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Antiproliferative activity of Verteporfin in Embryonal and Alveolar Rhabdomyosarcoma cell lines

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Luca Sanna
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...to everyone who believed in me...

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1. Abstract

Rhabdomyosarcoma (RMS) is a pediatric tumor, which arises from muscle precursor cells.
Recently, it has been demonstrated that Hippo Pathway (Hpo) is involved in tumorigenesis of RMS. Thanks to its kinase cascade, which activates Yes-Associated Protein 1 (YAP1-YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), Hpo is able to activate several physiological and biological features. YAP and TAZ are the heart of Hpo and they showed to have both cytoplasmic and nuclear role. In the nucleus, YAP is able to bind TEAD factors and constitute a complex that activates the transcription of several genes such as MYC, Tbx5 and PAX8 or maintains the stability of others like p73.
The key role of YAP and TAZ in cancer is leading to the development of new compounds able to block their action. One of these drugs is called Verteporfin (VP). This molecule is able to stop the formation of YAP/TEAD complex in the nucleus.
Considering that RMS is an aggressive tumor and that YAP recovers an important role on it, the aim of this project was to understand if VP is able to have a specific effect on RMS cell lines.
This work showed that VP has an antiproliferative action on RMS cell lines. VP perturbs cell cycle in a different manner depending on RMS cell lines. Through its action, VP modifies also the phenotype of RMS cells. Moreover, this drug is able to induce the activation of apoptosis mechanism through the cleavage of PARP protein in RMS cell lines. Furthermore, siRNA-induced knock down of YAP clarifies that VP induces anti proliferative action through other mechanism.

2. Introduction

2.1 Rhabdomyosarcoma

Soft tissue sarcomas (STS) are well-known and rare mesenchymal neoplasms which possess specific morphologic characteristics (Mariño-Enríquez and Bovée, 2016). According to the World Health Organization more than 50 different histological types of STS are known. STS may arise in every part of the body, but prevalently in the extremities such as in the thighs but also in the retroperitoneum and in the trunk. Because of the rarity of these tumors, the majority of patients dies for metastasis found preferably in the lungs (Singer et al., 2000).

Rhabdomyosarcoma (RMS) belongs to the STS. According to the Soft Tissue Sarcoma Committee (STSC) it is the most common STS in pediatric age accounting more than 50% of the cases of this type of cancer (Shern et al., 2017).

Table 1

Type/ Age	<5	5-9	10-14	15-19
Embryonal Rhabdomyosarcoma	4.4	2.7	1.6	1.8
Alveolar Rhabdomyosarcoma	0.8	0.8	0.6	0.8
Rhabdomyosarcoma NOS/pleomorhpic, etc…	1.2	0.9	0.9	0.9

Table 1: Age-specific incidence rates per millions of eRMS, aRMS and NOS/pleomorphic cases. In the data, all races and both sexes are included.

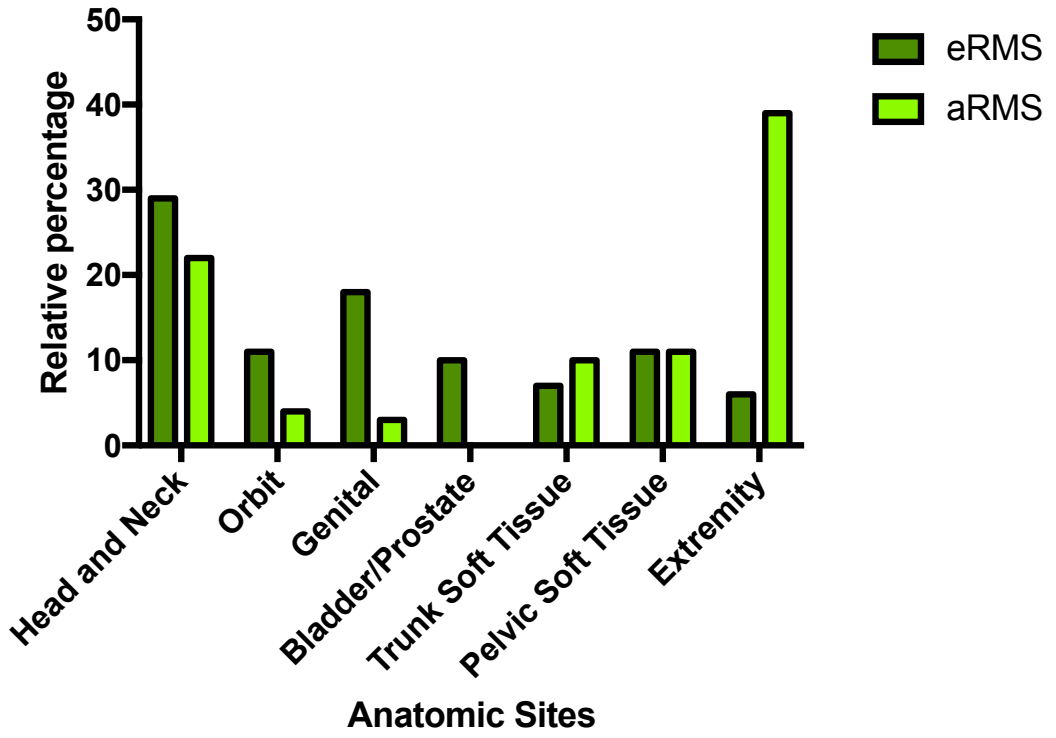
Histologically, the RMS cells appear small, round and dark blue with the hematoxylin-eosin stain. They express early markers of myogenic process like MyoD, desmin, myogenin and actin. Diagnostically, these features help to discriminate these RMS tumor cells from others STS cells. For example, bone or others STS are able to express late markers of myogenesis; instead, RMS cells fail to fuse into myotubes and to transcript late markers genes (Tsokos, 1994; Sebire and Malone, 2003; Saab et al., 2011). From the histological side, RMS can be divided into two different subtypes: embryonal rhabdomyosarcoma (eRMS) and alveolar rhabdomyosarcoma (aRMS).

According to statistics, eRMS arises predominantly in children <5 years old (4.4%), and in smaller percentage in adolescences (1.6 %) (Table 1). aRMS instead, is less common and appears in all ages

from 0 to 19 years old. The uncommon histological subtypes like sclerosing/spindle cells and pleomorphic have a very low percentage of incidence (Parham and Barr, 2013).

Depending on the different subtypes of RMS, the localization of the cancer can change. As shown in Graphic 1, head/neck and the extremities of the body are the predominant parts in which RMS can occur. Specifically, most cases of head and neck RMS are of eRMS type. Instead, the cases of cancer in the extremities are more common in the aRMS (Parham and Barr, 2013).

Often, the rarity of this tumor renders an early diagnosis difficult. The outcome for these patients depends by several factors. In the beginning, it is important the histological subtype, secondly the tumor size and the localization of the tumor. The presence of metastasis and eventual response of the patients to the surgery are also important factors (Meza et al., 2006; Saab et al., 2011).



Graphic 1: Distribution (on percentage) of anatomic site of embryonal and alveolar rhabdomyosarcoma. In the data both sexes and all races are included.

2.1.1 Embryonal Rhabdomyosarcoma

Embryonal Rhabdomyosarcoma is considered a malignant and primitive STS. eRMS cells have features ascribable to embryonic skeletal muscle. Considering this, cells of eRMS are spindle laid down on a collagenous stroma. These cells are present in different stages of myogenesis and appear with a stellate shape, with a central cytoplasm and an egg-shaped nucleus (Figure 1A). Thanks to several studies, it has been possible to help these cells to differentiate ***in vitro***. During these studies, cells appear more stretched (Marchesi et al., 2017) with a cytoplasm which appears eosinophil. Furthermore, thanks to the use of myogenic stimuli drugs, eRMS cells can appear fused into semi-myotubes, forming syncytia and showing more than one nucleus. All of these are signs of terminal differentiation (Marchesi et al., 2017). The histological structure of this tumor is peculiar. Indeed, cells are similar to embryonic muscle in which myoblasts are immersed in a mesodermal tissue (Newton et al., 1995). eRMS tissue shows these characteristics but mixed with denser areas and compacted cellularity. In some cases, the tissue appears with a mucoid stroma with

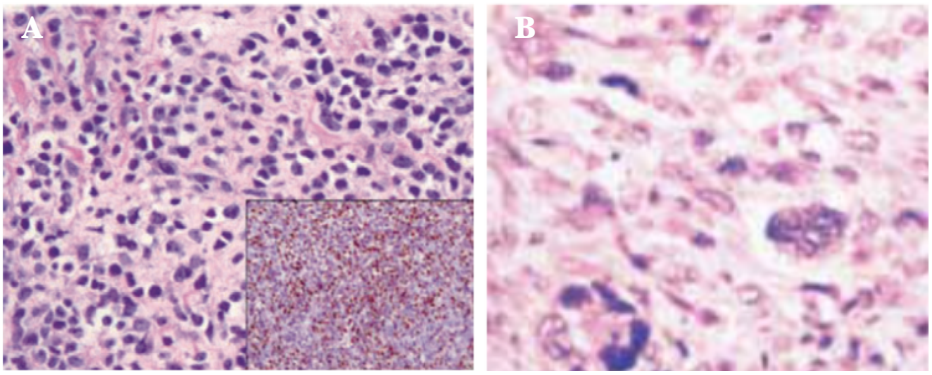


Figure 1: A. eRMS section stained with hematoxilin-eosin. B. Anaplastic eRMS showing hyperchromatic nuclei.

rhabdomyoblasts and myoblasts with a more stretched shape. Depending on the different features of the nuclei, the diagnosis can be more accurate. For example, some cells show rare hyperchromatic nuclei (Figure 1B) helping with the diagnosis (Kodet et al., 1993). This characteristic is present in eRMS and aRMS, but it is more common in the first subtype.

Concerning the immunophenotype, the markers of myogenesis help understanding the degree of differentiation in which these cells are found. For example, vimentin is a cytoplasmic protein of primitive cells, while desmin and actin are important for the arising of RMS. Differentiated cells express marker such as myosin, myoglobin and creatine kinase M. A panel of these markers is used in order to classify the degree of RMS and to understand the subtypes (Kapadia and Barr, 2006).

2.1.2 Oncogenesis of Embryonal Rhabdomyosarcoma

Several genetic abnormalities are involved in eRMS carcinogenesis. Specifically, gains and losses of function have been found. Recurrent gains have been studied in HCST, LRFN3 and ALKBH6 in 19q13.12 (Liu et al., 2014; Sun et al., 2015). Studies found also the involvement of chromosome 11 in the process. Indeed, the loss of heterozygosity is an important feature and it involves a small region in the position 11p15.5 (Koufos et al., 1985; Scrable:1987em Visser et al., 1997). Moreover, it has been demonstrated a wide frequency of loss of heterozygosity in chromosomes 11p, 11q and 16q (Visser et al., 1997; Sun et al., 2015). The alteration in chromosome 11 can be found also in others pathologies such as Beckwith-Wiedemann syndrome, Wilms tumor and hepatoblastoma (Ping et al., 1989; Rainier et al., 1995; Besnard-Guérin et al., 1996). eRMS cell lines are helping to understand the mechanisms involved in this tumor. Indicatively, the oncogene RAS resulted activated in several RMS samples and eRMS (Chardin et al., 1985; Stratton et al., 1989). N-RAS is mutated in the 20% of the RMS cases while H-RAS and K-RAS are less involved (Takahashi et al., 2004; Martinelli et al., 2009). The different kinds of mutations involved in eRMS oncogenesis are shown in Table 2.

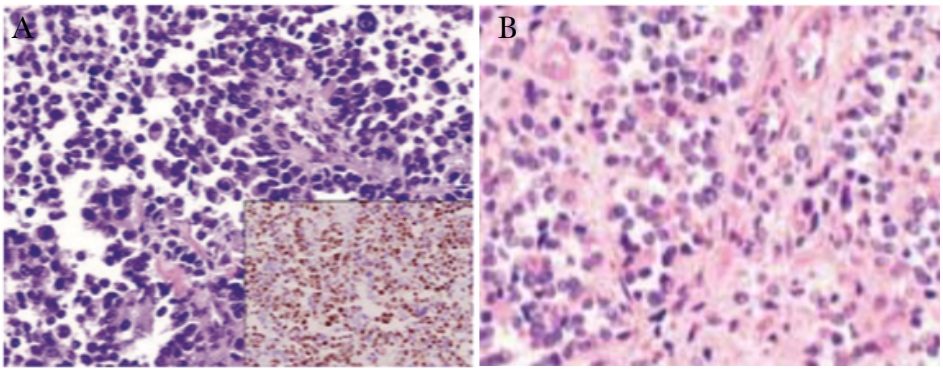


Figure 2: A. aRMS section stained with hematoxilin-eosin. B. The septa of collagenous stroma create a niche in which cells are laying down.

2.1.3 Alveolar Rhabdomyosarcoma

Alveolar Rhabdomyosarcoma (aRMS) can be defined as a malignant, primitive neoplasm which is cytologically similar to lymphoma, showing partial myogenic differentiation cells (Kapadia and Barr, 2006). The histopathology of aRMS is complicated because it includes different kinds of subtypes such as: RMS with typical features, the one with a particular pattern and a mix between

these two subtypes (Newton et al., 1995). Cells of aRMS have a peculiar feature that remind to lymphoma in which they are round but with primitive myoblasts differentiation characteristics. Tissue of aRMS appears with cells included into little niches. Inside of these niches cells are forming clusters which have lost the cohesion with the rest of the tissue (Kapadia and Barr, 2006). The three variants mentioned before show different features. For example, the solid variant of aRMS does not have the fibrovascular stroma and cells, which build the tumor, and show rhabdomyoblasts in various stages of differentiation. The mix type instead, can show the presence of embryonal characteristics with a myxoid stroma and spindle cells. As shown for eRMS, thanks to the staining with specific antibodies such as MyoD and myogenin, it is possible to classify this tumor. Some tissues could not show the presence of these typical markers from differentiation. Indeed, aRMS cells can be in an undifferentiated state which does not show the expression of some proteins involved in late myogenesis process (Dias et al., 2000; Kapadia and Barr, 2006).

2.1.4 Oncogenesis of Alveolar Rhabdomyosarcoma

Among the most common aberrations in the onset of aRMS, there is the involvement of chromosomes 1 and 2. Specifically, translocations t(1;13) and t(2;13) encoding for two transcription factors PAX7 and PAX3 respectively, translocate on chromosome 13 near FOXO1 gene that has a role in the family of Forkhead transcription factors (Barr et al., 1993; Galili et al., 1993; Davis et al., 1994; Saito et al., 2003; Saab et al., 2011). The result of this translocation is the formation of a chimeric gene, which encodes for a fusion protein that contains C-terminal FOXO1 and PAX3, PAX7 DNA binding domain. This fusion protein has a powerful transactivating function compared to PAX3 and PAX7 alone (Davis et al., 1995; Fredericks et al., 1995; Bennicelli et al., 1996; Barr et al., 1998; Fitzgerald et al., 2000; Saab et al., 2011).
Several studies have shown the role of this fusion protein. Specifically its expression in murine mesenchymal stem cells promotes RMS in association with other oncogenic stimuli (Ren et al., 2008; Saab et al., 2011).

Table 2

	GENE	aRMS	eRMS	REFERENCES
Fusion Transcript	PAX3-FKHR or PAX7-FKHR	+	-	{Barr:1993kh, Galili:1993ez, Davis:1994wt}
Locus	11p15	-	+	{BesnardGuerin:1996ux, Visser:1997co}
Oncogenes	RAS	-	+	{Chardin:1985vs, Stratton:1989ul, Wilke:1993vg, Yoo:1999vs}
	N-MYC	+	+	{Dias:1990vq, Driman:1994vw, Bayani:1995uy, Hachitanda:1998to}
	IGF2	+/-	+	{ElBadry:1990wq, Zhan:1994gy, Minniti:1994vo, Makawita:2009fp}
	C-MET	+	+	{Ferracini:1996uk}
	EGFR	+	+	{Ganti:2006gp, Armistead:2007kf}
	FGFR4	+	+	{Taylor:2009dl}
P53 Pathway	MDM2	+	+	{Keleti:1996vf, Taylor:2009dl}
	ARF	+	+	{Iolascon:1996hj}
	P53			{Felix:1992tv, Diller:1995hi, Taylor:2009dl}
RB Pathway	CDK4	+	+	{Khatib:1993ud, Berner:1996ci, Knudsen:1998wj}
	CDKN2A	+	+	{Iolascon:1996hj}
	RB	+	+	{Kohashi:2008hy}

Table 2: In the table are listed common genetic modification involved in RMS.

2.2 Hippo Pathway

Hippo Pathway (Hpo) was firstly observed in Drosophila Melanogaster (Hong et al., 2016). At a later time, studies identified Hpo also in mammals (Meng et al., 2016) uncovering its preservation during evolution. Depending on the species, the components which constitute Hpo have different names. In mammals, Hpo is made up of mammalian STE20-like protein kinase 1 and 2 (MST1 and MST2); large tumor suppressor 1 and 2 (LATS1 and LATS2); Salvador homologue 1 (SAV1), MOB Kinase activator 1A and 1B (MOB1A and MOB1B) and two Yki homologues Yes-Associated protein 1 (YAP1) and the transcriptional co-activator with PDZ-binding motif (TAZ) (Hong et al., 2016; Meng et al., 2016).

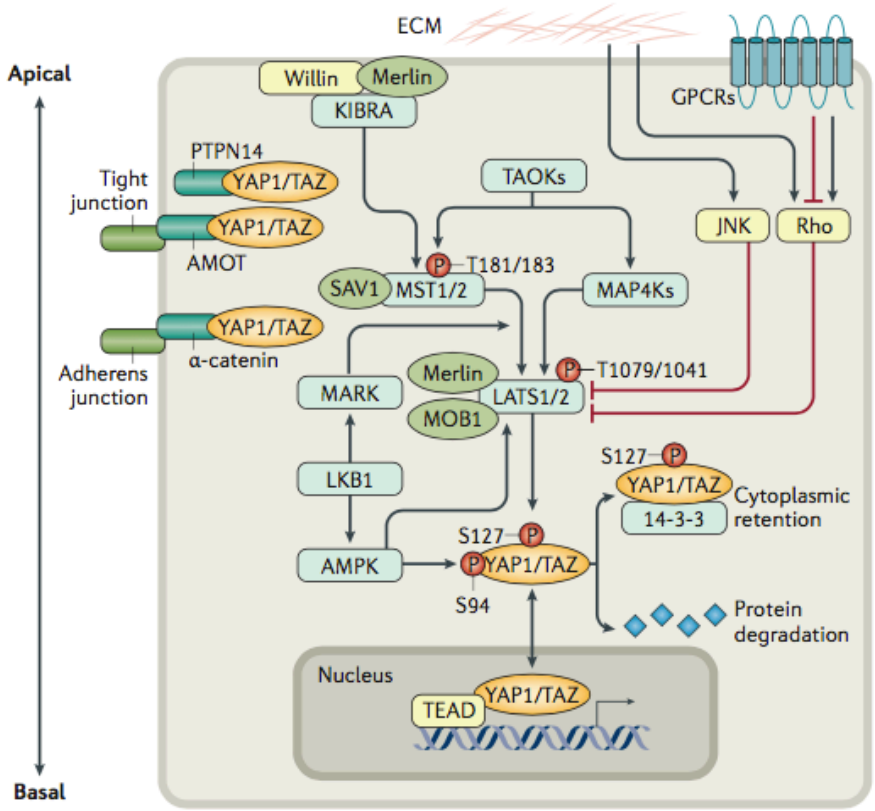


Figure 3: Schematic representation of the Hippo Pathway.

MST1 and MST2 are able to constitute homodimer through C-terminal Sav/RassF/Hpo (SARAH) domains and to activate an auto-phosphorylation feedback (Glantschnig et al., 2002; Lee and Yonehara, 2002; Hwang et al., 2007; Hong et al., 2016). LATS1 and LAT2 are activated through the phosphorylation by MST1 and MST2; furthermore, the activity of LATS1 and LATS2

is stimulated by SAV1 and MOB1 activation. (Chan et al., 2005; Callus et al., 2006; Ni et al., 2015; Hong et al., 2016). When LATS1 and LATS2 are activated they can phosphorylate in turn YAP1 and TAZ causing the retention of both in the cytoplasm through the 14-3-3 family proteins (Hong et al., 2016). When YAP1 and TAZ are in the cytoplasm they can withstand to phosphorylation and ubiquitylation-dependent degradation (Zhao et al., 2007, 2010b; Hong et al., 2016). The role of YAP1 and TAZ in the cytoplasm has been widely studied. Indeed, the sequestering in the cytoplasm prevents the interaction between them and the TEA domain family member 1 (TEAD1), TEAD2, TEAD3 and TEAD4. The interaction between YAP1 and TEAD activates the transcription of several genes such as MYC, BIRC5 and CTGF. These genes are involved in different biological functions like survival and cell growth (Hong et al., 2016). Studies found that degrading YAP1 through the phosphorylation by LATS1 and LATS2 causes the reduction of the expression of these genes (Dong et al., 2007; Zhao et al., 2007; Hong et al., 2016).

2.2.1 Hippo Pathway and Cancer

Hpo is regulated from several cellular properties, which can lead to carcinogenesis. (Harvey et al., 2013). Initially, studies in Fruit Flies of YAP counterparty (YKI) were performed and specifically, cells that showed an higher activity of YKI proliferated faster compared to wild-type cells (Tapon et al., 2002; Huang et al., 2005). Also in murine, the overexpression of YAP causes abnormal proliferation in several tissues such as gastrointestinal tract, heart, liver and skin (Camargo et al., 2007; Dong et al., 2007; Heallen et al., 2011; Schlegelmilch et al., 2011). Moreover, an important correlation between cell cycle and Hpo has been observed. Indeed, the senescence program, which regards the irreversible exit from cell cycle, is a well-known tumor suppressor feature. For this, studies about a short hairpin RNA of kinases uncovered a link between LATS2 and RB tumor suppressor. As a matter of fact, partial knock out of LATS2 can suppress RB-induced senescence markers. On the contrary, the overexpression of LATS2 increases the silencing of E2F target genes mediated by RB pathway (Tschöp et al., 2011; Harvey et al., 2013;). The activation of p53 by LATS2 is another important feature. p53 can increase the transcription of LATS2 that makes stronger G1/S checkpoint. The G1/S checkpoint is fundamental for the arresting of the cycle for those cells which show a tetraploid DNA content (Aylon et al., 2006). Therefore, all these researches showed that unstoppable activity of YAP and TAZ could perturb classical checkpoint of cell cycle. Cell survival is affected by Hpo in D. melanogaster. An overgrowth of tissues caused by the suppression of Hpo generates also insensitivity to apoptosis (Tapon et al., 2002). Pointedly in this species, Hpo controls apoptosis through regulation of inhibitor of apoptosis 1 (IAP1, or DIAP1) (Tapon et al., 2002). In mammals instead, evidences are still poor but the overexpression of YAP promotes cell survival ***in vitro*** and ***in vivo*** studies. In mouse liver for example, the tumor necrosis factor-induced and CD95 are blocked by an overexpression of YAP (Dong et al., 2007).

Deregulation and disruption of Hippo Pathway have been observed in several human carcinomas, such as colorectal, liver, lung, ovarian and prostate cancers (Dong et al., 2007; Zhao et al., 2007;

Steinhardt et al., 2008). Several studies have shown that the deregulation of this pathway is correlated with poor patients prognosis (Xu et al., 2009; Hall et al., 2010; Zhang et al., 2011), and moreover that various tumor suppressor genes, such as RB1, BRCA1, BRCA2 TP53, and PTEN are mutated. In addition, oncogenes like MET and KIT are involved in carcinogenesis (Harvey et al., 2013). For what concerns somatic mutations, a human cancer syndrome can arise from one gene associated with the mutation of the Hpo elements. An autosomal dominant syndrome called Type 2 Neurofibromatosis is caused by inherited mutation in neurofibromin 2 (NF2 also known as Merlin). Type 2 Neurofibromatosis is a syndrome which involves acoustic neuromas and also schwannomas of the dorsal roots of the spinal cord (Evans, 2009; Harvey et al., 2013). Moreover, NF2 has been discovered to be mutated in mesothelioma, meningioma and peripheral nerve sheath (Harvey et al., 2013). Genes involved in Hpo are rarely mutated, nevertheless an epigenetic silencing of MST1, MST2 and LATS1 and LATS2 has been demonstrated in several kinds of cancer. Interestingly, all these genes showed a tumor suppressor role in animal models (St John et al., 1999; Zhou et al., 2009; Lu et al., 2010; Song et al., 2010; Harvey et al., 2013).

Chromosomal translocations are still poorly studied in Hpo. Recently a translocation that involves TAZ and calmodulin-binding transcription activator 1 (CAMTA1) has been reported in a very rare sarcoma called Epithelioid Haemangioendothelioma (Errani et al., 2011; Tanas et al., 2011). Studies showed that TAZ is strongly expressed in endothelial cells and the fusion gene called TAZ-CAMTA1 is slightly expressed in this sarcoma. In addition, the expression of the fusion gene is under the control of TAZ promoter. Going more deeply, the fusion gene is the result of the translocation between the first two to three exons of TAZ that have the TEAD transcription factor binding domain, including the 14-3-3 binding site and the WW domain, with the last 15 exons of CAMTA1. The oncogenic mechanism is still unclear but it could be related to the transcriptional activity of both TAZ and CAMTA1 and furthermore the expression of this one in an ectopic tissue. Indeed, CAMTA1 is usually expressed only in the brain (Errani et al., 2011; Tanas et al., 2011; Harvey et al., 2013;).

All these recent discoveries uncovered how Hpo is deeply involved in the carcinogenesis of different tumors. Therefore, new studies will clarify the mechanisms regarding the Hippo Pathway and the relation with other pathways involved in carcinogenesis

2.2.2 Hippo Pathway as a therapeutic target in cancer

As said before, the mechanisms related to Hippo Pathway in carcinogenesis are still poorly understood. Nevertheless, several studies are fining-tune different therapeutic strategies in order to fight some kinds of cancer but also to understand how deeply Hpo is involved in carcinogenesis of specific diseases. Additionally, the synthesis of new drugs is always a very strong challenge in research.

Nowadays, kinases are considered one of the best targets for the development of new compounds in cancer research. Unfortunately, the majority of kinases involved in Hpo are tumor suppressor genes; consequently, Hpo represents a challenging target for the design of new drugs. (Harvey et al., 2013). Recently, Harvey and colleagues identified a novel role of a kinase called homeodomain-interacting protein kinase 2 (HIPK2) that although it exhibits a tumor suppressor function by interacting with p53 following DNA damage, is able to activate YAP (Puca et al., 2010; Harvey et al., 2013). Additionally, in ***Drosophila Melanogaster***, HIPK, the HIPK2 homologue, promotes tissue growth, confirming the dual role, although still unclear, of this kinase. (Chen and Verheyen, 2012; Poon et al., 2012). Further studies will explain if HIPK2 is involved in carcinogenesis by increasing YAP activity and enhancing its oncogenic activity. For all these reasons, HIPK2 represents a potential drug target for the development of new anticancer therapies.

2.3 Yes-Associated Protein 1

2.3.1 Yes Associated Protein 1 in Hippo Pathway

The Yes-Associated Protein 1 (YAP) was observed for the first time in the middle of the 90s from Marius Sudol (Sudol, 1994) and nowadays it has become a fascinating target in cancer research. When we talk about YAP, we always refer to it as related to another protein called TAZ and together they are the most important representatives of Hippo Pathway. Both of them are known to be the downstream effectors of Hpo where a kinases cascade finishes with the up-phosphorylation and inhibition of YAP/TAZ (Piccolo et al., 2014). Moreover, in the last decades YAP/TAZ are emerging for being very important in several human malignancies.

As previously reported, the kinases sequence belonging to Hpo is strongly correlated with the action of both YAP and TAZ. Specifically, the activated complex LATS1/2-MOB1A/B complex phosphorylates in turn YAP and TAZ (Dong et al., 2007; Zhao et al., 2007; Lei et al., 2008; Piccolo et al., 2014). The phosphorylation of YAP/TAZ causes sequestration in the cytoplasm and proteosomal degradation (Zhao et al., 2007; Lei et al., 2008; Liu et al., 2010; Zhao et al., 2010b). Studies have individuated in which sites YAP and TAZ are phosphorylated. Specifically, LATS1/2 phosphorylates YAP at five serine/threonine residues. TAZ instead, has four of these sites (Zhao et al., 2010a; Piccolo et al., 2014). Once these residues have been discovered, researchers focused their attention on the relevance of them. For instance, both YAP and TAZ are inhibited at serine S127 (that corresponds to S89 in TAZ) and in S381 (S311 in TAZ) (Zhao et al., 2010a). Consequently, mutations in these sites can cause dysfunctional in Hpo and insensitiveness of YAP/TAZ to the pathway itself. The phosphorylation occurred by LATS1/2 represents a fundamental step to maintain YAP/TAZ in the cytoplasm. Specifically, phosphorylation in the S127 creates a binding consensus for 14-3-3 proteins (Basu et al., 2003; Dong et al., 2007; Zhao et al., 2007) that could keep both proteins in the cytoplasm. Moreover, the interaction of YAP/TAZ with other proteins contributes to the activity in the cytoplasm or in the nucleus. A great example is the COOH-terminal PDZ binding domain (ZO2) that is extremely important for the regulation of YAP. Specifically, the deletion of this terminal domain causes the relocation of YAP in the cytoplasm inhibiting its activity. The interaction of ZO2 and YAP happens thanks to a PDZ-dependent manner localizing both in the nucleus (Oka et al., 2010; Remue et al., 2010; Piccolo et al., 2014).

2.3.2 YAP/TAZ and their role in the nucleus

As previously said, YAP/TAZ can be found both in the cytoplasm and in the nucleus. Depending on their localization, they are able to activate different features.

Indicatively, regarding the tumor setting, using immunohistochemical detection it has been possible to visualize the position of YAP in several cancer tissues. Indeed, YAP is present mostly in the nucleus of tumor tissues cells. This localization, can be explained by the fact that when YAP is in the nucleus it is able to induce growth tissues regulating several transcriptional program (Steinhardt et al., 2008). Statistically, YAP has a nuclear localization in 15% of ovarian cancers (Zhang et al., 2011), 60% of hepatocellular carcinoma (Xu et al., 2009) and 65% of non-small-cell lung cancers (Wang et al., 2010). For this reason, in normal tissues, it is hypothesized that Hpo regulates TAZ and YAP activity blocking their entry in the nucleus.

The transcriptional coactivation of YAP/TAZ in the nucleus has been hypothesized in mammals when it was demonstrated their binding with TEAD transcription factors (Piccolo et al., 2014). Mass-spectrometry analysis showed that TEADs interact with YAP and TAZ (Vassilev et al., 2001; Zhang et al., 2009; Chan et al., 2009). Several evidences showed the mediation of different functions of YAP and TAZ from TEAD factor. For instance, the loss of TEADs causes changes in the phenotype of mammalian cells, which are similar to those caused by the absence of YAP/TAZ. Moreover, there is a modification in the activation of the Hpo cascade (Nishioka et al., 2008; Ota and Sasaki, 2008; Sawada et al., 2008; Nishioka et al., 2009; Piccolo et al., 2014). Furthermore, Co-IP experiments demonstrated that YAP and TEAD bound a different genes that showed an overlap (Zhao et al., 2009). Several research groups have been studying the interaction of YAP/TAZ with important targets genes such as CYR61, ANKRD1, BIRC5, Col8a1 and CTGF (Cordenonsi et al., 2011; Calvo et al., 2013). Furthermore, the interaction of YAP/TAZ and TEAD factor can involve others proteins. For example, the interaction between YAP/TAZ and p73 is really important. p73 is known for having pro-apoptotic function during DNA damage context, specifically, c-Abl directly phosphorylates YAP which associates with p73 (Strano et al., 2005; Levy et al., 2008; Piccolo et al., 2014). Moreover, YAP maintains the stability of p73 and co-activates p73 pro-apoptotic genes. This pathway interaction is involved as a tumor suppressor pathway in hematological cancer. The expression of YAP is surely reduced in these tumors inhibiting the p73 activation by c-Abl (Levy et al., 2007; Cottini et al., 2014; Piccolo et al., 2014). YAP/TAZ can bind others transcription factors such as Tbx5 in colorectal cancer cells, TTF1/Nkx-2.1 in epithelial cells of lung alveolar type II and also PAX8 in thyroid development (Park et al., 2004; Di Palma et al., 2009; Mitani et al., 2009; Rosenbluh et al., 2012; Piccolo et al., 2014). Furthermore, recent studies revealed a role of YAP/TAZ on chromatin, specifically, it has been observed that the remodeling chromatin complex Brg1 mediates the activation by TAZ of vimentin and C44 transcription in MCF10A cells (Skibinski et al., 2014).

Nevertheless these information, it is not fully understood how YAP/TAZ can translocate from the cytoplasm to the nucleus and vice versa.

2.3.3 YAP/TAZ and their role in Rhabdomyosarcoma

Considering the aggressiveness of eRMS and aRMS, several studies are trying to find new treatments in order to fight these tumors. Specifically, recently, researchers are focusing on the role of YAP and TAZ in RMS. YAP and TAZ can be regulated by others pathway such as KRAS and β-catenin (Kapoor et al., 2014; Shao et al., 2014; Mohamed et al., 2016). These pathways are also involved in eRMS carcinogenesis (Shern et al., 2014).

The relevance of YAP in tumorigenesis has opened several doors in recent researches also in RMS. Thanks to its activity and capacity in modulating organs size and growth (Piccolo et al., 2014) it is easy to think that its role in RMS could be really heavy considering all the features quoted before (Marchesi et al., 2017). For example, the hyperactivity of YAP in activated muscle satellite stem cells causes eRMS in mice with an high penetrance (Tremblay et al., 2014). This great work from Tremblay and colleagues also showed the cellular localization of YAP in both aRMS and eRMS. Specifically, higher protein levels of YAP were detected more in eRMS cases compared to aRMS ones. Regarding the cellular localization in aRMS, YAP is more expressed in the cytoplasm compared to the nucleus. In eRMS instead, YAP can be found more in the nucleus (Tremblay et al., 2014). The authors concluded that the expression and activity of YAP are stronger in eRMS compared to aRMS; moreover, the activity of YAP can be associated to a poor prognosis in human eRMS cases at the contrary of aRMS (Tremblay et al., 2014).

Other researchers focused their attention specifically on aRMS. It has a worst outcome compared to eRMS since the survival rate for the patients who show the paired box 3-forkhead box protein O1 (PAX3-FOXO1) fusion gene, is less than 10% (Crose et al., 2014). Crose and colleagues showed that PAX3-FOXO1 helps the initiation of aRMS bypassing the cellular senescence checkpoint (Linardic et al., 2007). This event happens because a member of Ras-association domain family member (RASSF4) is upregulated by PAX3-FOXO1. Moreover, RASSF4 is upregulated in PAX3-FOXO1 positive aRMS cells and an increased expression of RASSF4 leads to evasion of senescence, cell cycle progression and carcinogenesis through the suppression of MST1 of the Hpo. Consequently, a dysregulation of Hpo causes an upregulation of YAP in RMS cells. All these evidences underline how the Hpo pathway is involved in RMS carcinogenesis (Crose et al., 2014).

Apart from YAP, a big role in the carcinogenesis of RMS is played by TAZ. This protein instead is involved in the promotion of myogenesis process while YAP inhibits it. Recent studies showed that a higher expression of TAZ is associated with a poor survival rate in eRMS patients. In addition, the murine model of C2C12 showed an increased proliferation when TAZ S89A resulted active (Mohamed et al., 2016) remarking again the important role of phosphorylation residues of both YAP/TAZ. Considering the aggressiveness of RMS and the emerging role of YAP/TAZ in this tumor, it is important to study their roles in order to uncover how deep is their involvement in this cancer.

2.4 Verteporfin

The development of new drugs able to fight carcinogenesis is one of the most important leading factors in cancer research. Verteporfin, called Visudyne by Novartis, (VP) is a new compelling drug, which is giving positive results in several kinds of tumors. VP belongs to the family of porphyrins that are aromatic heterocyclic molecules. They are made up of four modified pyrrole units that are linked between them thanks to a methane bridges in the α carbon atoms (Liu-Chittenden et al., 2012a). VP is already used in clinic as a photosensitizer in the photodynamic therapy for a pathology

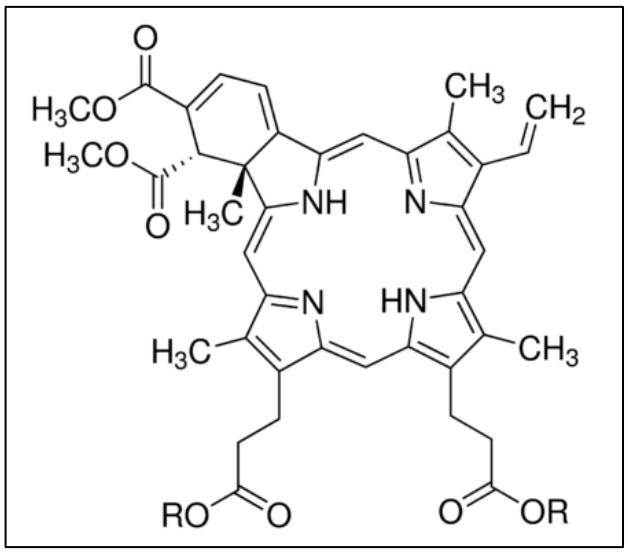


Figure 4: Structure of Verteporfin.

called neo-vascular macular degeneration. The drug's mechanism of action is really peculiar. Indeed, the photochemical light activation of VP generates reactive oxygen radicals which are able to completely eliminate abnormal blood vessels (Michels and Schmidt-Erfurth, 2001; Liu-Chittenden et al., 2012a). Nevertheless this feature, VP is having a great success thanks to its important action on Hpo.

2.4.1 Verteporfin and cancer

Nowadays, VP is considered one of the stronger drugs able to inhibit the activity of YAP. Based on this ability, both ***in vitro*** and ***in vivo*** models have been used to study VP in different types of cancers, in order to uncover its potential role in cancer therapy (Zhang et al., 2015). As previously reported, the interaction between YAP and TEAD factor in the nucleus is extremely important for

the activation of transcription of a large number of genes. Several studies have demonstrated that the perturbation of Hpo can cause tumorigenesis through different ways (Piccolo et al., 2014).

In the beginning, VP was used in photochemical light activation (Schmidt-Erfurth and Hasan., 2000) and nowadays others researches are helping to understand its mechanism of action (Mimouni et al., 2016). At a later time, studies uncovered its role in the disruption of YAP-TEAD interaction (Liu-Chittenden et al., 2012) and the drug was tested in several types of cancer. VP gave great results in breast, prostatic and pancreatic cancers (Donohue et al., 2013; Pellosi et al 2017), revealing also growth inhibition in colorectal cancer cells independently of its effects on YAP1 (Zhang et al., 2015).

It is recent instead, its potential anti-proliferative activity in Leukemia. Indeed, ***Chen and colleagues*** showed that VP is able to inhibit the proliferation of NB4 cells at different concentrations and time-dependent manner. Furthermore, VP inhibits the protein expression of YAP, c-myc, p-AKT and others and induces the protein expression of cleaved PARP (Chen et al., 2017) involved in apoptosis process.

In relation to the main role of VP on YAP-TEAD complex, the treatment with this drug blocks their interaction. Moreover, VP can also cause conformational changes in the binding sites of YAP and TEAD but the mechanism of action is not completely understood (Liu-Chittenden et al., 2012b). Several authors hypothesize that VP causes conformational changes in the structure of YAP without affecting the structure of TEAD, instead. These changes block the formation of the complex YAP-TEAD inhibiting therefore the oncogenic activity of YAP. Moreover, considering the similar structure of YAP and TAZ it has been hypothesized but not widely demonstrated that VP is able to decrease protein levels of TAZ (Zhang et al., 2015).

3. Aim of the project

Rhabdomyosarcoma is an aggressive pediatric tumor which arises from muscle precursor cells (Marchesi et al., 2012). It can manifest through two different subtypes Alveolar and Embryonal Rhabdomyosarcoma. Both of them show peculiar characteristics in phenotype and genotype, rendering them two distinctive types of cancers (Saab et al., 2011).

Recent studies showed the important role of Hippo Pathway in the carcinogenesis of RMS. Specifically YAP and TAZ, the main genes involved in the Hpo, have shown to be involved in the carcinogenesis of RMS.

YAP and TAZ have a dual role both in the cytoplasm and in the nucleus. It is not clear how these two proteins can shuttle from the cytoplasm to the nucleus. In the nucleus, YAP is able to bind TEAD factor and create a complex called YAP/TEAD. This complex can activate the transcription of several genes such as MYC, p73 and others (Piccolo et al., 2014).

Considering the aggressiveness of RMS and the involvement of YAP and TAZ in the carcinogenesis process, the aim of this thesis is to understand the effect of a compound called Verteporfin (VP), which is able to disrupt the formation of YAP/TEAD complex. Moreover, this drug showed to have an antiproliferative action in cancer research (Liu-Chittenden et al., 2012b) and for this reason, we decided to investigate the role of VP on RMS cell lines in order to understand its mechanism of action and to find a new treatment for RMS.

4. Materials and Methods

4.1 Cell culture

RD (Embryonal Rhabdomyosarcoma) (ATCC) and A204 (Embryonal Rhabdomyosarcoma) (CLS) were cultured with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco), supplemented with 10% of Fetal Bovine Serum (FBS) (Gibco), 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco) and 1% of L-glutamine.

RH30 (Alveolar Rhabdomyosarcoma) (ATCC) and RH4 (Alveolar Rhabdomyosarcoma) (ATCC) instead, were cultured in RPMI 1640 medium (Gibco), supplemented with 10% of FBS, 100 units/mL penicillin, 100 µg/mL streptomycin (Gibco) and 1% of L-glutamine.

All four cell lines of RMS were incubated at 37°C with 5% of CO_2 in a humidified incubator.

4.2 Proliferation Assay (XTT)

In order to study the anti-proliferative activity of Verteporfin (Sigma Aldrich), XTT proliferation assay (Cell proliferation Kit II, Roche) was performed.

Every cell line was seeded in a 96-well plate at a density between 1500 and 3000 cells/well depending on the different size, phenotype and population doubling of the cells. Cells were treated with different concentrations of Verteporfin in a range between 0,1 and 10 µM in a final volume of 100 µl and were incubated for 72 hours. Dimethyl sulfoxide (DMSO) is the solvent in which Verteporfin is resuspended and it has been used as a control.

After 24, 48 and 72 hours XTT assay was performed. 0,5 µl of electron coupling reagent and 24,5 µl were resuspended in 74,5 µl of medium (final volume of 100 µl/well) and incubated for 4 hours at 37°C.

At a later time, the absorbance was measured using a microplate reader at a wavelength of 490 nm. The experiment was performed in sestuplicate for every concentration and it was repeated for more than three times. The Verteporfin concentration that inhibits 50% of cells growth (IC50) was determined from a dose-response curve.

4.3 Flow Cytometry Analysis

Flow Cytometry analysis has helped to determine the distribution of cell cycle in treated cells with VP, using Propidium Iodide (PI, Sigma Aldrich).

Cells were seeded on 10 cm dishes and after 24 hours were treated with different concentration depending on IC50 of Verteporfin in each cell line. In Table 3 the different concentrations used depending on the IC50 at 72 hours of every cell line are shown.

Table 3

Cell Line	IC50 at 72h	First concentration used	Second concentration used
A204	0,2 µM	0,05 µM	0,1 µM
RD	0,2 µM	0,05 µM	0,1 µM
RH4	0,5 µM	0,1 µM	0,2 µM
RH30	0,5 µM	0,1 µM	0,2 µM

Table 3: Different concentrations of VP used for the experiments performed after the calculation of the IC50.

After 24, 48 and 72 hours post-treatment, supernatants and adherent cells were collected. Thus, cells were centrifuged at 500 g for 5 minutes. Successively, pellets were washed and resuspended in PBS and centrifuged at the same conditions as before. After that, pellets were resuspended in 200 µl of PBS and every sample was fixed with 5 ml of 70% cold ethanol in agitation on a vortex. Later, samples were put at -20°C for a time between 1 and 15 days. At a later time, once all the samples were collected from time courses, cells were centrifuged and the obtained pellets were washed in PBS and resuspended in 200 µl of PBS with RNAsi (20µg/ml) and PI (5µg/ml) and incubated at 4°C in the dark.

4.4 Protein extraction

Cells were lysed using lysis buffer made up of:

- 20 mM Tris HCl pH 8.0;
- 137 mM NaCl;
- 10% glycerol;
- 1% Nonidet P-40;
- 2 mM EDTA

with Protease Inhibitors Cocktail (25x).

The concentration of protein was obtained using Bradford assay (Sigma Aldrich), following the manufacture's protocol. BSA was used as a standard.

4.5 Immunoblotting

15 µg of proteins were resolved in 8% SDS/PAA gel and successively transferred into a nitrocellulose membrane (GE, Healthcare, Whatman) at a temperature of 4°C at 100V for 1 hour.

In order to understand if the proteins were successfully transferred, the membrane was stained using a Ponceau solution made up of:

- ponceau 0,1%;

- trichloroacetic acid (TCA) 7%;

until the formation of a red stain indicating the presence of the bands. Consequently, the membrane was rinsed in a TBS-T for transient staining. At a later time, the membrane was blocked in a 5% Bovine Serum Albumin (BSA) in 0,5 % TBS-Tween for 1 hour at room temperature with gentle agitation.

In order to detect the levels of the proteins of interest, the membrane was exposed at first to antibodies resuspended in 5% of BSA in 0,5 % TBS-Tween. The incubation with the first antibodies was performed o/n at 4°C in cold room with gentle agitation. Protein levels were detected with the different antibodies listed below:

- Anti-PARP (Cell Signaling, rabbit) 1:1000;

- Anti-YAP/TAZ (Sigma Aldrich, mouse) 1:1000;

- Anti-GAPDH (Santa Cruz, rabbit) 1:1000.

After this incubation, membranes were washed 3 times for 5 minutes in 0,5% TBS-Tween at room temperature. At a later time, membranes were incubated at room temperature for 1 hour with secondary antibodies: anti-mouse and anti-rabbit (1:10000) resuspended in 5% BSA in 0,5% TBS-Tween. Thus, three additional washes were performed with 0,5% TBS-Tween, 5 minutes for three times.

For the detection of protein levels, the membranes were incubated for 1 minute in the darkness with Western Lightning Plus-ECL (PerkinElmer); X-ray film (Kodak) or VersaDoc machine were used for detection.

4.6 siRNA transfection

In a 6 multiwell were seeded:

2,5 x 10^5 RD;

2,5 x 10^5 A204;

2,0 x10^5 RH30;

2,0 x 10^5 RH4

24 hours before transfection.

After 24 hours, the media was changed and the transfection was performed. In the scramble (CTR) 10 µl of GlosiRNA was dissolved in 200 µl of DMEM without antibiotics. For the siRNA of YAP (Sigma Aldrich) (siYAP) 5 µl of siYAP (30 pmol) and 5 µl of GlosiRNA were resuspended in 200 µl of DMEM without antibiotics. For both scramble and siYAP a mix with 200 µl of DMEM and 10 µl of Lipofectamine RNAiMAX Reagent (Thermo Fisher) was prepared. At a later time, all diluted siYAP and scramble were added to the RNAiMAX mix in a 1:1 ratio and incubated for 5 minutes at room temperature in the dark. After that the mixes were spread drop to drop in all the multiwall, and cells were incubated for 48 hours at 37°C in incubator.

After 48 hours, cell reached confluence. They were detached using trypsin, counted and then centrifuged for obtaining the pellet for western blotting analysis.

5.Results

5.1 YAP and TAZ are expressed in Embryonal and Alveolar Rhabdomyosarcoma cell lines

In order to understand the protein levels of YAP and TAZ in RMS cell lines, western blotting has been performed. The levels of both YAP and TAZ have been quantified through densitometric analysis using GAPDH as a control.
As shown in Figure 5, regarding eRMS results, A204 and RD both showed high protein levels of YAP. Specifically, RD cells had a higher level of YAP compared to the other eRMS cell line, A204. Instead, aRMS cell lines showed both similar levels of YAP protein.
Protein levels of TAZ showed a difference between both tumor subtypes. Indeed, aRMS cells RH4 and RH30, showed lower level of TAZ protein compared to the eRMS cell lines. RH4 showed a slightly higher TAZ protein levels compared to RH30.

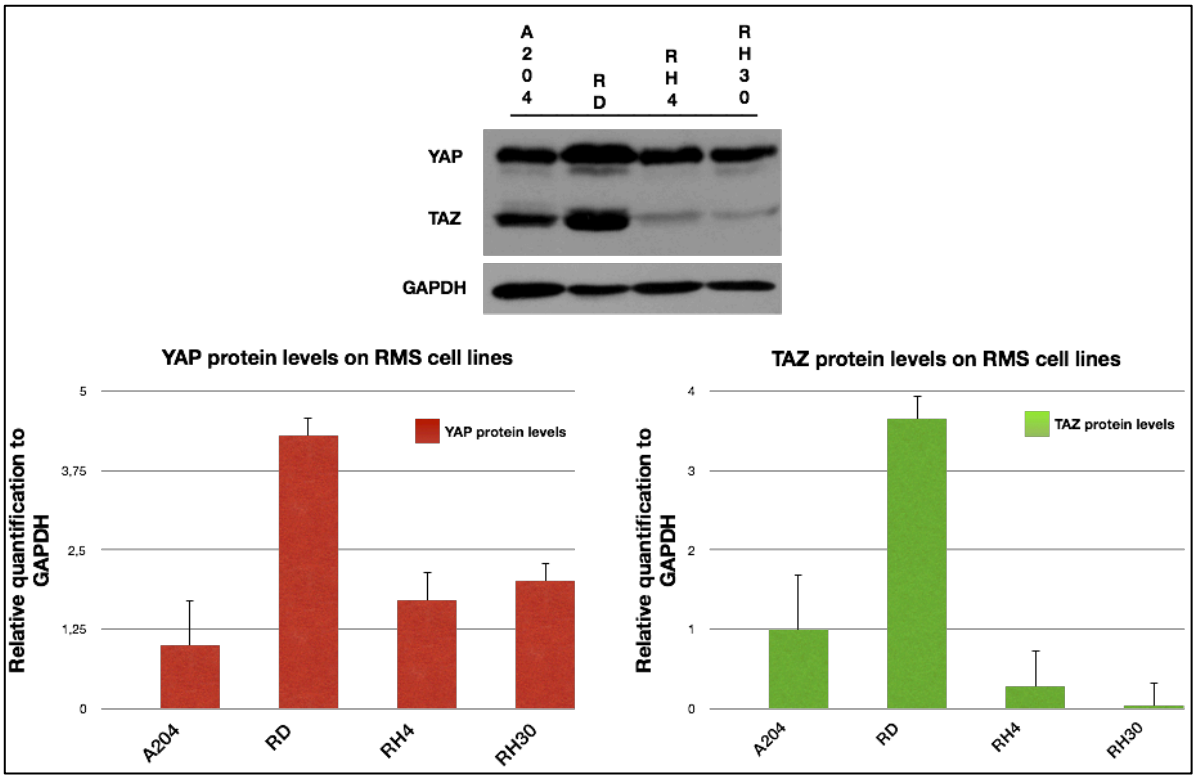


Figure 5: Protein levels of YAP and TAZ in RMS cell lines.

5.2 Verteporfin has an anti-proliferative effect and induces phenotyping changes in RMS cell lines

Considering the high levels of YAP protein in RMS cell lines and all the recent studies which are uncovering its important role in carcinogenesis, we decided to test the activity of a compound called Verteporfin (VP) on four difference cell lines of RMS. Studies on VP showed that it has a specific action on YAP-TEAD interaction, blocking the formation of this complex (Liu-Chittenden et al., 2012).

So as to understand if the VP has an anti-proliferative effect on RMS, XTT assay was performed. On 96-multiwell plates, one for each cell line, 1500 and 3000 cells were seeded. After 24 hours, cells were incubated for a total of 72 hours with 0,1/0,2/0,5/1 and 2 µM of VP or cultured medium (untreated cells) or DMSO (the solvent in which VP is resuspended). XTT assay was performed at 24, 48 and 72 hours in sestuplicate and at least 3 times for each cell line.

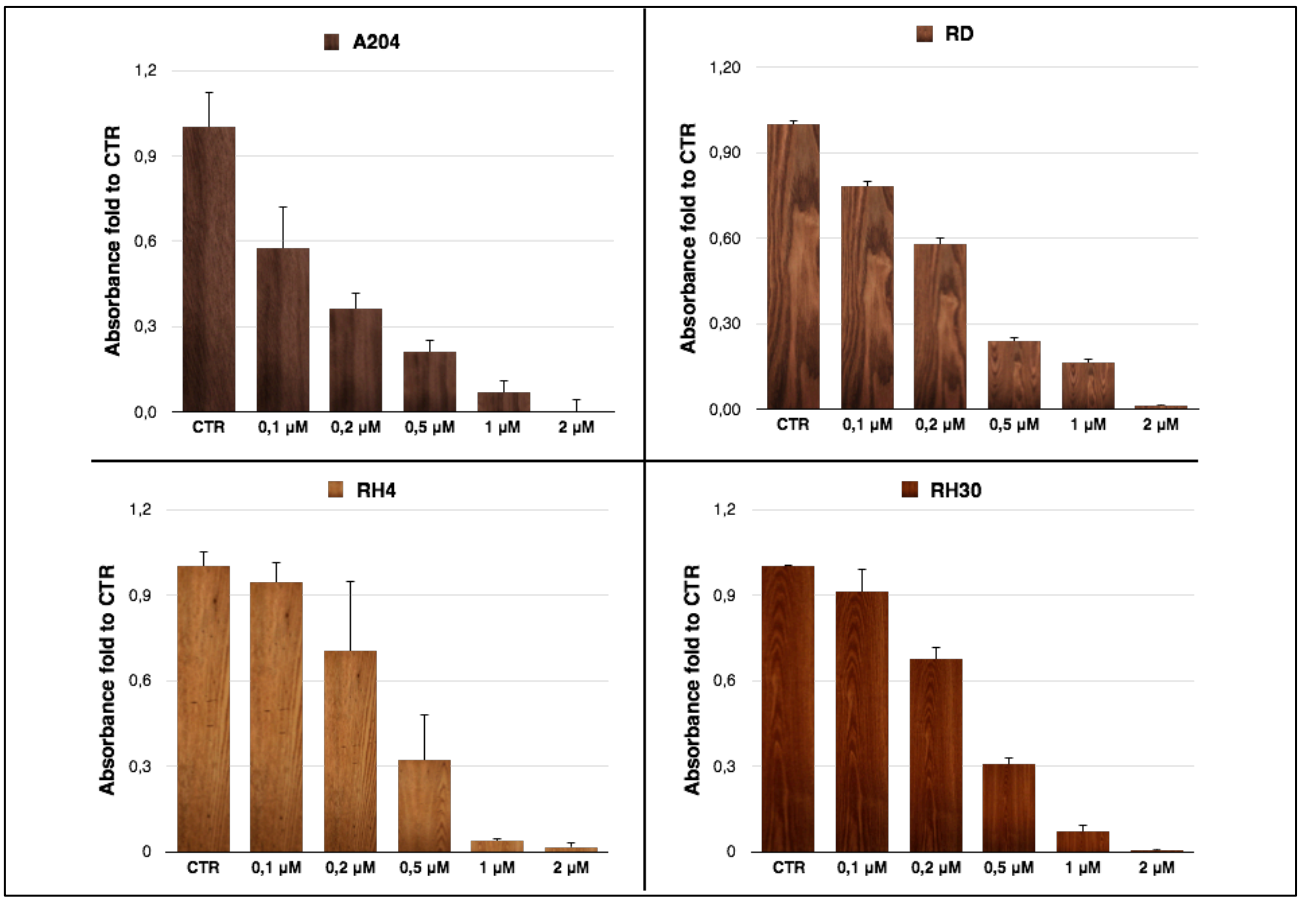


Figure 6: In the graphs are represented the results obtained by XTT assay at 72 hours after treatment with VP. Depending on the RMS subtype, VP has a different IC50. eRMS cell lines resulted more sensible to the treatment compared to aRMS.

Results at 72 hours after treatment showed a different IC50 depending on the RMS cell line. Specifically, RD and A204 have an IC50 around 0,1 and 0,2 μM after 72 hours. Instead, RH4 and RH30 showed an IC50 around 0,2 and 0,5 μM after 72 hours of treatment. Considering the results obtained with XTT, two additional concentrations were chosen for every cell line and for the next experiments.
In order to understand the effect of VP on RMS cell lines, RD and A204; RH4 and RH30 were seeded onto 10 cm plates at different density. Specifically:

- RD at 6 $x10^5$;
- A204 at 7,5$x10^5$;
- RH4 at 7$x10^5$;
- RH30 at 7$x10^5$.

After 24 hours, VP treatments were performed with concentrations of 0,05/0,1 and 0,2 μM for RD and A204; while 0,1, 0,2 and 0,5 μM concentrations were used in RH4 and RH30 cells.

5.2.1 Effects of VP on A204 phenotype

One of the main characteristics of a compound used in cancer treatment ***in vitro*** experiments is to cause a change in the phenotype of cells. For this reason, one of the first steps of this research was to understand if VP is able to cause a change in the phenotype.
As shown in Figure 7, A204 cells appear more stretched compared to the control (DMSO). Moreover, cells are not organized in bundles as in CTR. They show a quicker duplication in comparison to the other RMS cell lines and some cells can be found in the supernatant.

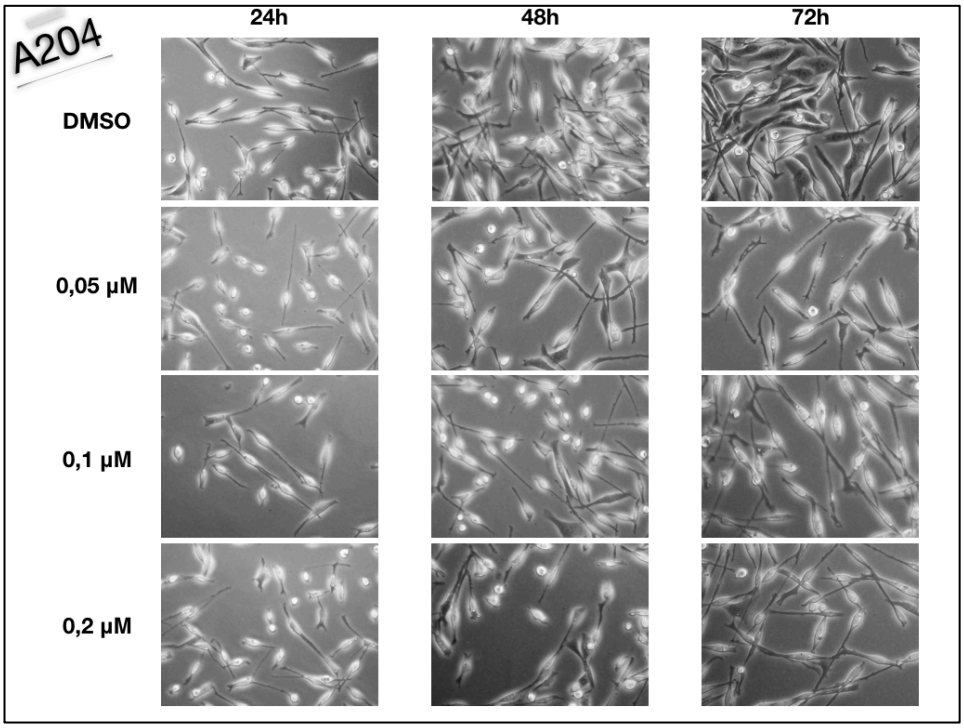


Figure 7: Phenotype of A204 cells after treatment with VP.

5.2.2 Effects of VP on RD phenotype

After the treatment with VP, also RD cells showed specific characteristics, which defer from the typical phenotype of these cells. As it is shown in Figure 8, in the DMSO at 72 hours, cells appear at a high confluence, which grows during the time course. In the treatment with VP instead, cells appear in a

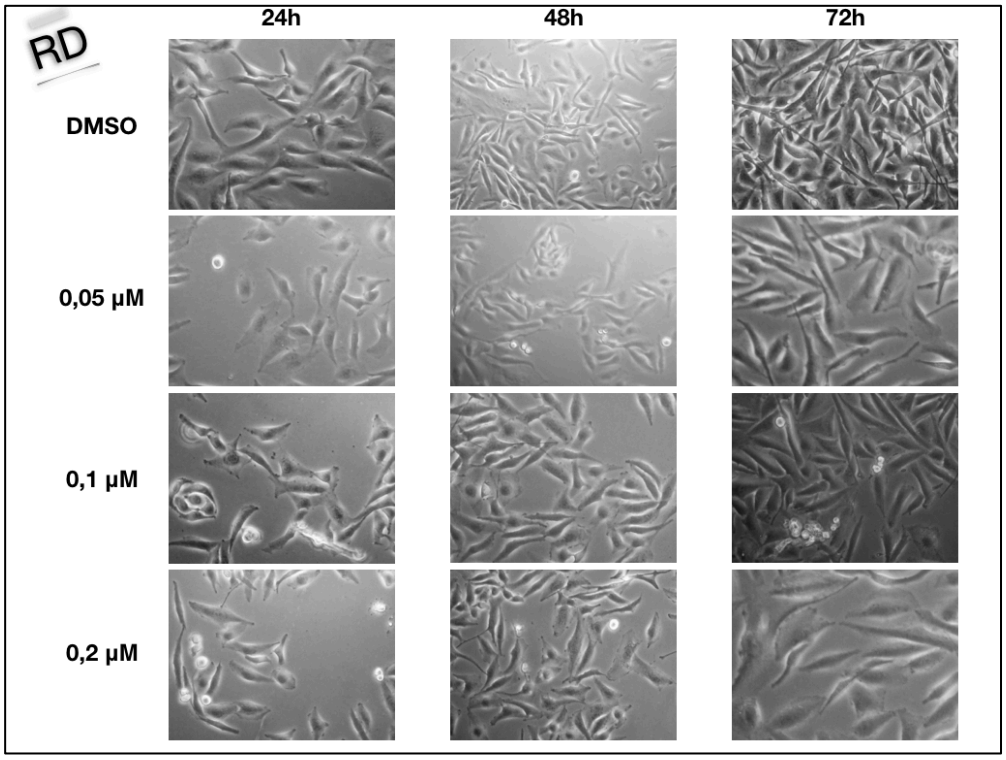


Figure 8: Phenotype of RD cells after treatment with VP.

clear suffering, losing the typical characteristics that they show in the CTR at 72 hours. Moreover, at 0,2 and 0,1 µM can be observed the presence of cells in the supernatant, which indicate cell death.

5.2.3 Effects of VP on RH4 phenotype

aRMS cells have a different phenotype compared to eRMS cells. They usually show a grape structure, which is less stretched, compared to the eRMS cells and are usually spread out in the entire surface in a disorganized way.

After the treatment with VP, RH4 showed peculiar features. In the CTR, cells are disorganized but showed stretching in order to communicate between them. This feature is not visible in the treatment with VP specifically after 72 hours at the highest concentration of 0,5 µM. Moreover, they change the phenotype becoming smaller because they are detaching from the plate. Indeed, the supernatant contains most of the small density cells.

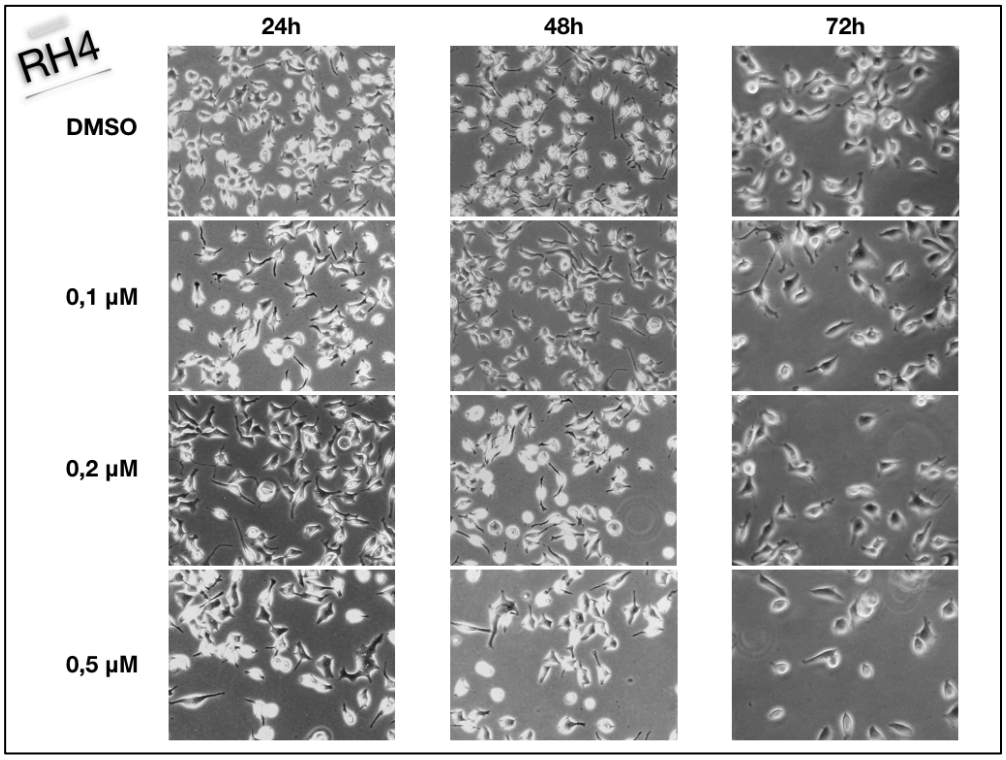


Figure 9: Phenotype of RH4 cells after treatment with VP.

5.2.4 Effects of VP on RH30 phenotype

The antiproliferative activity of VP on aRMS can be assessed also on RH30 cells. Indeed, compared to the CTR, cells change their phenotype showing to be smaller, less stretched and disorganized. Remarkably, several dead cells are present in the supernatant.

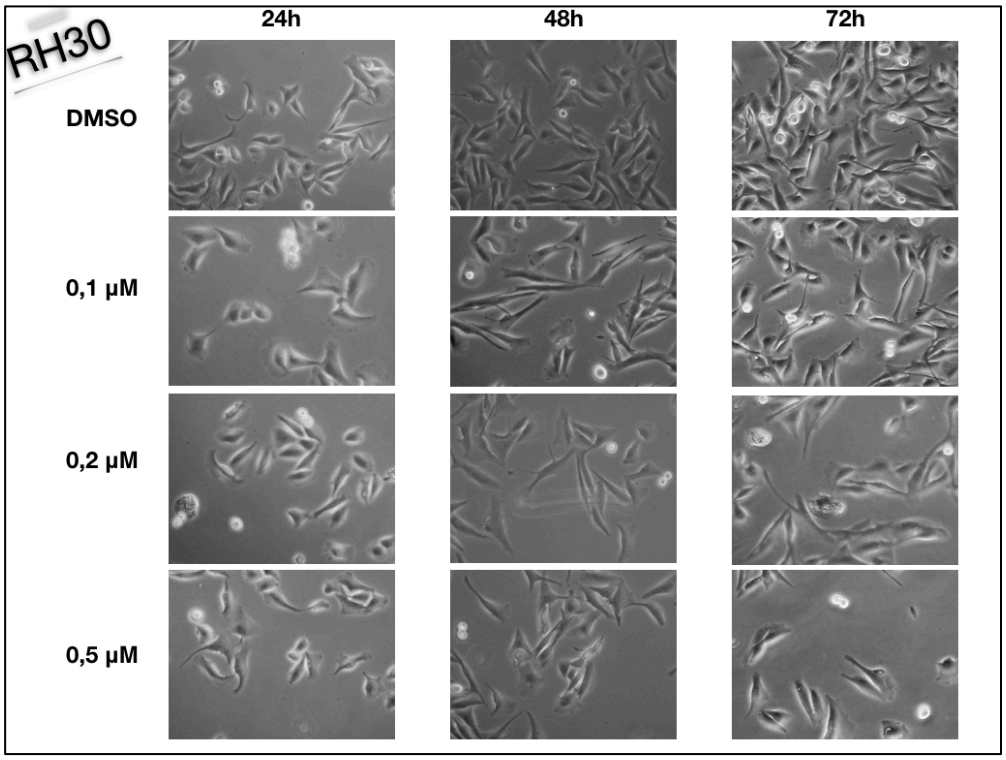


Figure 10: Phenotype of RH30 cells after treatment with VP.

5.3 Cell cycle analysis at flow cytometry showed different effects of Verteporfin on eRMS and aRMS cell lines

The anti-proliferative activity of VP is one of the main features, which are required to a compound for being used in cancer research. Another characteristic of a cancer drug is the ability to perturb cell cycle. In RMS, the activity of cell cycle is extremely important. Indeed, RMS cells fail to stop cell cycle and they are in an unrestrained proliferation status. In order to understand if VP has an effect on cell cycle, flow cytometry was performed. Cell cycle was analyzed after 24, 48 and 72 hours of treatment with VP.

5.3.1 A204 cell cycle analysis

A204 cells have been treated with IC50 at 72 hours (0,2 µM) and others two concentrations under the IC50. As the Figure 11 shows, A204 cells exhibit a great G2/M phase both in the control and in the treated samples. Nevertheless, VP treatment decreases the G2/M phase and increases S and G0/G1 phases. After 48 hours of treatment, there is a reduction of G2/M phase and an increase of G0/G1 phase. Moreover, at the highest concentration of 0,2 µM this result is more emphasized.
At 72 hours A204 showed a stronger decrease of G2/M phase compared to the control and cells in S phase increases in the treated compared to the control.
Moreover, the analysis of cell cycle did not show the presence of cells with less content of DNA. These results showed that VP has a cytostatic activity on A204 eRMS cell line and not a cytotoxic one.

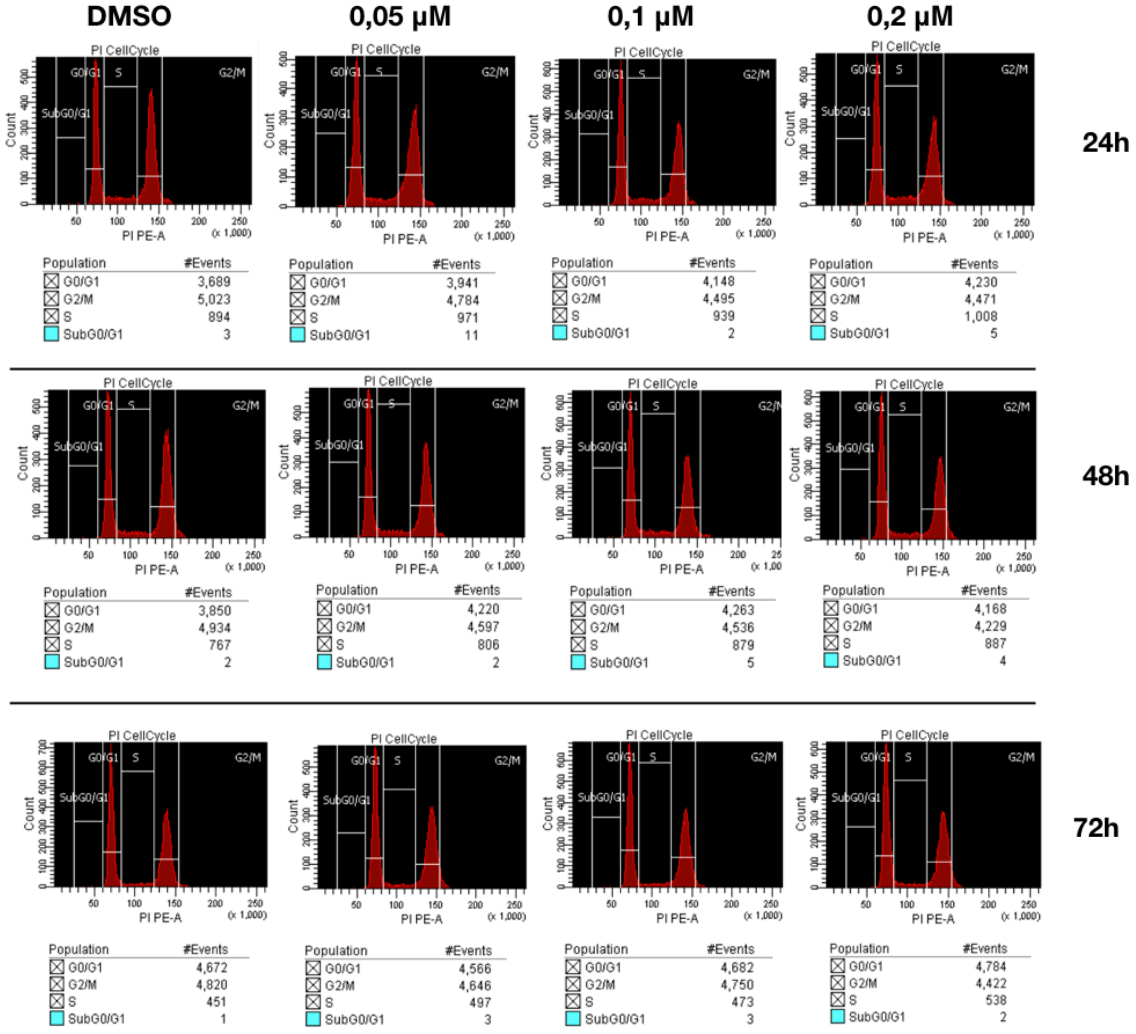


Figure 11: Cell cycle analysis of A204 cells at 24, 48 and 72h after treatment with different concentrations of VP.

5.3.2 RD cell cycle analysis

Flow cytometry of RD cells showed a different result compared to the other eRMS cell line. Specifically, the G2/M phase is different from A204 and RD showed to be in a less proliferation status compared to the other eRMS cell line. At 24 hours and at the highest concentration there is a reduction of G2/M phase. After 48 hours instead, the cell cycle showed an increase of G0/G1 phase going to the detriment of S and G2/M phases. 72 hours of treatment with VP showed a sensible increase of G0/G1 phase compared to the control at highest concentration of VP of 0,2 µM.

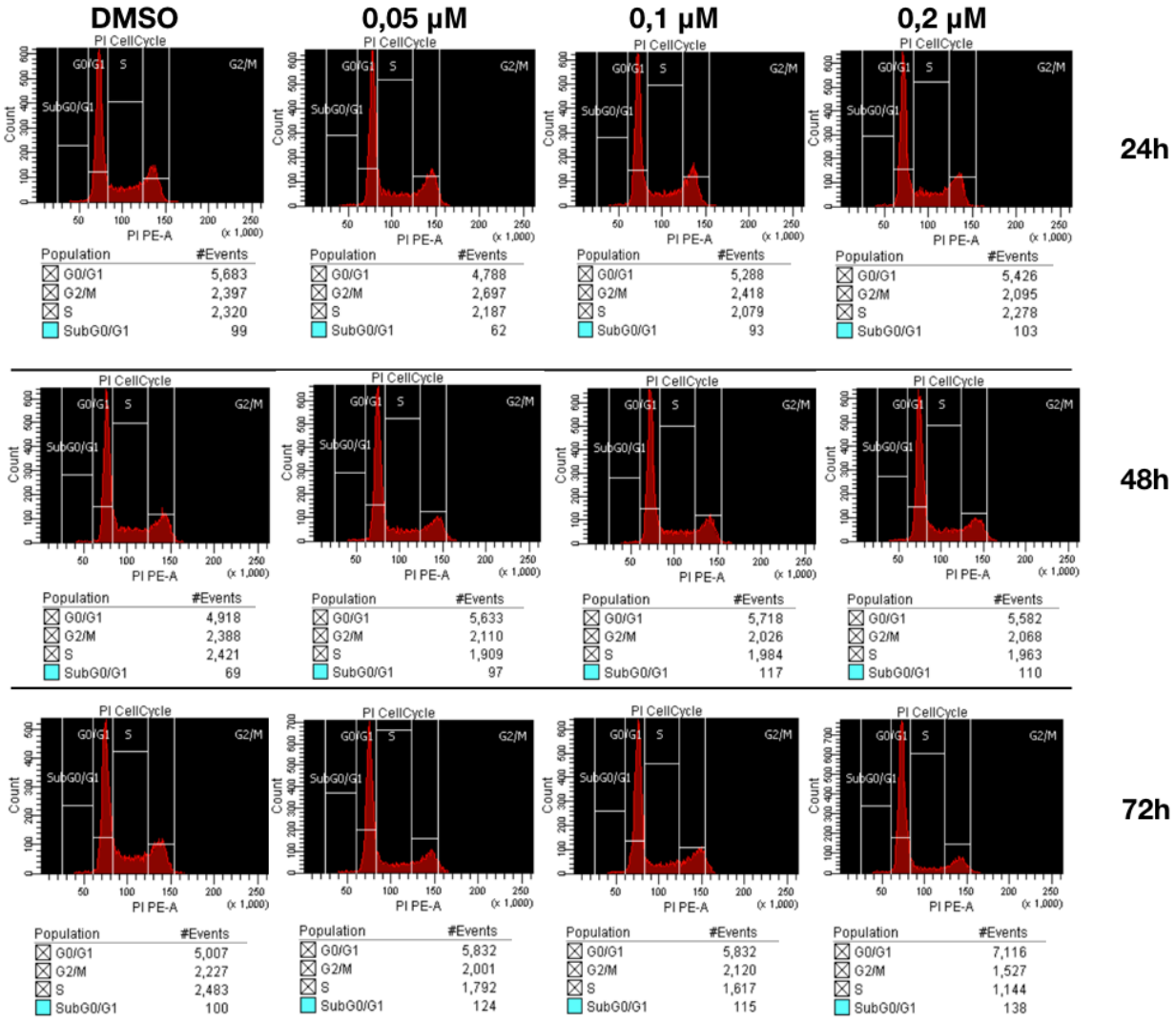


Figure 12: Analysis of cell cycle of RD cells through Flow Cytometry at 24, 48 and 72h after treatment with different concentrations of VP.

5.3.3 RH4 cell cycle analysis

aRMS showed different sensibility at the VP in the cell cycle analysis. Indeed, RH4 cells have a great S phase both in the CTR and in treatment. During the first 24 hours after treatment there is a decrease of the G2/M phase and an increase of cells in the G0/G1 phase in the highest treatment with VP.
In the others 48 hours instead, there is a decrease of G2/M and S phases with an increase of sub G0/G1 phase and particularly of the G0/G1 phase.
72 hours of treatment with VP causes a decrease of S phase and a slightly increase of sub G0/G1 phase.

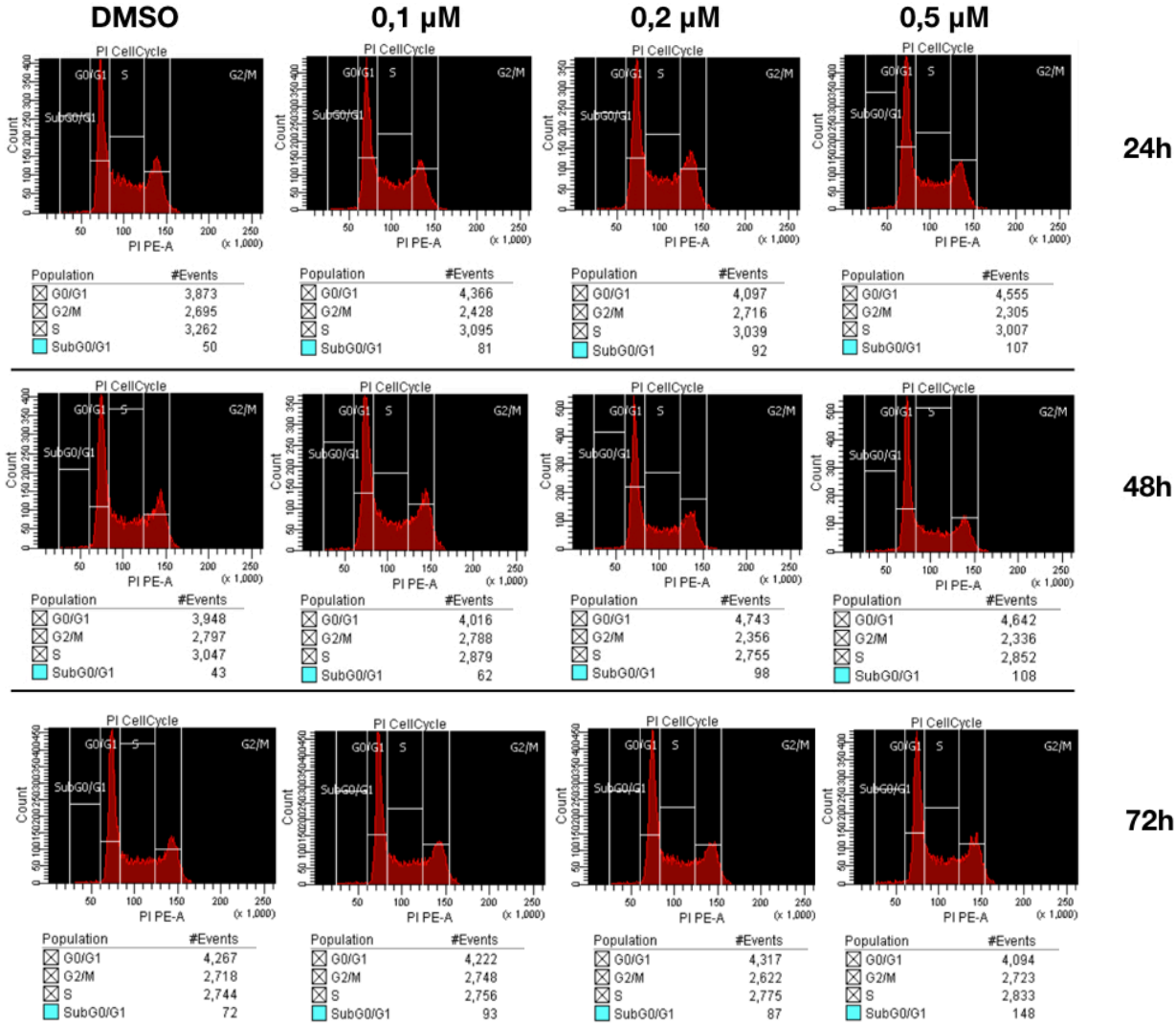


Figure 13: RH4 cell cycle analysis at 24, 48 and 72 hours.

5.3.4 RH30 cell cycle analysis

Cell cycle analysis of RH30 showed an important increase of G0/G1 phase at 24 and 48 hours at 0,1 µM concentration with an increase of sub G0/G1.
The increase of G0/G1 and of sub G0/G1 phases can be appreciated specifically comparing the CTR at 24 hours and the highest treatment (0,5 µM) at 72 hours.

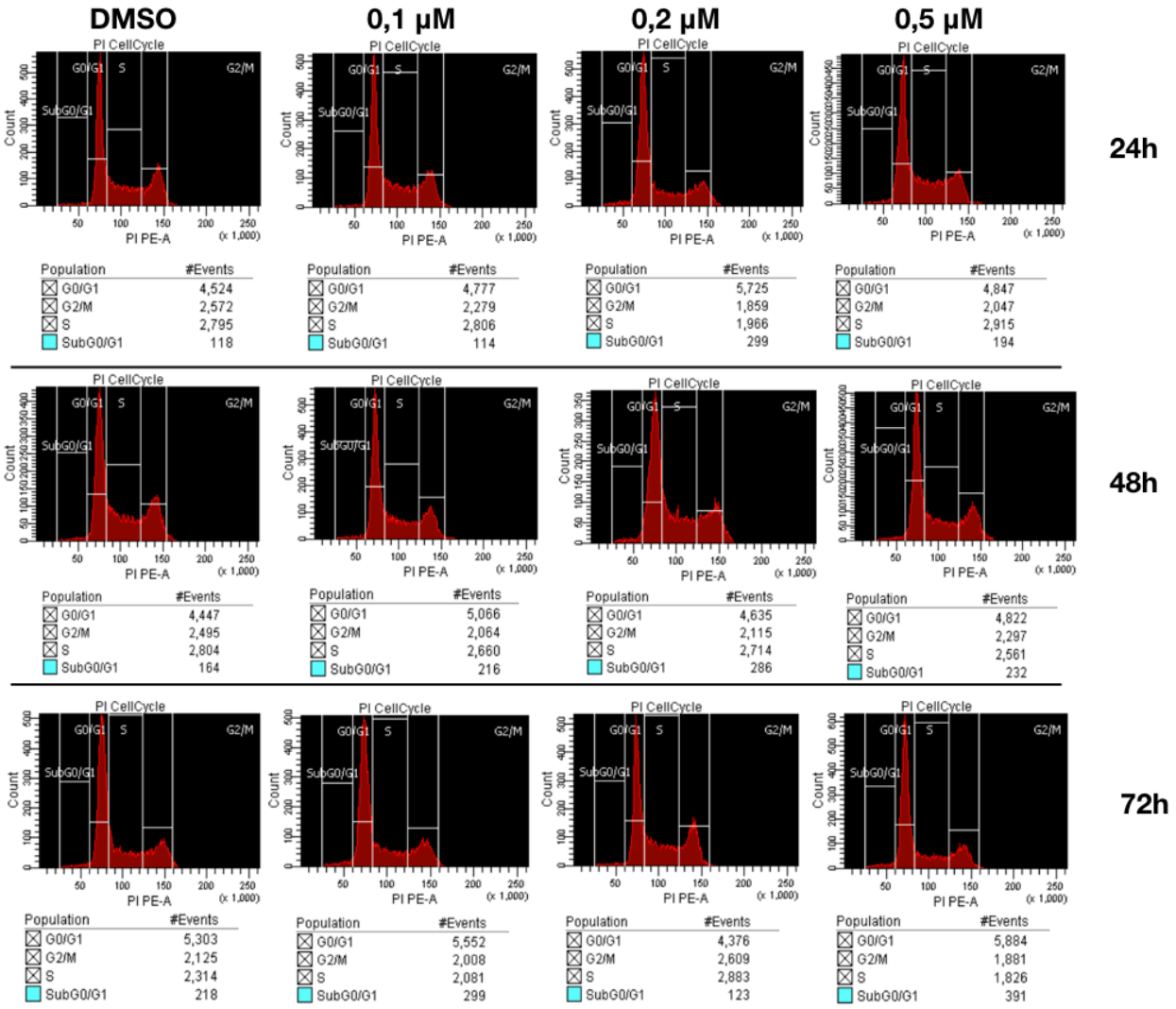


Figure 14: RH30 cell cycle analysis showed the increase of G0/G1 and sub G0/G1 phases.

5.4 Western Blotting analysis showed the involvement of apoptosis pathway and the reduction of protein levels of YAP in eRMS and aRMS cell lines

As said before, one of the main characteristics of a compound that can be used in cancer research is the ability to cause a block of the proliferation of tumor cells or a decrease of the number of cells. Based on the results obtained with XTT assay and in cell cycle analysis, next step of this work has been to understand if could be hypothesized the involvement of apoptosis pathway. Moreover, by using western blotting it could be possible to understand if VP has an effect on the protein levels of YAP and TAZ also in RMS.

5.4.1 A204 western blotting

After 24 hours of treatment with VP, the protein levels of YAP resulted still high in A204 cell line. At the strongest concentration of 0,2 μM the protein levels of YAP decreased compared to the control. Thanks to the administration of VP at 48 and 72 hours a decrease of the YAP protein levels is recognizable. The highest concentration is able to cause a strong reduction of YAP protein levels compared both to the control at 24 and at 72 hours.
Protein levels of TAZ, which resulted higher since the beginning of the treatment, remain the same during the entire time course.
The XTT assay showed a strong reduction of number of cells at a concentration of 0,2 μM after 72 hours of treatment. Nevertheless this, flow cytometry did not show the presence of a sub G1 phase. Probably, the action of VP helps to decrease the G2 phase blocking cells and leading them to a G1 phase and to a beginning of apoptosis process. The involvement of apoptosis can be revealed from the presence of cleaved-PARP 1 which grows during the time course.

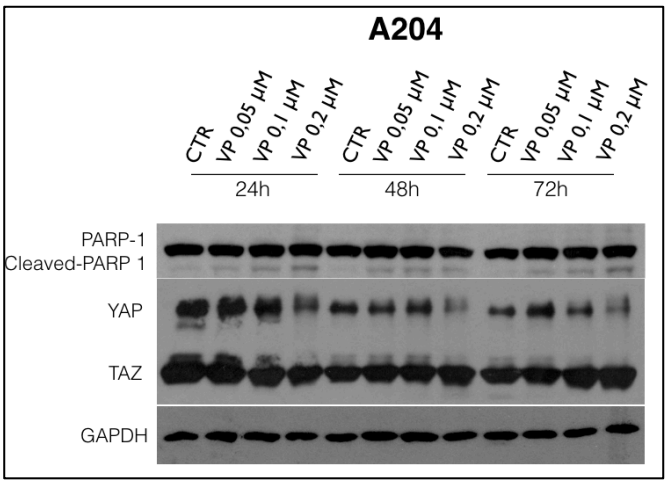


Figure 15: Protein levels of YAP and TAZ after the treatment with VP in A204 cells. Analysis showed the involvement of PARP. All the results have been normalized using GAPDH.

5.4.2 RD western blotting

The other line of eRMS showed different results. The activation of apoptosis is indicated also in this case from the cleavage of PARP. As to the protein levels of YAP, there is a sensible decrease of protein levels since the first 24 hours in the strongest concentration of VP. At 72 hours, YAP decreases compared to the control.
In this cell line, the protein level of TAZ decreased during the time course but a recovering of the levels at 72 hours has been observed. Probably, the decrease of YAP levels activates the expression of TAZ, which has to compensate the action of its orthologue.

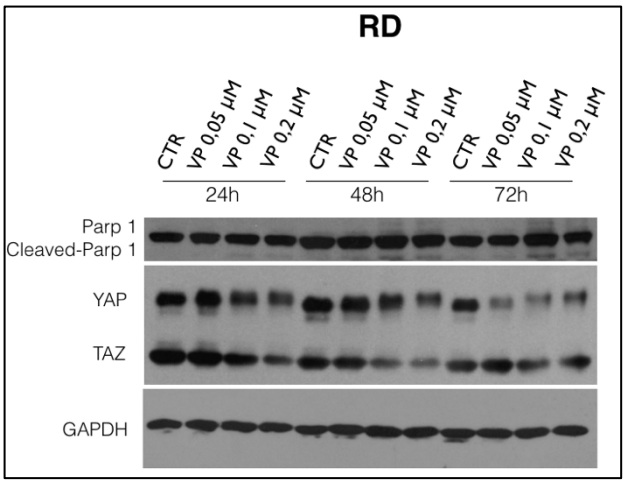


Figure 16: RD cells showed the activation of apoptosis process through PARP analysis and a sensible decrease of YAP levels.

5.4.3 RH4 western blotting

In RH4 cell line the action of VP can be revealed in the activation of PARP1 at the highest concentration, 0,5 µM, both at 48 and 72 hours after the administration.
YAP protein levels are always higher during the time course in all the concentrations of VP used, apart from the IC50 at 72 hours in which the levels are strongly down compared to the control. Moreover, TAZ protein levels increase during the time course.

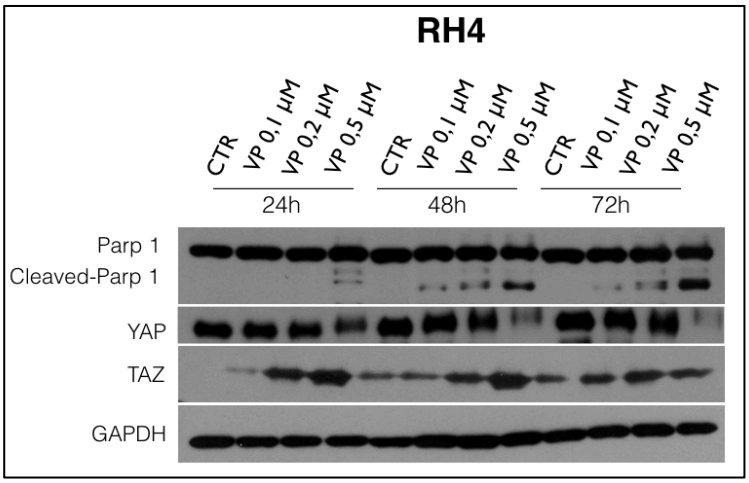


Figure 17: RH4 protein levels of YAP and TAZ and the involvement of PARP1 in apoptosis mechanism.

5.4.4 RH30 western blotting

As shown in figure 18, also in RH30 the activation of cleaved-PARP1 is present at 0,2 and 0,5 µM.
On the contrary of the other aRMS cell lines, RH30 shows the decrease of YAP at 48 hours in all the concentration of VP used. Instead, there is a recovery of protein levels at the end of time course apart from the highest treatment in which levels are sensibly low.
TAZ protein instead, shows a decrease at 48 and 72 hours compared to the control.

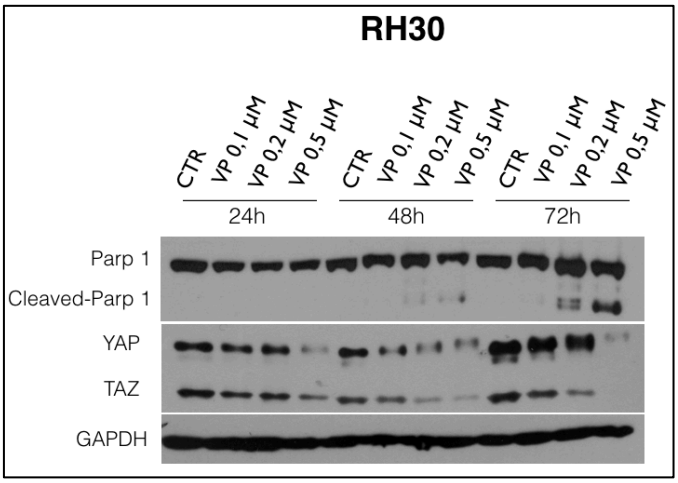


Figure 18: RH30 western blotting analysis of protein levels of marker of apoptosis PARP1 and of YAP and TAZ.

5.5 siRNA-induced knock down of YAP does not have antiproliferative effects on eRMS and aRMS cell lines

Several studies showed that VP has an effect on the protein and gene levels of YAP. Furthermore, VP showed to have an antiproliferative activity (Liu-Chittenden et al., 2012a). In addition to this, western blotting analysis showed that protein levels of YAP decrease during the administration of VP in RMS cell lines. Therefore, in order to understand if the simple inhibition of YAP has antiproliferative effects on RMS cell lines, siRNA transfection of YAP (siYAP) was performed.
24 hours before transfection, A204, RD, RH4 and RH30 were seeded at different density, depending on their phenotype, and so as to be at 70% confluency the day after. Later on, siYAP transfection was performed obtaining different efficiency depending on cell line. Globally, eRMS cell line was easier to transfect compared to aRMS. The experiments were performed three different times in triplicate.
After 48 hours from transfection, cells reached confluency and they were trypsinized, counted and centrifuge for pellet collection.

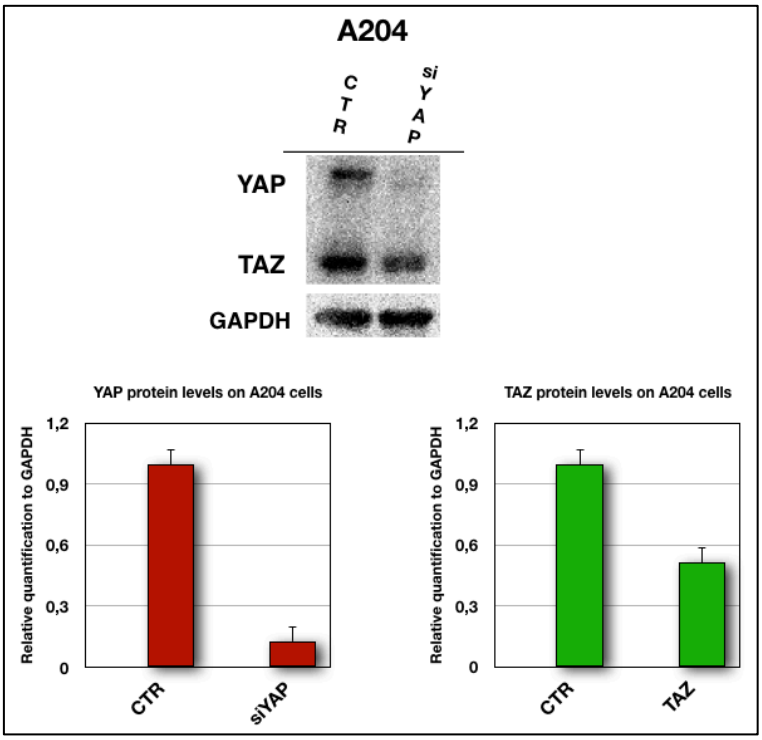


Figure 19: Protein levels of YAP and TAZ after siYAP transfection.

In order to see if the transfection succeeded, western blotting analysis was performed in all the cell lines. As shown in Figures 19 and 20, siYAP worked greatly in eRMS cell lines. Protein levels showed to be higher for what concerns TAZ, which decreases in A204 only in siYAP compared to the control. This decrease cannot be seen in RD (figure 20).

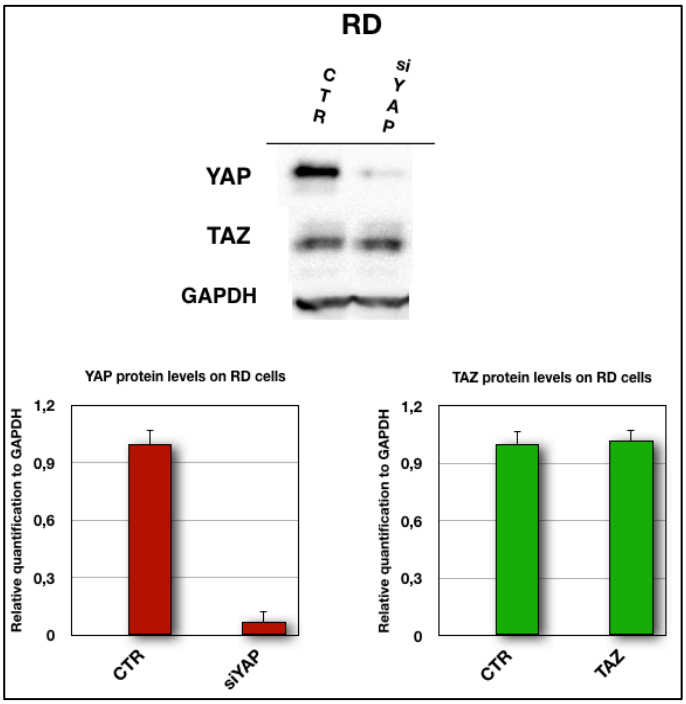


Figure 20: Protein levels of YAP and TAZ after siYAP transfection. TAZ protein levels remain the same after transfection.

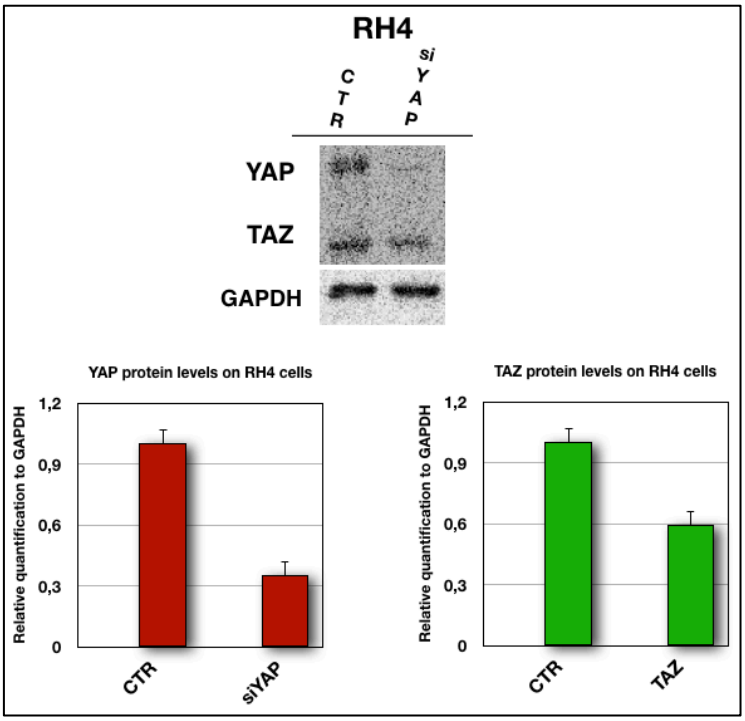


Figure 21: Protein levels of YAP and TAZ after siYAP transfection on RH4.

In relation to the aRMS transfection, results showed that YAP is knocked down in RH4 (figure 21) compared to RH30 (figure 22). Moreover, the protein levels of TAZ decrease compared to the control in RH4 cells.
The western blotting of RH30 showed that YAP is still present at high levels compared to the other cell lines. Nevertheless, TAZ levels increase after the transfection in a percentage, which is similar to the decrease of YAP.

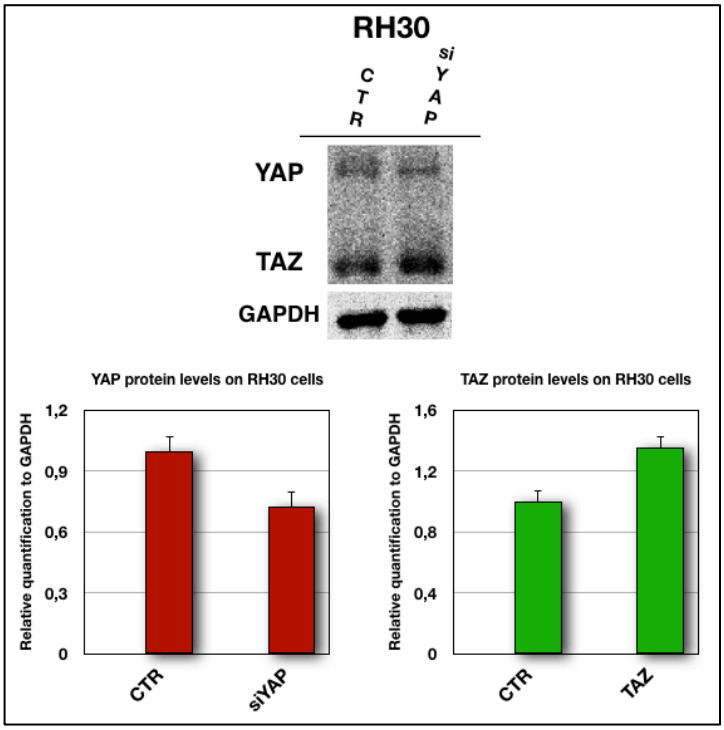


Figure 22: siYAP western blotting in RH30. TAZ levels increase after the silencing of YAP.

After transfection experiments, all RMS cell lines did not show any differences concerning the numbers of cells or phenotype features (figure 23).

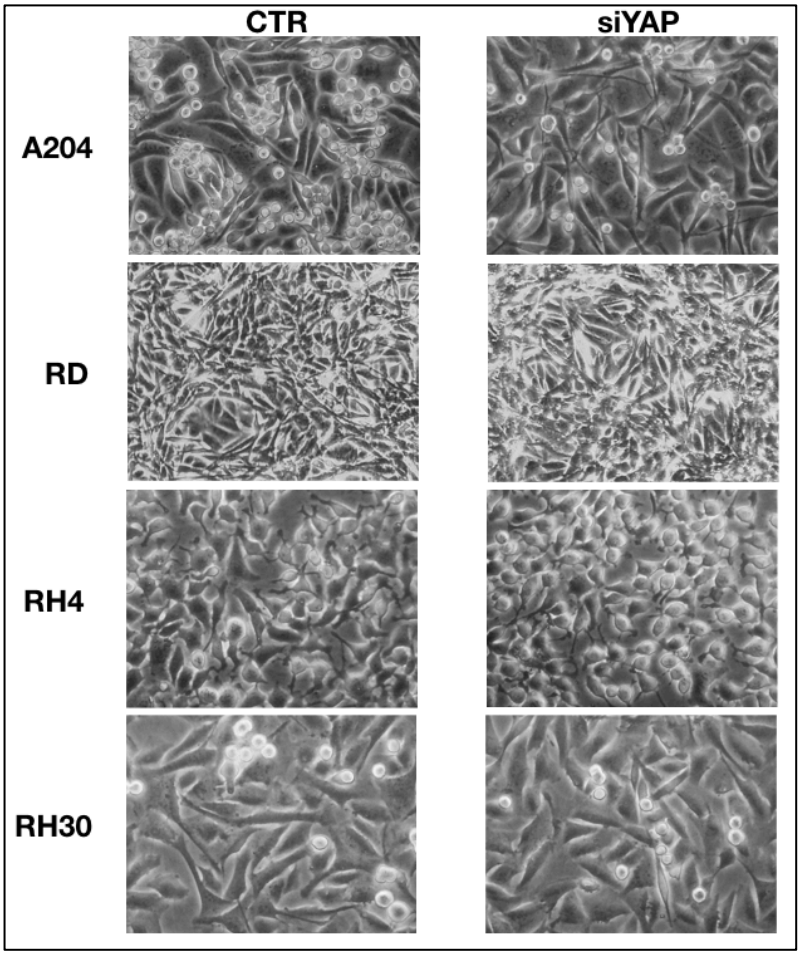


Figure 23: Phenotype features of RMS cell lines after transfection of siYAP.

The number of cells did not show any differences between the control and the siYAP transfection cells, as shown in Figure 24.

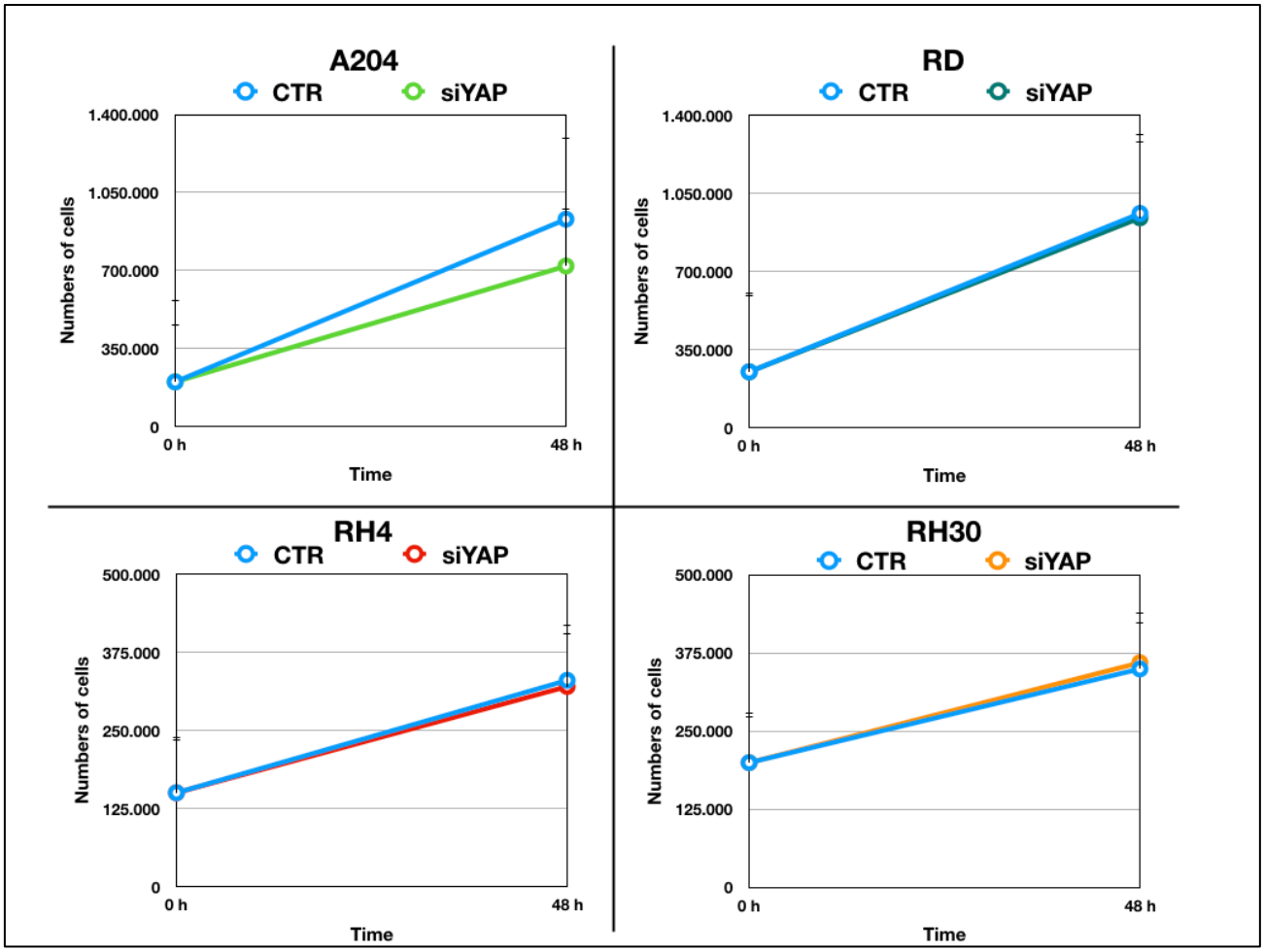


Figure 24: Cell counting of RMS cells after siYAP transfection.

6.Discussion and conclusions

Rhabdomyosarcoma belongs to soft tissue sarcoma and it is the most common sarcoma in its category (Shern et al., 2017). RMS can manifest generally through two subtypes: embryonal rhabdomyosarcoma (eRMS) and alveolar rhabdomyosarcoma (aRMS). These subtypes are assumed to arise from the same embryonic skeletal muscle cells. RMS can arise also in other parts of the body such as nerves, skin or vessels (Ruiz-Mesa et al., 2015). Fortunately, RMS is considered a rare tumor and statistics speak of 300 new cases in the United States every year. Around half of these patients are less than 10 years old. Instead, adult patients are extremely rare but show an invasive tumor with distance metastasis. Moreover, these patients have more unlucky and worst diagnosis compared to younger patients (Ruiz-Mesa et al., 2015).

According to the age of insurgence of the tumor, the treatment approach can vary. In adults with localized lesions, it has been observed that chemotherapy is not the best approach for children patients. Children patients usually undergone surgery, chemotherapy and radiation (Ruiz-Mesa et al., 2015). Considering that all these features can cause secondary pathologies and strong pain, several studies are trying to uncover the mechanisms involved in carcinogenesis of RMS. Furthermore, it is known that eRMS cells express early marker of myogenesis process (Saab et al., 2011). These features associated to an aggressive outcome for RMS patients, have pushed researchers to find new ways to fight this tumor. For instance, in our laboratory we demonstrated that a methyl-transferase called Enhancer of Zeste Homolog 2 (EZH2) (Marchesi and Bagella, 2013) is extremely involved in the carcinogenesis of this tumor. Indeed, a depletion of EZH2 in eRMS cells can help the recovery of myogenesis process decreasing the gene and protein expressions of early markers of myogenesis and increasing the ones involved later in the process (Marchesi et al., 2012). This kind of approach for restoring myogenesis process is challenging and full of surprises. For this, different drugs have been tested on RMS cells in order to push the recovery of myogenesis (Ciarapica et al., 2014; Marchesi et al., 2017).

Carcinogenesis of RMS is an extremely complicated process which involves different pathways (Saab et al., 2011). One of these pathways is called Hippo Pathway (Hpo) and with its two compelling genes YAP and TAZ, it is arising as one of the most important pathways in tumorigenesis (Piccolo et al., 2014). First identified in ***Drosophila Melanogaster***, it is conserved also in human. It is made up of kinase cascade with the activation of two orthologous YAP and TAZ. These two genes, which share several domain sites, demonstrated to be extremely involved in tumorigenesis (Piccolo et al., 2014). Recent studies demonstrated that the phenotype of YAP/TAZ double mutants shows severe changes compared to the phenotype with a single mutation (Varelas, 2014; Moroishi et al., 2015). The role of YAP and TAZ is at the center of scientific community discussions for their dual role in cytoplasm and nucleus. Thanks to their shuttling role inside cells, these proteins are able to activate several physiological process binding other proteins or activating other important pathways (Piccolo et al., 2014; Moroishi et al., 2015).

As often happens in research, compounds used for treating a specific pathology showed a dual or triple role on other worst pathologies. This is what happened with Verteporfin, which belongs to the porphyrins family. This compound has an aromatic and heterocyclic structure and is made up of four pyrrole units bound between them with methane bridges (Liu-Chittenden et al., 2012). VP has been used for photodynamic therapy but it showed to have a particular action on YAP-TEAD interaction in the nucleus (Liu-Chittenden et al., 2012). Indeed, it is able to disrupt the formation of this complex, consequently aborting the binding of YAP-TEAD with other important transcription factors, and to block subsequent processes (Liu-Chittenden et al., 2012). Some of these processes have not been clarified yet.

Studies on RMS showed the nuclear and cytoplasmic ratio of YAP and TAZ. In aRMS, YAP is more expressed in the cytoplasm, while in eRMS is more expressed in the nucleus (Tremblay et al., 2014).

Considering these data and the role of YAP in RMS, the aim of this project was to evaluate the activity of VP in eRMS and aRMS cell lines, and particularly, analyze the potential different actions that the compound could display between aRMS and eRMS or between cell lines in the same subtype of RMS.

At the beginning the protein levels of YAP and its orthologous TAZ were evaluated in eRMS cell lines (A204 and RD) and in aRMS cell lines (RH4 and RH30). The protein levels resulted higher in A204, and in RD cells, confirming the results already published regarding eRMS (Tremblay et al., 2014). RH4 and RH30 showed important levels of YAP proteins while the presence of TAZ was lower compared to eRMS cell lines.

Successively, the goal of the research was to identify a drug with a potential inhibition of YAP expression, in order to investigate its action in RMS. Therefore, VP was identified and XTT assay was performed in eRMS and aRMS cell lines. The XTT assay showed that at 72 hours eRMS cell lines have an IC50 around 0,2 μM while aRMS around 0,5 μM at 72 hours post VP treatment. The slight differences between the two subtypes could be given by the different oncogenesis mechanism involved in the tumor that are able to give more resistance to the VP at aRMS instead of eRMS cell lines.

Consequently, to investigate if VP has an effect on cell cycle, flow cytometry was performed.

As measured by flow cytometric analysis, during the time course, at 72 hours, A204 showed the decrease of G2/M phase. Moreover, cell cycle exhibited an increase of both S and G0/G1 phases.

In RD cell line instead, flow cytometry registered the decrease of S phase. Alternatively, aRMS cell lines displayed the decrease of all phases of cell cycle, and a distinct increase of the hypodiploid sub-G1 phase (apoptosis) of the cell cycle.

These results demonstrated that VP exhibits a different action compared to the various RMS cell lines. Specifically, VP is capable to perform cytostatic activity on A204 compared to all the other RMS cell lines tested where it seems to display cytotoxic activity.

Probably, cells, which are in the G0/G1 at the end of the treatment with VP, are slowly dying or they have the mitochondrial respiratory chain enzymatic activities, which results blocked. For this, the XTT assay showed a low number of cells after 72 hours post administration of VP. Indeed,

some works showed that VP can have a role also on mitochondria activities (Belzacq et al., 2001; Peng et al., 2005; Chiou et al., 2010). Moreover, western blotting analysis of PARP1 showed the cleavage of the protein indicating the activation of apoptosis in all cell lines both in eRMS and aRMS.

Western blotting helped also to analyze the protein levels of YAP and TAZ during the time course. As to the YAP protein levels, they sensibly decrease during the time course in all eRMS and aRMS cell lines at the higher concentration of 0,2 µM for A204 and RD; 0,5 µM for RH4 and RH30. TAZ protein levels instead are not completely clear and showed to have a tendency to increase or decrease during all the treatment of VP and in all RMS cell lines apart from A204. In this cell line the level of TAZ remains the same during the entire time course. Some studies hypothesized that considering the several domains which are in common between YAP and TAZ, VP could act on both protein levels (Liu-Chittenden et al., 2012). Compensation between the two proteins could be taken in exam since the absent or the blocked activity of one of them could increase the levels of the other.

In order to understand if the knocking down of YAP could have an effect on RMS cell lines, siRNA transfection was performed. The results showed that there are no changes in phenotyping or in the number of cells in RMS cell lines.

The purpose of this thesis was to test the efficacy of Verteporfin on RMS cell lines, in order to understand if this drug could be a valid compound for fighting RMS cancer. Indeed, nowadays several researchers are focusing their attention on the use of different drugs both in combination between them or with others technique such as radiation and surgery. This work showed that

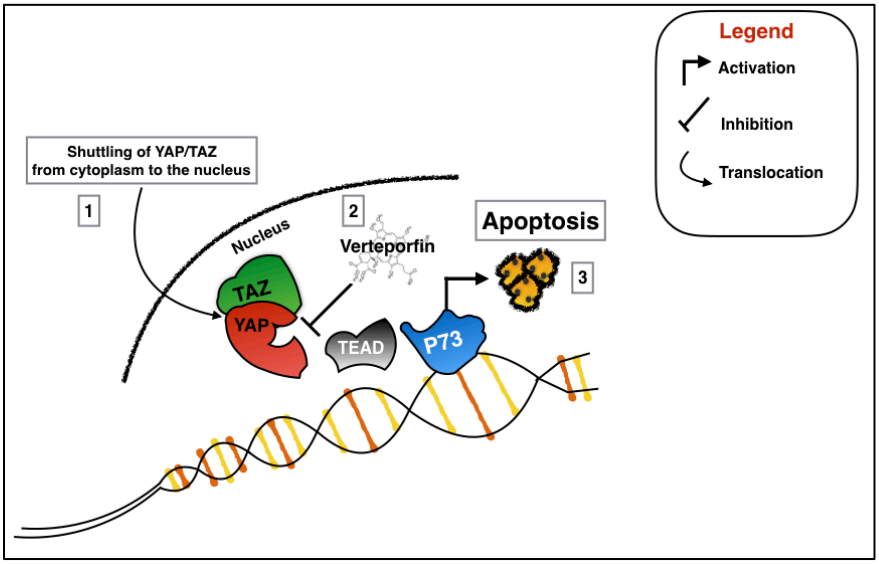


Figure 25: Representation of the role of Verteporfin in the inhibition of the binding between YAP and TEAD. 1. Thanks to phosphorylation, YAP and TAZ can enter in the nucleus. 2. Administration of Verteporfin blocks the binding between TEAD and YAP. 3. P73 cannot be bound by YAP/TEAD complex causing the activation of apoptosis process.

Verteporfin is a valid compound and it is able to decrease the protein levels of YAP at rather concentrations (0,2 and 0,5 μM). Moreover, it is able to change also the phenotype both in aRMS and in eRMS cell lines. Cell cycle analysis showed that the action of VP could vary depending on the cell lines. In aRMS cell lines, VP is able to increase sub G0/G1 phase (RH30) and to decrease S phase (RH4), whereas in eRMS cell lines the effect is different. Indeed, there is a reduction of G2/M phase in A204, which have a high rate of proliferation, while in RD the administration of VP increases the G0/G1 phase. Western blotting instead, showed in all the RMS cell lines the activation of apoptosis process through the cleavage of PARP1. Furthermore, this work showed that the inhibition of YAP does not affect phenotype or number of cells in RMS cell lines, hypothesizing that VP acts in a different way.

According to these results, further studies will have to be performed in order to understand how VP acts in RMS cell lines. Specifically, a model of what could happen is shown in Figure 25. As explained before, YAP and TAZ have both cytoplasmic and nucleus role (Piccolo et al., 2014). When they enter in the nucleus, they are able to bind TEAD factor creating a complex called YAP/TEAD. This complex can activate the transcription of several genes such as MYC, BIRC5 and others (Hong et al., 2016). Moreover, this complex represses the transcription of p73, which is an apoptotic protein that belongs to p53 family. YAP/TEAD also maintains the stability of p73 which is activated during DNA damages (Cottini et al., 2014; Piccolo et al., 2014). Therefore, thanks to the action of Verteporfin, which blocks the formation of YAP/TEAD complex, p73 cannot be stabilized and consequently activates apoptosis.

Concluding, we can say that Verteporfin is a promising compound that can probably have several mechanisms of action on RMS cell. Further studies will be necessary in order to understand how this molecule is able to act not only in RMS but also in the other type of tumors. Additional experiments will clarify if Verteporfin can block the binding between YAP/TEAD also in RMS and if this blocking is really able to activate p73-apoptotis mechanism.

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