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Verteporfin exhibits anti-proliferative activity in Embryonal and Alveolar Rhabdomyosarcoma cell lines

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Running head: Inhibition of growth in eRMS and aRMS cell lines through Verteporfin administration.

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Figures: 5

Table: 1

Abstract

Rhabdomyosarcoma (RMS) is a pediatric tumor, which arises from muscle precursor cells. Recently, it has been demonstrated that Hippo Pathway (Hpo), a pathway that regulates several physiological and biological features, is involved in RMS tumorigenesis. For instance, an upregulation of the Hpo downstream effector Yes-Associated Protein 1 (YAP) leads to the development of embryonal rhabdomyosarcoma (eRMS) in murine activated muscle satellite cells. On the other hand, the YAP paralog transcriptional co-activator with PDZ-binding motif (TAZ) is overexpressed in alveolar rhabdomyosarcoma (aRMS) patients with poor survival.

YAP and TAZ exhibit both cytoplasmic and nuclear functions. In the nucleus, YAP binds TEADs (TEA domain family members) factors and together they constitute a complex that is able either to activate the transcription of several genes such as MYC, Tbx5 and PAX8 or to maintain the stability of others like p73. Due to the key role of YAP and TAZ in cancer, the identification and/or development of new compounds able to block their activity might be an effective antineoplastic strategy. Verteporfin (VP) is a molecule able to stop the formation of YAP/TEAD complex in the nucleus.

The aim of this study is to evaluate the action of VP on RMS cell lines.

This work shows that VP has an anti-proliferative activity on all RMS cell lines analyzed. Depending on RMS cell lines, VP affects cell cycle differently. Moreover, VP is able to decrease YAP protein levels, and to induce the activation of apoptosis mechanism through the cleavage of PARP-1. In addition, Annexin V assay showed the activation of apoptosis and necrosis after VP treatment.

In summary, the ability of VP to disrupt RMS cell proliferation could be a novel and valuable strategy to improve the therapeutic approaches in treating rhabdomyosarcoma.

1. Introduction

Rhabdomyosarcoma (RMS), a common childhood cancer, is the most widely known Soft Tissue Sarcoma (STS), accounting more than 50% of the cases as malignant tumors [1]. According to the American Cancer Society, about 400-500 new cases of RMS are diagnosed every year in the United States of America [2]. RMS is histopathologically classified into 5 subtypes: embryonal rhabdomyosarcoma (eRMS) and alveolar rhabdomyosarcoma (aRMS) which are the most common [3] accounting respectively 60% and 25% of all RMS cases. The other 15%, instead, regards spindle cell RMS, undifferentiated cell RMS and botryoid cell RMS [4]. Particularly, eRMS occurs predominantly in children while aRMS arises in both children and adolescents. [5]. Although RMS derives from mesenchymal cells that express early marker of myogenesis the capabilities of these cells to fuse into myotubes and encode late muscle-specific transcription factors are impaired both *in vitro* and *in vivo* [6-8]. Treatments for RMS differ depending on several factors such as the histological subtype, localization and tumor size [9]. Considering the age of the patients and the difficult effectiveness of cancer treatments [3], numerous research efforts are currently focusing in identifying new therapeutic target genes and drug combination strategies to fight RMS.

Hippo Pathway (Hpo) was firstly recognized in *Drosophila Melanogaster* [10]. Afterwards, numerous studies have allowed to identify mammalian orthologs [11], disclosing its preservation during evolution. Hpo regulates cell growth [10], apoptosis [12] and cell differentiation [13]. All these processes are possible both for the complexity of Hpo network and for the regulation of several genes in the pathway. Yes Associated Protein 1 (YAP) belongs to Hpo, representing the core of the pathway [14]. YAP and the transcriptional co-activator with PDZ-binding motif (TAZ) [11], which is the YAP paralog [14], are the pivotal downstream effectors of Hpo [15]. Indeed, Hpo kinase cascade increases YAP/TAZ phosphorylation leading to its inhibition [14,16], since this event is crucial to regulate several biological processes [17]. Nevertheless, it is still unknown how YAP/TAZ are able to undergo tissue specific-inhibition/activation [14]. Hpo activation occurs through the phosphorylation on specific serine residues, which allows the sequestration of YAP/TAZ in the cytoplasm and consequent proteasomal degradation [15,18-20]. In the nucleus instead, YAP/TAZ

interacts with TEADs, causing the transcription of genes involved in cell proliferation and inhibition of apoptosis [21-23].

The wide range of actions of Hpo led scientists to investigate its potential role in carcinogenesis, for example, in human carcinomas, such as lung and pancreas, characterized by activating K-RAS mutations. In Kras-mutant mice, YAP is fundamental for the progression of pancreatic ductal adenocarcinoma (PDAC) [24]. Furthermore, in Kras-driven murine models of lung cancer and PDAC, YAP causes, in the absence of KRAS, the recurrence of both tumors [25-27]. On the other side, TAZ showed oncogenic features in malignant mesothelioma (MM). Studies found that TAZ is expressed and activated through phosphorylation in MM cells and its knockdown suppresses cell proliferation, invasion and cell motility [28].

Lately, aberrations of Hpo in RMS have been identified, mainly consisting of the dysregulation of both YAP and TAZ. The upregulation of YAP was detected in RMS and its hyperactivity causes eRMS in murine activated muscle satellite cells with a high penetrance [29]. Most recently, new studies have uncovered an important role of TAZ in aRMS. The formation of a chimeric gene (P3F) which encodes for a fusion protein made up of C-Terminal FOXO1 and PAX3-PAX7 DNA binding domain is at the base of aRMS oncogenesis [3,30-34]. Furthermore, *Mohamed* and colleagues showed that a higher expression of TAZ is associated with a poor survival rate in eRMS patients [35]. Moreover, *Deel* and colleagues demonstrated that TAZ is upregulated in P3F-initiated aRMS mouse model and it accumulates in the nucleus of cancer cells. A suppression of TAZ in subcutaneous (SQ) xenografts attenuates aRMS tumor growth [36].

The aggressiveness of RMS, along with the emerging evidences of the involvement of YAP/TAZ in tumorigenesis, are encouraging researchers to discover new drugs able to inhibit the activity of these proteins as a potential add-on therapy for the treatment of RMS [37,38]. Verteporfin (VP) also known as Visudyne (*Novartis*) is a drug that is providing positive results in fighting several experimental tumor types. VP belongs to the family of porphyrins and it has been clinically approved by FDA [39] as a photosensitizer in the photodynamic therapy for macular degeneration [39]. *Liu-Chittenden* and colleagues discovered the ability of VP to destroy the interaction between YAP and TEAD. Indeed, VP acts by modifying the structure of YAP, which then fails to complex with TEAD in the nucleus

[39]. The efficacy of VP has been evaluated in breast, prostatic, pancreatic [40,41] and colorectal cancers [42]. Moreover, novel studies uncovered anti-proliferative action in leukemia and endometrial cancers [43,44].

In the present study, two eRMS and two aRMS cell lines were used to delve into the effect of VP on RMS cell lines. The results demonstrated that VP has an anti-proliferative effect on RMS cell lines, suggesting that YAP/TAZ inhibition could be a promising novel approach for the treatment of RMS.

2. Material and Methods

2.1 Cell culture

aRMS cell lines RH30 (ATCC, Rockville, MD) and RH4 (RRID:CVCL_5916) were cultured in RPMI 1640 medium (Gibco). eRMS cell lines RD (ATCC) and A204 (CLS) were cultured with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco). Both media have been supplemented with 10% of Fetal Bovine Serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin (Gibco) and 1% of L-glutamine. Verteporfin (Sigma-Aldrich) treatments were performed at concentrations between 0.05 µM and 2 µM. Cells were incubated at 37°C with 5% of CO_2 in a humidified air [45].

2.2 Proliferation assay (XTT)

Cells were seeded at a density between 1000 and 1500 cells/wells in a 96-wells plate based on different sizes, population doublings [46] and phenotypes. After 24 hours from seeding, cells were treated for 24, 48 and 72 hours with different concentrations of VP in a range between 0.1 µM and 2 µM in a final volume of 100 µl. Dimethyl sulfoxide (DMSO), the vehicle of VP, has been used as a control. After 24, 48 and 72 hours, XTT assay was performed using the Cell proliferation Kit II (Roche, Basel, Switzerland). 0.5 µl of XTT electron coupling reagent and 25 µl of labeling reagent were resuspended in 74.5 µl of medium (final volume 100µl/well) and incubated for 4 hours at 37°C. After incubation, the absorbance was measured at 490 nm with a spectrophotometric plate reader (SPECTRAMax 384 PLUS) and the achieved results were used to calculate the IC_{50} values. Based on IC_{50} value for each cell line, three different concentrations were selected for subsequent experiments [47].

2.3 Cell Cycle Analysis

Cells were seeded at 50% of confluence on 6 cm dishes. After 24 hours, cells were treated for 72 hours using three different concentrations of VP depending on the IC_{50}. After that, floating (necrotic and in late apoptosis cells) and adherent cells were firstly centrifuged at 3000 rpm for 5 minutes; washed in PBS and then fixed with ice-cold ethanol at 70% and incubated at -20°C overnight [48]. Fixed cells were washed twice with cold PBS and then resuspended with 200µl of a solution of PBS and 20µl/test of 7-AAD (Bioscience, San Diego, CA) and incubated for 20 minutes at room temperature. Cell Cycle assay was performed by Flow Cytometry using BD FACS CANTO II and collecting around 20000 events for each sample. Data were analyzed by BD FACS DIVA software.

2.4 Protein extraction and Western Blot

Total proteins were extracted using lysis buffer (20 mMTris HCl pH 8; 137 mM NaCl; 10% glycerol; 1% Nonidet P-40; 2 mM EDTA) with the addition of Protease Inhibitors Cocktails (Roche, Basel, Switzerland). Fifteen µg of proteins were dissolved in 8% SDS/PAA and successively transferred into a nitrocellulose membrane (GE, Healthcare, Whatman) for 1 hour at 4°C and 100V. Nitrocellulose membrane was blocked with 5% of Albumin Bovine Serum (BSA) for one hour at room temperature. Blots were incubated overnight at 4°C with primary antibody listed below: anti-PARP (Cell Signaling, Boston, MA); anti-YAP/TAZ (Sigma-Aldrich, Saint Louis, MO) and anti-GAPDH (Santa Cruz Biotechnology, CA). Membranes were incubated with secondary peroxidase conjugated antibodies and signal was detected with Western Lightning Plus-ECL (PerkinElmer, Waltham, MA, USA) and X-ray films (Kodak, Rochester, NY) [49].

2.5 Annexin V-propidium iodide assay

Annexin V-propidium iodide assay was carried out using FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen, San Jose, CA), following the manufacturer's protocol. After 18-24 hours, cells were treated at the same concentrations of VP used for cell cycle analysis. After 72 hours, floating and adherent cells were collected, centrifuged and washed twice with cold PBS. One ml of Binding Buffer was used to resuspend the cells. Five µl of Propidium Iodide (PI) and 5 µl of annexin V-fluorescein

isothiocyanate were added to the cells and incubated in the darkness for 15 minutes at room temperature. Flow cytometry was performed immediately using BD FACS CANTO II. 20000 events were analyzed using the BD FACS DIVA software.

3. Results

3.1 Yap and TAZ are differently expressed in eRMS and aRMS cell lines.

According to recent studies showing the potential role of YAP/TAZ in aRMS [50] and eRMS [29] carcinogenesis, the protein levels of both YAP and TAZ were tested through Western blotting in RMS cells using a specific antibody for YAP and TAZ. As shown in figure 1A, YAP is expressed in all cell lines analyzed. Specifically, RD cells displayed higher protein levels of YAP compared to A204, RH4 and RH30 cells. For what concerns TAZ, eRMS cells showed higher protein levels compared to aRMS cell lines, where TAZ expression was almost undetectable (Figure 1B).

3.2 Verteporfin has anti-proliferative activity on RMS cell lines.

Taking inspiration from prior studies on the role of YAP and TAZ in RMS carcinogenesis [29,50] and in order to understand whether YAP/TAZ inhibitors were able to exhibit anti-proliferative and/or cytotoxic effects on RMS cells, cell viability of RMS cells after VP treatment using XTT assay was evaluated. Cells were treated with concentrations of VP ranging between 0.1-2 μM, using DMSO as a control. Colorimetric assay was performed after 72 hours and recorded absorbance values were used to calculate IC_{50}. Results after 72 hours treatment are listed in Table 1. aRMS cell lines showed a IC_{50} value slightly higher than that of eRMS.

3.3 Verteporfin induces cell cycle deregulation in RMS cell lines.

RMS cells exhibit dysregulation of the cell cycle machinery entering in an unrestrained growth. In order to understand the impact of VP on RMS, a cell cycle analysis by measuring DNA content in flow cytometer in RMS cell lines was performed. Cell cycle analysis showed different effects of VP in all RMS cell lines examined. As shown in figure 2A, the treatment with VP on A204 cells at 0.2 μM caused an increase of G2-phase. In RD cell line (Figure 2B), there was a decrease of G1 phase

with a contemporary increase of G2 (32.6%) and S (21.5%) phases in the treated samples compared to the control. aRMS cell lines showed a similar profile. Indeed, in RH4 cells the 0.5 μM of VP was able to increase G2 phase in RH4 (32.7%) and in RH30 (26%) cell lines (Figure 2C/D). Summarizing, VP is able to cause a G2-phase block in all RMS cell lines with a clear increase of cells in S-phase after treatment (Figure 2E).

Furthermore, a microscopic observation of treated cells showed that VP in A204 cells caused a disruption of bundle structure present in control cells; while RD cells showed a typical sign of distress and evident presence of death cells in the supernatant (Figure 3A). At 0.5 μM, RH4 and RH30 cell lines became smaller, less stretched, and disorganized compared to respective controls (Figure 3B).

3.4 Annexin V assay uncovers the activation of apoptosis in RMS cell lines by Verteporfin treatment.

Based on cell cycle assay and XTT results, Annexin V-FITC/PI double staining kit was used in flow cytofluorimetric analyses. RMS cells were treated with VP at the following concentrations: 0.2 μM for eRMS and 0.5 μM for aRMS cell lines. After 72 hours, cells were stained with both FITC and PI and analyzed with BD FACS CANTO II. VP activated apoptosis in eRMS and aRMS cell lines (Figure 4A/B). In RD cells, after 72 hours, percentage of apoptotic cells increased from 7.6% in control cells to 39.3% in treated cells (Figure 4B). In RH4, VP caused apoptosis in 13.4% of cells compared to 6.5% in control cells (Figure 4C). RH30 showed a higher percentage of apoptotic cells (19.8%) compared to 7.3% of the control (Figure 4D). The rate of apoptosis in A204 increased slightly in treated cells compared to control, whereas the percentage of necrotic cells increased significantly (Figure 4A). Comparing Annexin V assay results, apoptosis is activated in all RMS cell lines whereas necrosis can be observed in A204 after VP administration (Figure 4E).

3.5 Verteporfin causes a reduction of YAP protein levels and the activation of PARP-1.

In order to investigate the mechanism of action of VP on RMS cells, the latter cells were treated with different concentrations of VP to measure the protein expression levels of YAP, TAZ, PARP-1 and cleaved PARP-1. After 24, 48 and 72 hours from treatment, pellets were collected, and Western

blotting was performed. Results are shown in figure 5. After 72 hours of treatment with VP, YAP protein levels decreased in both eRMS cell lines (Figure 5A/B/C/D). Specifically, RD showed a higher decrease of YAP protein levels at 72 hours at every concentration compared to A204. Moreover, the activation of PARP-1 was observed in both cell lines, confirming annexin V assay. Specifically, the cleavage of PARP-1 in A204 was activated after 24 hours from the treatment with VP (Figure 5A). TAZ protein levels did not exhibit a clear tendency as shown in figure 5A/B/C/D.

As concerns aRMS cell lines (Figure 5E/F/G/H), YAP protein levels decreased after 72 hours of treatment at 0.5 μM in RH4 and RH30. As seen in eRMS cell lines, TAZ protein levels displayed the same tendency in RH4. In RH30 instead, a clear reduction of YAP protein levels has been detected. In both cell lines the cleavage of PARP-1 was observed.

4. Discussion

As previously mentioned, among soft tissue sarcomas, RMS is the most common [51]. RMS presumably arises from embryonic mesenchyme like striated skeletal muscle [52]. According to statistics, half of the patients of RMS are children less than 10 years of age. In the rare cases of adults RMS, tumor shows aggressive features with distant metastases. Moreover, these patients have a worse prognosis if compared to younger patients [52].

Nowadays, children with localized RMS are treated with multidisciplinary treatment programs which include chemotherapy. In contrast, standard treatment for adult RMS is based on surgical resection, often in conjunction with radiotherapy. Currently, adjuvant chemotherapy has shown certain advantages in adults with RMS, but the level of benefits has not proved to be as high as for childhood RMS [53].

Considering the aggressiveness of chemotherapy and radiotherapy, numerous studies are focusing on the development of less toxic therapies and novel treatment approaches for patients with RMS. In the last decade, researchers have shown an increased interest in the fine tuning of new drugs with minimal side effects or, even better, without any [54,55], in the development of therapeutic strategies intending to restore myogenic differentiation [56], or in understanding the basic mechanisms of RMS carcinogenesis at the cellular and molecular level [8]. On the basis of what

previously described, today, the challenges faced by researchers encompass biological studies aimed to explore the molecular machineries that drive cell proliferation, differentiation and survival, leading to new targets and strategies for cancer prevention and treatment. In particular, alterations affecting the Hpo pathway and its downstream effectors, YAP and TAZ, are attracting the attention of many cancer researchers [14]. The perturbation of this kinase cascade has been demonstrated to cause alteration of the physiological process. Specifically, overexpression of YAP has been found to result in an abnormal proliferation of many tissues and organs, such as skin, gastrointestinal tract, heart, and liver in experimental conditions [16,57-59]. Thus, it is not surprising that a disruption or deregulation of Hpo has been detected in several malignancies such as liver, lung, colorectal, prostate and ovarian cancer [15,16,60].

Recent studies focusing on both TAZ [36] and YAP [29] proteins showed the upregulation of these Hpo members in RMS, paving the way for deeper investigations into the potential role of YAP/TAZ in this tumor entity. Specifically, a compound called VP, which has demonstrated great versatility in cancer research acting on leukemia [43] and colorectal cancer [42] models, was tested in the present study to evaluate its possible effects on the growth of aRMS and eRMS cell lines.

The first goal of the current research was to assess protein levels of both YAP and TAZ. As shown in figure 1A/B, YAP/TAZ protein levels were higher in RD and A204 cell lines, confirming the results already published by Tremblay [29]. RH4 and RH30 exhibited the same trend, while the levels of TAZ were lower if compared to those in eRMS cell lines. Consequently, these results prompted to assess whether VP could have a role in the inhibition of YAP or TAZ in RMS cells.

Previous investigations proved that VP is able to downregulate YAP expression exhibiting anti-proliferative activity [44,61]. In order to understand if VP has any effect on RMS cell lines, XTT assay was performed and the IC_{50} was calculated for every cell line (table 1). The anti-proliferative activity of VP showed an IC_{50} ranging from 0.15 to 0.25 μM against a panel of RMS cell lines. The slight differences between the IC_{50} of the two subtypes of RMS could be conferred by the oncogenic mechanism of the cancer subtype [3], which can give more resistance to aRMS compared to eRMS after VP administration.

At a later time point, considering that the perturbation of cell cycle is one of the main features of

cancer cells, cell cycle analysis was performed on RMS cells after treatment with VP. At 0.2 μM VP blocked A204 cells (Figure 2A) and RD (Figure 2B) in the G2 phase. aRMS cell lines showed the same tendency after treatment at 0.5 μM of VP. Furthermore, in all RMS cells an increase of S phase has been observed.

In order to test *in vitro* effects of VP, eRMS and aRMS cell lines were seeded and treated with different concentrations of VP, higher than IC_{50} to stress the effect of the compound on RMS cell lines. The results displayed that VP changed the phenotype of treated cells inducing different cytopathological features depending on the cell type (Figure 3).

To confirm the data obtained from cell cycle analysis and clarify the effects of VP on RMS cell lines, apoptosis was analyzed through the Annexin V assay. The results showed that the apoptosis process is activated in all RMS cell lines after the treatment with VP for 72 hours, with the exception of A204 cells, in which necrosis process showed higher percentage of activation (Figure 4A).

These data helped to move on the investigation of protein levels of YAP and TAZ after VP treatment and the potential activation of a mechanism of cell death. While higher concentrations of VP caused a reduction of YAP protein levels in all RMS cell lines, TAZ levels displayed a heterogeneous behavior that requires to be further investigated (Figure 5). Indicatively, some researches hypothesized that in light of several domains shared by both YAP and TAZ, VP could act on both protein levels and a compensation mechanism between the two proteins could be in place [39].

Furthermore, as previously explained, various studies found that VP is able to downregulate YAP protein levels and block YAP-TEAD binding [39]. Our research confirmed that treatment with VP decreased YAP protein levels in a dose-dependent manner in all RMS cell lines (Figures 5). Probably, after the administration of VP and the disruption of YAP-TEAD binding, the YAP half-life is affected, and YAP protein is sent to lysosome-dependent degradation, as recently shown [62].

Additionally, Western blot analysis revealed the cleavage of PARP-1 protein, which is involved in the apoptosis process, in all RMS cell lines (Figure 5) after treatment with VP. In contrast to the other RMS cell lines, although A204 showed the cleavage of PARP-1 by Western blotting, Annexin V analysis pointed out the activation of necrosis as a way of death. Presumably, in this cell line, the early apoptosis process after 72 hours has already been overcome.

Altogether, these results enrich the long list of cancers in which VP has shown to have an anti-cancer activity; its ability to inhibit YAP-TEAD complexes formation [39] can be used to deeply study mechanisms in which YAP/TAZ are involved. For example, a recent paper showed that VP has antiproliferative effect in another sarcoma. Indeed, Trautmann and colleagues hypothesize that anti-proliferative effect of VP in myxoid liposarcoma cells is due to a reduction of mitotic activity and disruption of YAP-TEAD complex [63]. Indicatively, a model of what could happen regards the role of YAP and TAZ in the nucleus [14]. Here, their entry allows the formation of a complex with TEAD factor. The complex YAP-TEAD activates the transcription of a different genes such as BIRC5, MYC and others [10]. On the other hand, this complex represses the transcription of p73 an apoptotic protein which belongs to p53 family maintaining also its stability [14,64]. Presumably, the disruption of YAP-TEAD complex after VP administration [39] causes the transcription of p73 which activates apoptosis cascade after DNA damages in RMS cell lines. Furthermore, our data lead us to claim a possible mitotic catastrophe caused by VP that could be taken in exam in future studies.

The positive data of this research lead to encourage further researches to investigate VP as a potential anticancer drug for the treatment of RMS and also to discover new biological small molecules with the greatest inhibitory potential against this specific target. All past studies and newer approaches will require ongoing clinical evaluation to allow constant progress in the treatment of RMS.

Figure and table legend

Table 1

Table 1: Cytotoxic activity of Verteporfin in RMS cell lines. IC_{50} obtained after 72 hours of treatment with VP on eRMS and aRMS cell lines. VP exhibited anti-proliferative activity on RMS cell lines in a range between 0.16 µM and 0.23 µM.

	Cell lines	IC_{50} (µM)
A204	Embryonal Rhabdomyosarcoma	**0.16** ± 0.03
RD	Embryonal Rhabdomyosarcoma	**0.15** ± 0.01
RH4	Alveolar Rhabdomyosarcoma	**0.24** ± 0.01
RH30	Alveolar Rhabdomyosarcoma	**0.23** ± 0.02

Figure 1

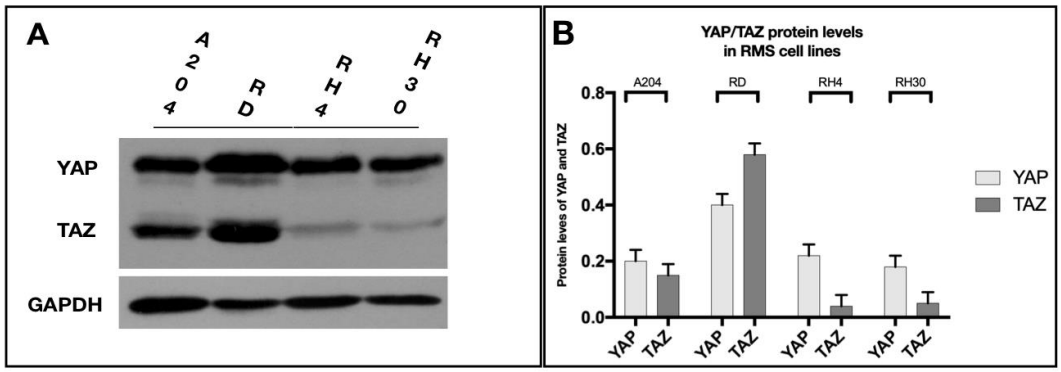
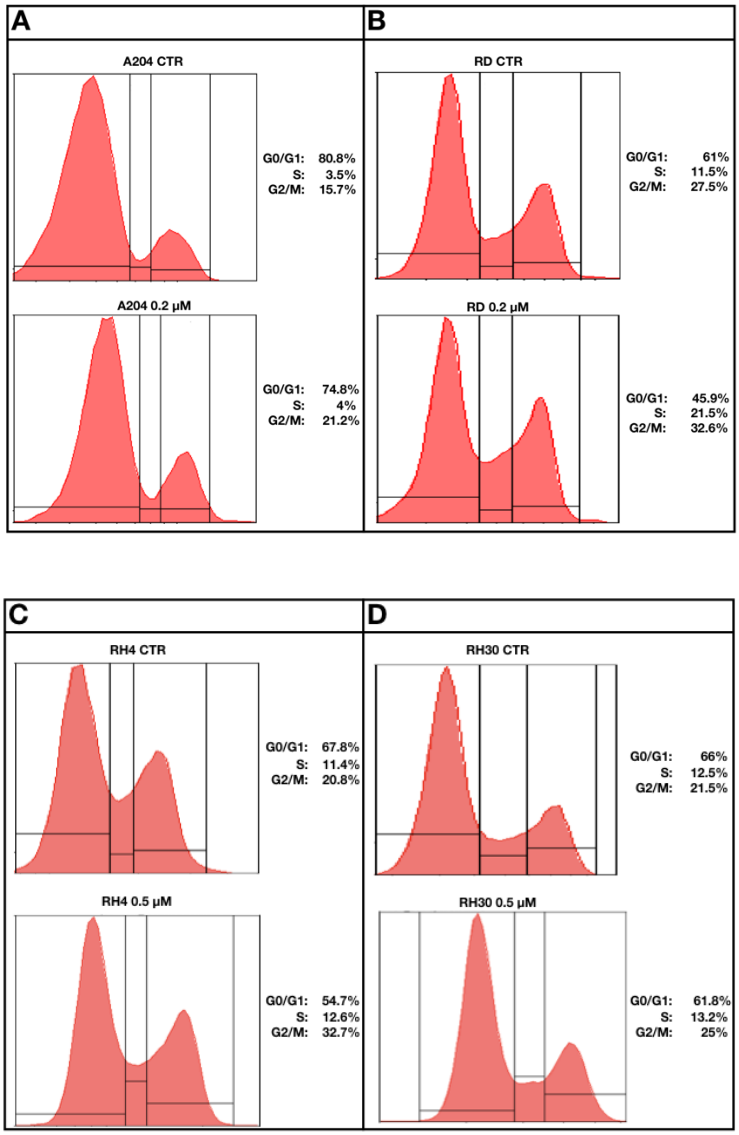


Figure 1: YAP and TAZ have different protein levels in RMS cell lines.

Protein levels of YAP and TAZ in eRMS and aRMS cell lines. YAP is expressed in both eRMS and aRMS cell lines, while TAZ showed higher protein levels in eRMS compared to aRMS cell lines. GAPDH was used as loading control **(A)**. YAP and TAZ band intensities were quantified through ImageJ (NIH, USA) and data were organized using Graphpad Prism software (San Diego, CA, USA) **(B).**

Figure 2


A
A204 CTR
G0/G1: 80.8%
S: 3.5%
G2/M: 15.7%
A204 0.2 µM
G0/G1: 74.8%
S: 4%
G2/M: 21.2%
B
RD CTR
G0/G1: 61%
S: 11.5%
G2/M: 27.5%
RD 0.2 µM
G0/G1: 45.9%
S: 21.5%
G2/M: 32.6%
C
RH4 CTR
G0/G1: 67.8%
S: 11.4%
G2/M: 20.8%
RH4 0.5 µM
G0/G1: 54.7%
S: 12.6%
G2/M: 32.7%
D
RH30 CTR
G0/G1: 66%
S: 12.5%
G2/M: 21.5%
RH30 0.5 µM
G0/G1: 61.8%
S: 13.2%
G2/M: 25%

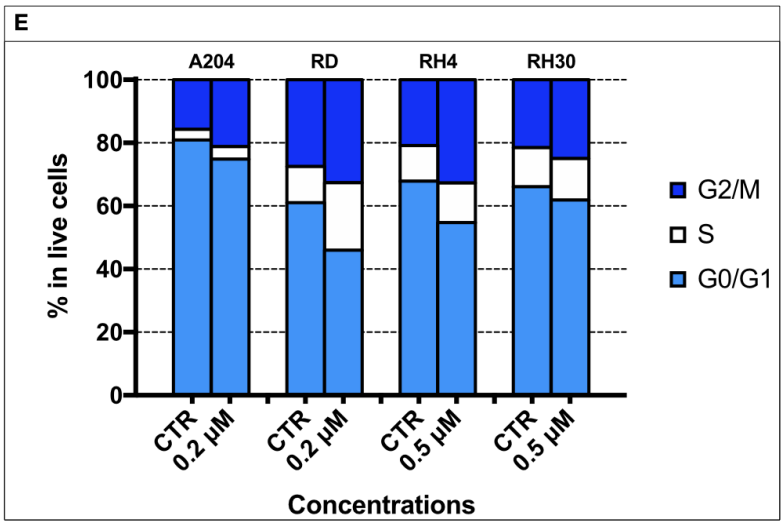


Figure 2: Cell cycle distribution in RMS cell lines after VP administration. Different effects on cell cycle after VP treatment in eRMS **(A/B)** and aRMS **(C/D)** cell lines. At a concentration of 0.2 µM, VP caused a block in G2 phase in A204 cells **(A)** and in RD cells **(B)**. A higher concentration of 0.5 µM blocked cells in G2 phases both in RH4 **(C)** and RH30 **(D)**. In addition to this, an increase of cells in S phase can be seen after VP administration in all RMS cell lines. **(E)** Different VP effects on cell cycle of RMS cell lines.

Figure 3

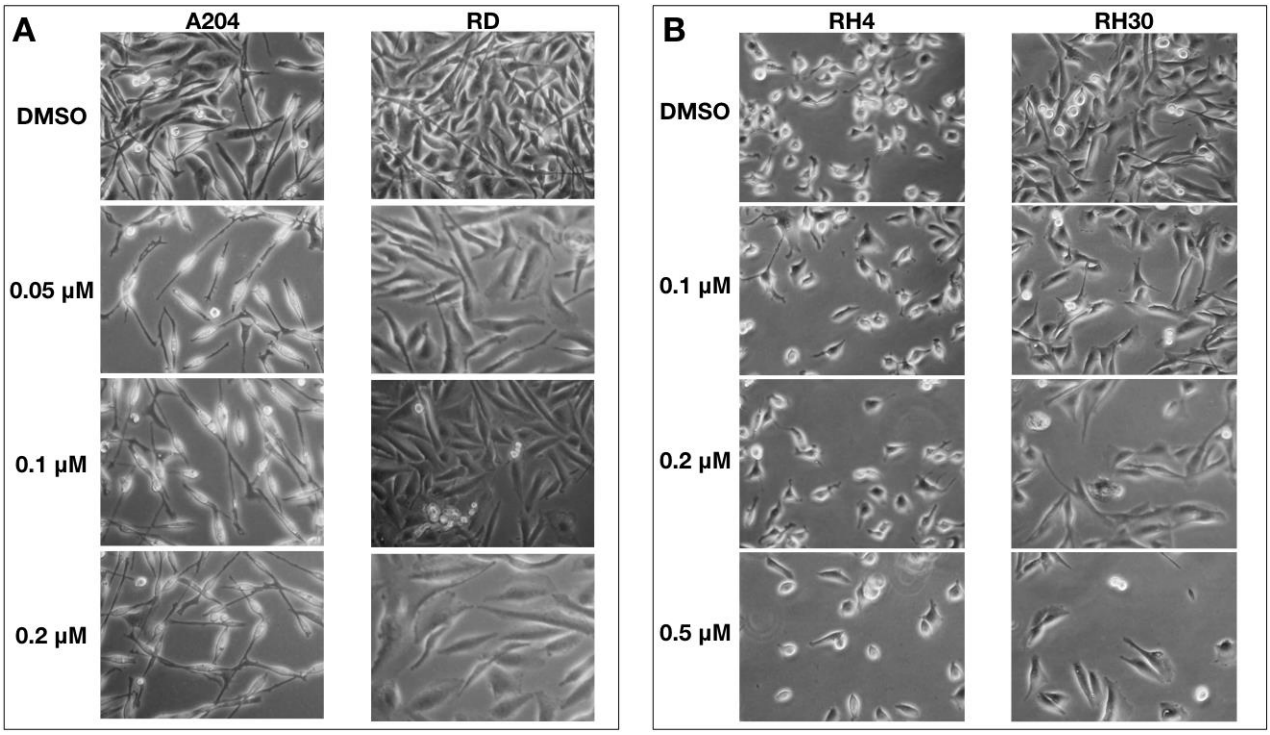
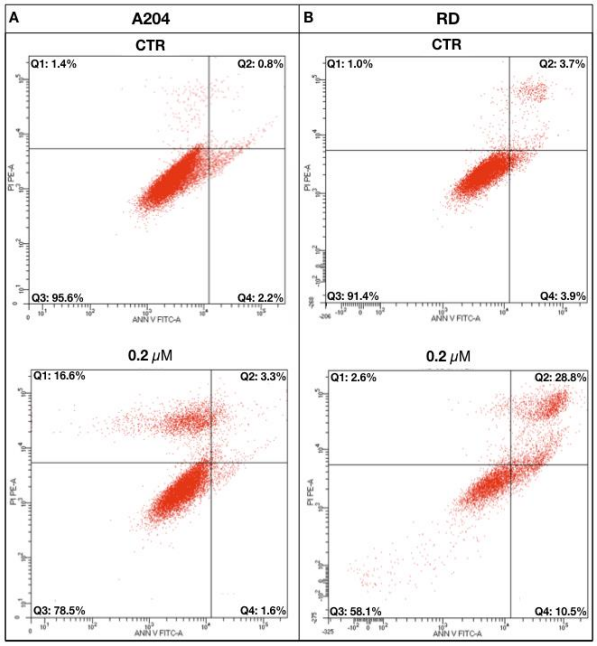
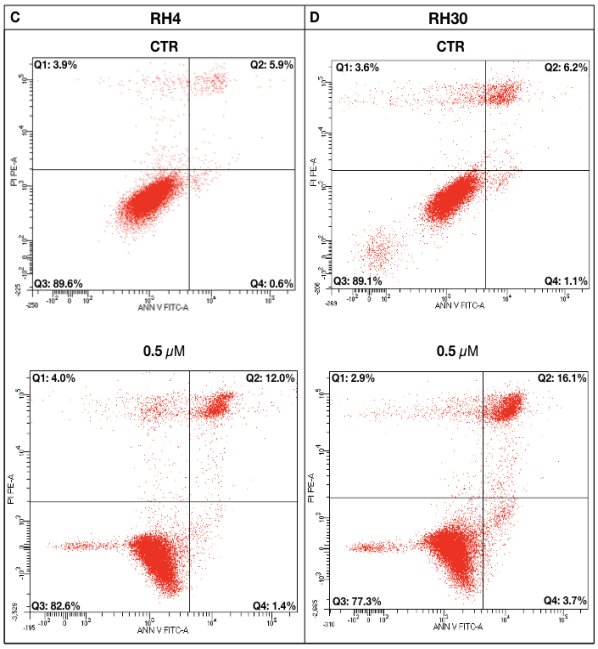


Figure 3: VP changed the phenotype of RMS cells.

Morphological changes induced by VP in eRMS **(A)** and aRMS cell lines **(B)**. VP showed different effects depending on cell types and concentration. Indeed, A204 cells appeared more stretched, while RD showed cytopathological characteristics of suffering cells and cell death **(A)**. After VP administration, RH4 cells became smaller, while RH30 cells showed disorganization **(B).** In all RMS cells, VP caused an increase of dead cells in the supernatant in a dose-dependent manner.

Figure 4


A
A204
CTR
Q1: 1.4%
Q2: 0.8%
Q3: 95.6%
Q4: 2.2%
0.2 μM
Q1: 16.6%
Q2: 3.3%
Q3: 78.5%
Q4: 1.6%
B
RD
CTR
Q1: 1.0%
Q2: 3.7%
Q3: 91.4%
Q4: 3.9%
0.2 μM
Q1: 2.6%
Q2: 28.8%
Q3: 58.1%
Q4: 10.5%
PI PE-A
ANN V FITC-A


C
RH4
CTR
Q1: 3.9%
Q2: 5.9%
Q3: 89.6%
Q4: 0.6%
0.5 μM
Q1: 4.0%
Q2: 12.0%
Q3: 82.6%
Q4: 1.4%
D
RH30
CTR
Q1: 3.6%
Q2: 6.2%
Q3: 89.1%
Q4: 1.1%
0.5 μM
Q1: 2.9%
Q2: 16.1%
Q3: 77.3%
Q4: 3.7%
PI PE-A
ANN V FITC-A

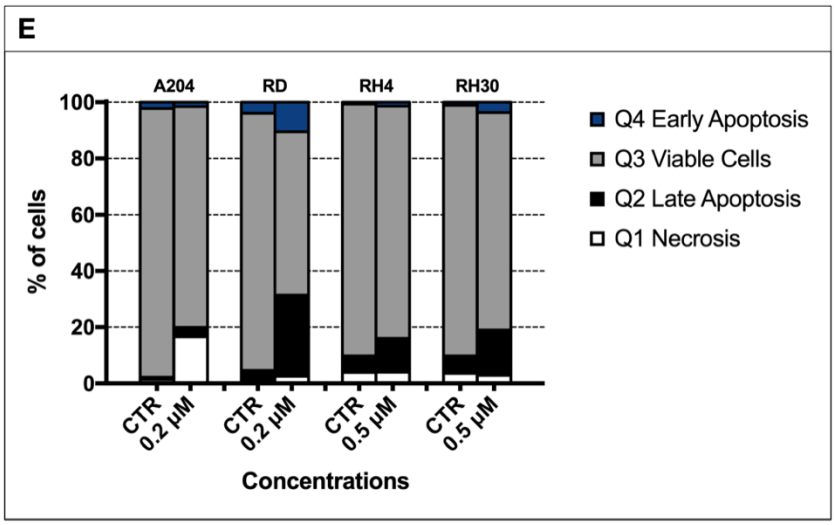


Figure 4: Annexin V analysis of RMS cell lines after treatment with Verteporfin. A204 **(A)** RD **(B)**, RH4 **(C)** and RH30 **(D)** cell lines were treated with different concentrations of VP for 72 hours. Apoptotic, necrotic and alive cells were stained with Annexin V FITC/PI and then analyzed through the BD FACS DIVA software. **(E)** Histograms showed activation of death process on RMS cell lines after VP administration.

Figure 5

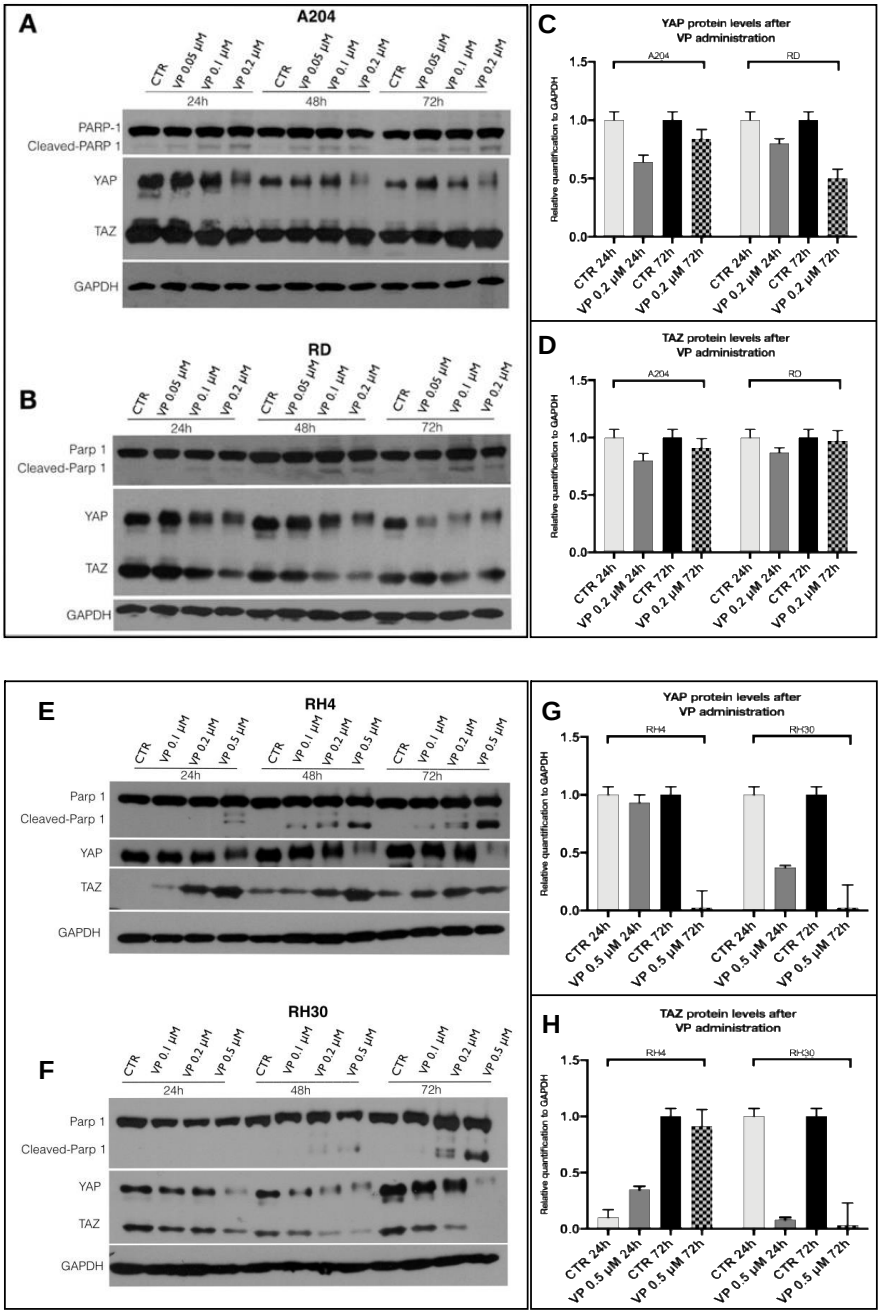


Figure 5: Protein levels of YAP, TAZ and the cleavage of PARP-1 in eRMS and aRMS cell lines after treatment with Verteporfin. YAP protein levels decreased after administration of VP in eRMS (**A/B**) and aRMS **(E/F)** cell lines. TAZ protein levels showed instead a heterogeneous response in all cell lines. Cleavage of PARP-1 indicated the activation of cell death in A204 **(A)**, RD **(B)**, RH4 **(E)** and RH30 **(F)** cell lines. All results have been normalized using GAPDH. YAP and TAZ proteins band intensities during VP administration were analyzed and normalized using GAPDH in eRMS **(C/D)** and aRMS cell lines **(G/H)**.

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Competing interests

All the authors confirm that they have the guidance on competing interests and none of the authors have any competing interests in the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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