some extent) signaling pathways such as NGF, SCF-KIT, interleukins, and FGFR. Immune response processes involving interferon (alpha/beta), PI3K/AKT; as well as B-cell activation and phosphorylation cascades. With so many complex processes interacting one may wonder what the driving forces (and what the 'side-effects') are.

In order to acquire a closer understanding of these processes, we performed computational and data-mining analyses in both protein-protein interactions and biochemical pathway enrichment. Particular emphasis has been paid to molecular interactions related with the turn-over between apoptosis and autophagy since these are the main mechanisms of cell death observed in NB cells after Cas treatment.

6.1. Protein interaction networks

Computational mining in protein-protein interaction databases (String) was performed in a curated list of molecules associated with apoptosis. The results may be seen in Figure 2. Panel A presents the associated protein-protein (physical) interaction network. Panel B renders a visualization of the same network color- and size-coded according with their connectivity degree: big red circles represent protein that are highly connected within such network (i.e. proteins that may interact in a large number of macromolecular assemblies and other functional roles) while small green nodes are less connected proteins.

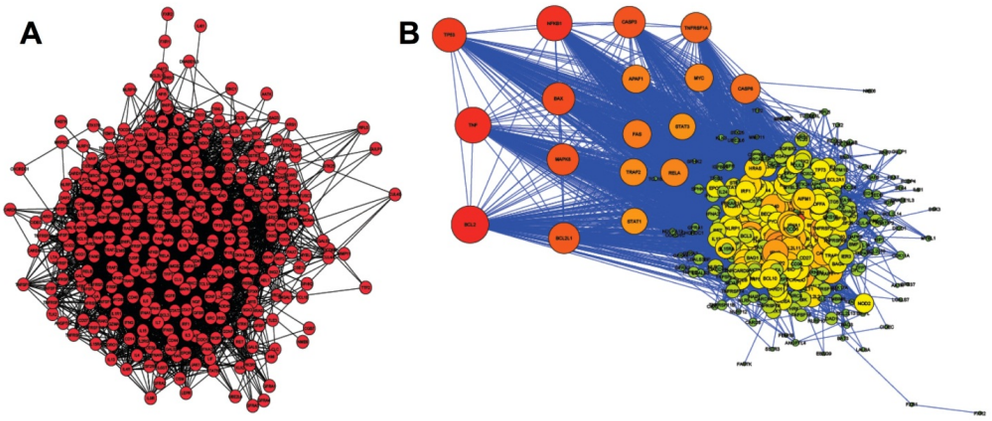

Figure 2. Protein interaction network of apoptotic molecules Panel A displays the protein-protein (physical) interaction network for molecules playing a role in apoptotic processes. Panel B renders a visualization of the same network color- and size-coded according with their number of known interactions. Big, red circles are thus highly interacting apoptotic molecules.

One may hypothesize that highly connected proteins (or hubs) perform central roles in their corresponding biochemical pathways [73]. In fact, by considering the hubs in Figure 2 B we may see this. Molecules such as TP53, TNF, BCL2, NFKB1, BAX, MAPK8, BCL2L1, CASP3, APAF1, FAS, TRAF2, STAT1, TNFRSF1A, MYC, STAT3, RELA, and CASP8 seem to be the main players in the pro-apoptotic/anti-apoptotic molecular switching. These general trends may vary from one tissue/phenotype to another (for instance CASP8 is usually absent or mildly expressed in NB cells) yet their compact interaction structure allows for biological robustness, as displayed for instance in the complementary roles of intrinsic and extrinsic apoptosis.

A similar analysis was performed for molecules associated with autophagy; in Figure 3 we can see the protein interaction network for these molecules. Again panel A shows the protein-protein network. Panel B also displays the network color- and size-coded according with the individual connectivity degree. The key players in this process seem to be MTOR, TP53, UBA52, BCL2, BECN1, AKT1, FAM48 A, and to a lesser extent PTEN, ULK1, PIK3C3, ATG5, HSP90AA1 and JUN. Interestingly enough this network is not so densely connected as the one corresponding to apoptosis (Figure 2) a fact that may result in important outcomes: the network is less robust to removal of one of its key players hence the regulatory mechanisms should be more strict. This may be a reason for apoptosis (and not autophagy) as the main mechanism of cell death.

Given the fact that treatment by Casiopeínas in NB may involve a turn-over between apoptotic and autophagic cell death, it results appealing to analyze the protein interaction network of molecules common to both processes since these molecules may serve as switches between both regimes. In Figure 4 we can see the depiction of a (much simpler) protein interaction network involving molecules related both to apoptosis and autophagy: main players in both processes are BCL2, TP53, and AKT1 with TNFSF10, TRAF6, BNIP3 and BECN1 also associated. Again, response to oxidative stress, DNA damage and cell signaling are represented in

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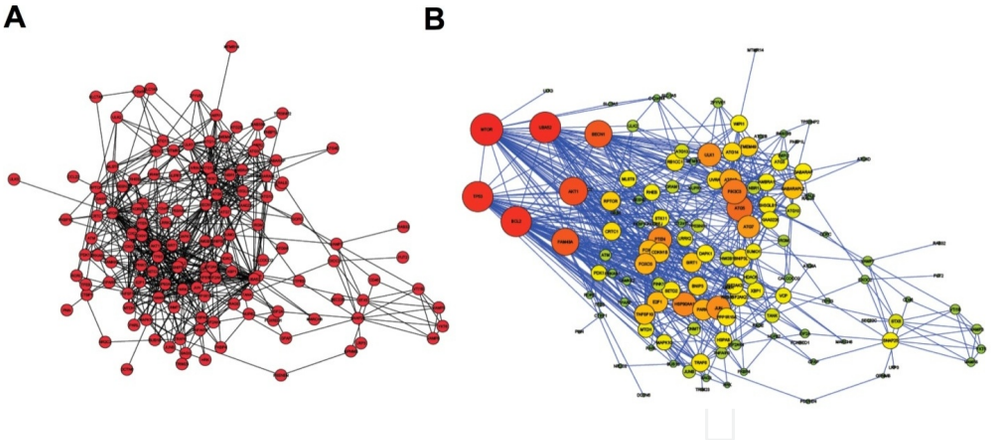

Figure 3. Protein interaction network of autophagic molecules Panel A displays the protein-protein (physical) interaction network for molecules playing a role in authphagy. Panel B renders a visualization of the same network color- and size-coded according with their number of known interactions. Big, red circles are highly interacting autophagic molecules.

this set. Especially intriguing results the role of AKT1 since it is known that some survival factors, induce transcription-independent anti-apoptotic behavior by activation of the oncogene-homolog RAC. AKT1 in turn, phosphorylates inactivating components of the apoptotic machinery.

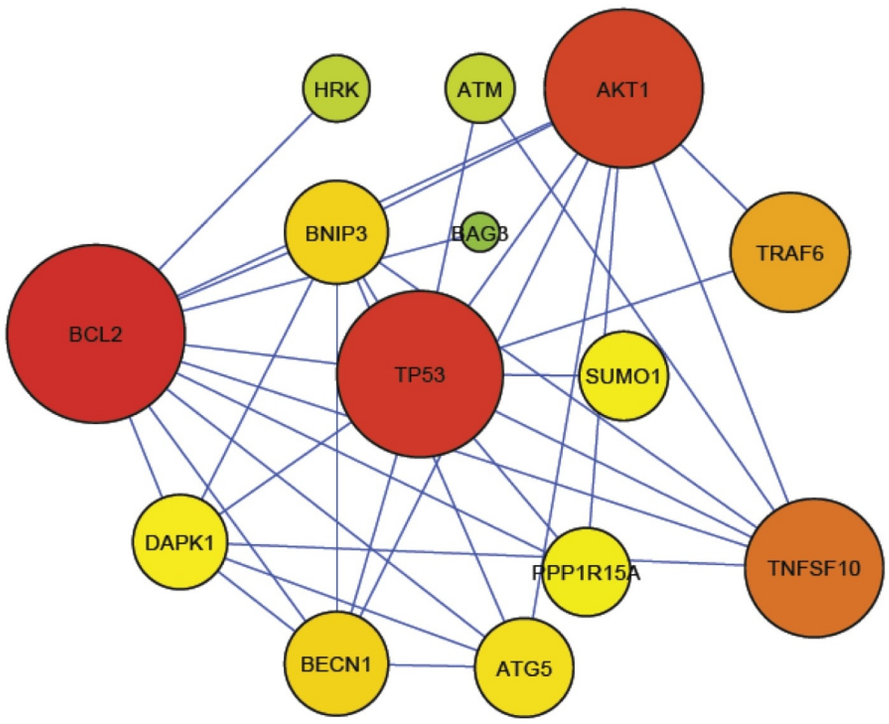

Figure 4. Protein interaction network of molecules involved both in apoptotic and autophagic pathways Molecules are again size and color-coded according with the number of interactions. The role of BCL2, TP53 and AKT1 as strongly interacting molecules centrally-involved both in apoptosis and autophagy may point to these molecules as concertators for the transition between these processes.

6.2. Coupled biochemical pathways

After considering physical interactions between proteins related both to apoptosis and autophagy, we may wonder which are the most common cellular processes involving such molecules. Resorting to data-mining and enrichment analyses in the REACTOME database we found the following pathways (see Fig.4).

7. Conclusions

In the search for new keys to undermine neuroblastoma, our knowledge of the pathways limiting tumor growth, as well as a deeper understanding of the molecules involved in these processes result essential. Based on years of research, our experience is that chemotherapeutic interventions based in Copper compounds as an essential metal, have opened new possibilities to understand not only the pharmacology but indeed some of the biology behind neuroblastoma's behavior. In this sense, we consider of extreme importance to understand, for instance, how Casiopeínas affect neuroblastoma cells in such a way that they finally undergo cellular death by apoptosis, or either -in what resembles a failed attempt of cellular rescue- resort to autophagy. By identifying key molecules for these processes by means of functional genomics and similar analyses we may be able to determine if there are other routes involved in neuroblastoma's final-fate decision processes.

With regards to apoptosis associated molecules, these also take part in biological functions such as: NGF-signaling (TRIB3, MAPK7, RAF1, PRKAR1A, MDM2, BCL2L11, DUSP6, NFKBIA, NGFR, RIPK2, BRAF, NGFRAP1, MYD88, **MAPK8**, NFKB1, **AKT1**, PIK3R1, CASP9, CHUK, GSK3B, SRC, CASP2, MLLT7, AKT3, FOXO1A, **STAT3**, FOXO3A, MAGED1, TRAF6, MAPK1, BAD, CDKN1A, RELA, **CASP3**, HRAS, NTRK1, AATF, YWHAE, NGFB, GRB2), Interleukin-signaling (IL6ST, IL18, STAT5A, RAF1, IL3, CARD15, CSF2RB, HRAS, RIPK2, MYD88, STAT5B, NFKB1, **STAT1**, PIK3R1, CARD4, CHUK, IL1A, JAK1, IL2RG, IL6, STAT3, CASP1, TRAF6, IL1R1, MAPK1, GRB2, IL7), SCF-KIT signaling (BAD, TRIB3, CDKN1A, STAT5A, RAF1, MDM2, HRAS, STAT5B, **AKT1**, **STAT1**, PIK3R1, CASP9, CHUK, GSK3B, SRC, MLLT7, FOXO1A, AKT3, STAT3, FOXO3A, MAPK1, GRB2) and activation of BAD and translocation to the mitochondria (BAD, **AKT1**, **BCL2**, BID); amongst others (full enrichment analysis –more than 500 statistically significant ($p<0.05$) processes- available upon request).

In the case of autophagic molecules, they also participate in the following biological processes: PI3K/AKT activation (PTEN, PDK1, Lst8, FoxO3, PKB, FoxO1, **MTOR**), adaptive immune system (TAK1, **ATG5**, LRSAM1, **BCL2**, ATG7, ATG12, PKR, Rab7, PDK1, FoxO1, CD46, HMGB1, **PTEN**, RAGE, JNK1, FoxO3, Lst8, Vps34, PKB, TRAF6, **MTOR**), as well as in the regulation of apoptosis (JNK1, **BCL2**, ZIPK, PKB, TRAIL, HMGB1, DAPK) by activation of BAD and translocation to mitochondria (**BCL2**, PKB) also among many others [full enrichment analysis of more than 200 statistically significant ($p<0.05$) pathways available upon request].

One interesting case for study in the analysis of apoptotic and autophagic pathways as an effect of Cas therapy, is the biochemical processes (other than apoptosis and autophagy) involving

apoptotic/autophagic molecules, these involve autodegradation of the E3 ubiquitin ligase COP1 (**ATM**, **TP53**) related to DNA damage repair, NOD-like receptor signaling (**BCL2**, **TRAF6**) closely associated with inflammatory processes, and immune system preparation (**AKT1**, **BCL2**, **SUMO1**, **TRAF6**, **ATG5**). In all the biological response of NB to treatment with Cas seem to involve oxidative stress causing immune response, cytokine inflammation and DNA damage leading to cell death initiation either by apoptosis or autophagy depending on the specific kinase signaling present.

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