Anti-tumour activity of Panobinostat in oesophageal adenocarcinoma and squamous cell carcinoma cell lines

Nair Lopes[1], Sofia Salta[1,2], Bianca Troncarelli Flores[1], Vera Miranda-Gonçalves[1,3], Margareta P. Correia[1,3], Davide Gigliano[4], Rita Guimarães[4], Rui Henrique[1,3,4] and Carmen Jerónimo[1,3]*

Abstract

Background Oesophageal cancer remains a challenging disease with high mortality rates and few therapeutic options. In view of these difficulties, epigenetic drugs have emerged as potential alternatives for patient care. The goal of this study was to evaluate the effect and biological consequences of Panobinostat treatment, an HDAC (histone deacetylase) inhibitor already approved for treatment of patients with multiple myeloma, in oesophageal cell lines of normal and malignant origin, with the latter being representative of the two main histological subtypes: adenocarcinoma and squamous cell carcinoma.

Results Panobinostat treatment inhibited growth and hindered proliferation, colony formation and invasion of oesophageal cancer cells. Considering HDAC tissue expression, HDAC1 was significantly upregulated in normal oesophageal epithelium in comparison with tumour tissue, whereas HDAC3 was overexpressed in oesophageal cancer compared to non-malignant mucosa. No differences between normal and tumour tissue were observed for HDAC2 and HDAC8 expression.

Conclusions Panobinostat exposure effectively impaired malignant features of oesophageal cancer cells. Because HDAC3 was shown to be overexpressed in oesophageal tumour samples, this epigenetic drug may represent an alternative therapeutic option for oesophageal cancer patients.

Keywords Oesophageal cancer, Panobinostat, HDAC

*Correspondence:
Carmen Jerónimo
cljeronimo@icbas.up.pt; carmenjeronimo@ipoporto.min-saude.pt
[1] Cancer Biology and Epigenetics Group, Research Center of IPO Porto (CI-IPOP) – CI-IPOP@RISE (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto)/Porto Comprehensive Cancer Center Raquel Seruca (Porto.CCC), Research Center-LAB 3, F Bdg, 1st Floor, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal
[2] Doctoral Program in Pathology and Molecular Genetics, ICBAS – School of Medicine and Biomedical Sciences – University of Porto (ICBAS-UP), Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
[3] Department of Pathology and Molecular Immunology, ICBAS-School of Medicine and Biomedical Sciences, University of Porto (ICBAS-UP), Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
[4] Department of Pathology, Portuguese Oncology Institute of Porto (IPO Porto)/Porto Comprehensive Cancer Center Raquel Seruca (Porto.CCC), Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal

Background

Oesophageal cancer is a major health burden worldwide, with high mortality rates [1]. This is a heterogenous disease, composed of two main histological subtypes: squamous cell carcinoma (SCC) and adenocarcinoma (AC). Although these tumours represent different biological entities with distinct molecular features, they share poor survival rates [1–3]. This is presumably a consequence of the disease being diagnosed at advanced stages due to difficulties in screening and the lack of specific targeted therapies [4]. Therefore, considering the high mortality rates of oesophageal cancer, research is now focused on new therapeutic options and also biomarkers allowing for early diagnosis.

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In this regard, epigenetic alterations have recently drawn attention, because of their potential use as diagnostic and therapeutic tools. Indeed, the reversible nature of this type of alterations has made them attractive alternatives for clinical patient management. Histone deacetylation is a frequent event in cancer [5] that reduces chromatin accessibility and is catalysed by histone deacetylases (HDAC), which comprise four classes of proteins, grouped according to their homology [6]. Histone deacetylation is involved in multiple biological processes, such as autophagy, apoptosis, cell cycle control, angiogenesis and metastasis, among others, and, thus, HDAC inhibitors became interesting clinical options and have been extensively studied as anti-cancer agents [7, 8].

Panobinostat is one of such HDAC inhibitors, displaying activity at low concentrations (nanomolar range) [9], and it has already been approved in 2015 for the treatment of multiple myeloma [10, 11]. Additionally, this compound has demonstrated anti-tumour effects in pre-clinical studies in various cancer models, alone or in combination with other drugs [12–17] and several clinical trials are ongoing evaluating its efficacy in the medical setting.

Here, we evaluated the effect of Panobinostat treatment and its biological consequences in oesophageal cell lines of normal and malignant origin. Oesophageal cells were treated with Panobinostat, and its functional effects were assessed through in vitro assays. Furthermore, the expression of HDAC class I members (HDAC1, HDAC2, HDAC3 and HDAC8) was evaluated in a series of oesophageal cancer (comprising SCC and AC) and normal epithelium tissue samples.

Material and methods
Cell culture and treatments
Normal oesophageal cell line HET1A was a kind gift by Professor Raquel Almeida (i3S, University of Porto, Portugal), the AC cell line OE-19 was a kind gift of Professor Filomena Botelho (CIMAGO, University of Coimbra, Portugal), and the SCC cell line KYSE-30 was kindly gifted by Professor Fátima Baltazar (ICVS, University of Minho, Portugal). All oesophageal cells were maintained at 37°C and 5% CO_2 at low passages (maximum passage was 20). HET1A was cultured in DMEM culture medium (PAN-Biotech, Aidenbach, Germany), whereas the other cell lines were grown in RPMI culture medium (PAN-Biotech) and both media were supplemented with 10% foetal bovine serum (Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (GRiSP, Porto, Portugal).

Panobinostat (Selleckchem, Houston, TX, USA) was dissolved in PBS, which was used as treatment control. For three consecutive days (72 h) cells have undergone treatment with Panobinostat: the culture medium was discarded daily and fresh medium containing the drug was added. In every assay, HET1A, OE-19 and KYSE-30 were treated with 28 nM, 24 nM and 38 nM of Panobinostat, respectively.

Cell viability assay
Oesophageal cells were seeded (1×10^4) in 96-well plates and on the following day were treated with various Panobinostat concentrations (5–100 nM). Treatments were repeated for 3 consecutive days. Cell viability was assessed using the MTT (3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) (Sigma-Aldrich/Merck, Darmstadt, Germany) assay. Evaluation was performed before the first treatment and 24 h after the last treatment. Cells were incubated with 0.5 mg/mL of MTT dissolved in culture medium and placed at 37°C and 5%CO_2 in the dark for 3 h. The resulting formazan crystals were dissolved using DMSO (dimethyl sulphoxide), and absorbance was measured in a microplate reader (FLU-Ostar® Omega, BMG Labtech, Ortenberg, Germany) at 540 nm.

Western blotting
Protein lysates were extracted from oesophageal cells and separated in a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and blocked with 5% (w/v) non-fat milk in TBS containing 0.5% (v/v) Tween 20 for 1 h. Membranes were incubated with cyclin D1 (1:200 ON, clone DCS-6, Santa Cruz, Dallas, TX, USA), p21 (1:125 ON, clone SX118, Pharmingen/BD Biosciences, Franklin Lakes, NJ, USA) and with β-actin antibodies (clone A1978, Sigma, Germany) 1:10,000 ON. Following washes with TBS-T, they were incubated with horseradish secondary antibodies (Cell Signaling, USA) 1:1000 1 h and after washes with TBS-T, proteins were detected using ECL (Bio-Rad, USA). Images were analysed using the ImageJ software.

Proliferation assay
Oesophageal cell lines were seeded (5×10^3) in 96-well plates and treated daily with Panobinostat for the following 3 days. Assays were performed at the 0 h time point and 24 h after the final treatment using the Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche/Sigma–Aldrich/Merck, Darmstadt, Germany) following manufacturer's guidelines. Absorbance was evaluated using a microplate reader (FLUOstar® Omega, BMG Labtech) at 450 nm with background subtraction at 690 nm; all values were normalised for the first time point (0 h) and the vehicle condition.

Apoptosis assay

Oesophageal cell lines were seeded (2×10^5) in flasks and treated daily with Panobinostat for the following 3 days. The FITC Annexin V Apoptosis Detection Kit with 7-AAD (Biolegend, San Diego, CA, USA) was used according to manufacturer's protocol to identify the apoptotic oesophageal cells. Data were acquired by flow cytometry using a FACS Canto™ II Cell Analyzer (BD Biosciences) and analysed using the FlowJo™ software (BD Biosciences).

Colony formation assay

Oesophageal cells were seeded (5×10^3 cells) in 6-well plates and treated daily with Panobinostat for the following 3 days. Cells were incubated for an additional 10 days post-treatment for OE-19 and 3 days for KYSE-30 to allow colonies to grow. Upon colony formation, cells were washed in PBS (phosphate-buffered saline), followed by fixation with methanol (Supelco/Sigma–Aldrich/Merck, Darmstadt, Germany) for 10 min and another wash with PBS. Subsequently, colonies were stained with Hemacolor solution 2 (Sigma–Aldrich/Merck) for 5 min, washed with PBS, incubated with Hemacolor solution 3 (Sigma–Aldrich / Merck) for 5 min and washed with PBS. Finally, colonies were washed with running water for 2 min and allowed to dry overnight. The survival fraction (SF) was calculated considering the plating efficiency (PE) of the control, using the following equations: PE = number of colonies counted on control ÷ number of cells plated × 100 and SF = number of colonies counted ÷ (cells plated × [PE ÷ 100]).

Cell invasion assay

Oesophageal cell lines were seeded (2×10^5) in flasks and treated with Panobinostat for 72 h. 24 h after the final treatment, cells were harvested and seeded (1×10^5 for OE-19 and 5×10^4 for KYSE-30) in Matrigel® invasion chambers (Corning, Corning, NY, USA) in serum-free medium. After a defined time period (72 h for OE-19 and 24 h for KYSE-30), the chambers were washed with PBS, the cells in the lower part of the chamber were incubated with paraformaldehyde 4% (ChemCruz/Santa Cruz Biotechnology) for 2 min, while the cells in the upper part of the chamber were removed. After washing with PBS, cells were fixed using methanol (Supelco/Sigma-Aldrich/Merck) for 20 min, followed by another wash with PBS. Finally, cell staining was performed with Crystal Violet (Active Motif, Carlsbad, CA, USA) for 10 min and cells were washed with PBS. The whole membrane was photographed under a stereomicroscope (model S2X16,

Olympus) and the invasive cells were counted using the ImageJ software Cell Counter Plugin.

Patients' selection and tissue microarray (TMA) construction

Oesophageal tissue samples (n = 161) were obtained from the archives of the Department of Pathology of IPO Porto, Porto, Portugal. The series comprised 112 cases of oesophageal cancer, including 70 SCC and 42 AC, as well as 49 normal mucosae (derived from the oesophageal margin of gastrectomies performed for gastric cancer treatment). The cases included in this series were collected between 2007 and 2018.

Representative areas in all oesophageal tissue samples were selected by a pathologist using hematoxylin and eosin-stained sections and marked on the corresponding paraffin blocks. Three tissue cores of 2 mm in diameter were extracted from each selected donor block and deposited in a recipient paraffin block, using a TMA workstation (Abcam, Cambridge, UK). Twenty-four TMAs were constructed (10 for SCC, 7 for AC and 7 for normal tissue), each containing 24 samples, arranged in a 6 × 4 sector. Each TMA included 3 cores used for orientation purposes only: in the cancer TMAs, liver and normal oesophagus were used, whereas liver and colon mucosa were used in the normal tissue TMAs. In order to homogenise the paraffin of the recipient blocks and the paraffins of the cores extracted from the donor blocks, the TMAs were kept overnight at 37ºC. They were subsequently placed for 1 h at room temperature, followed by two cycles of 1 h at 37ºC plus 1 h at room temperature.

This study was approved by the Institutional Review Board (Comissão de Ética para a Saúde) of IPO Porto (CES 202/017). All the procedures involving the use of human samples were performed in accordance with the national ethical standards and following the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Immunohistochemistry

Immunohistochemistry was performed on 3-µm-thick sections, using the Novolink Max Polymer Detection System (Leica Biosystems, Newcastle, UK). Briefly, after deparaffinisation, epitope retrieval was performed in the microwave for 20 min, using a 10 mM citrate buffer solution (pH = 6) (Vector Laboratories, Newark, CA, USA) for HDAC1 and HDAC2 and a 1 mM EDTA buffer solution (pH = 8) (Sigma-Aldrich/Merck) for HDAC3 and HDAC8. Endogenous peroxidase activity was blocked in a 0.6% H_2O_2 solution for 20 min, and non-specific binding was hindered in a 1:50 solution of horse serum in antibody diluent for 20 min. Primary antibody incubation was performed as follows: HDAC1 (1:1000, clone 5C11,

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Sigma-Aldrich/Merck), HDAC2 (1:50, clone C-8, Santa Cruz Biotechnology, Dallas, TX, USA), HDAC3 (1:1000, clone Y415, Abcam) and HDAC8 (1:200, clone 48, Sigma-Aldrich/Merck) all for 1 h at room temperature. Afterwards, tissue slides were incubated with post-primary block for 30 min, followed by the polymer solution for 30 min and stained with 3,3′-diaminobenzidine (Sigma-Aldrich). Finally, slides were counterstained with haematoxylin, dehydrated and cover-slipped using Entellan (Sigma-Aldrich/Merck). Gastric mucosa, prostate (normal and malignant), endometrial carcinoma and colon carcinoma were used as positive controls for HDAC1, HDAC2, HDAC3, and HDAC8, respectively.

Immunohistochemical staining was evaluated considering the percentage of positive cells (extension) and intensity of staining. The score for extension was defined from 0 to 10 (10 corresponding to 100% of positive cells), and intensity was set from 1 to 3 (corresponding to weak, moderate and strong expression, respectively). The final score was obtained by multiplying the extension and intensity scores.

Statistical analysis

GraphPad Prism software version 7 was used to perform statistical analyses. Differences between conditions were assessed using Mann–Whitney or Kruskal–Wallis nonparametric tests, with Dunn's correction. In all analyses, statistical significance was considered when p values were lower than 0.05: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. All results are presented as mean ± standard deviation for each group and are representative of at least three independent experiments.

Results

Panobinostat inhibits oesophageal cell viability

The cytotoxic effect of Panobinostat was evaluated in oesophageal cell lines HET1A, OE-19 and KYSE-30 after 3 days of treatment, and the corresponding EC_{50} (half maximal effective concentration) values were calculated. The EC_{50} values for Panobinostat in HET1A, OE-19 and KYSE-30 were 28.3 nM, 24.4 nM and 37.9 nM, respectively (Fig. 1). The normal oesophageal cell line HET1A and the AC cell line OE-19 displayed comparable EC_{50} values, being more sensitive than the SCC cells (KYSE-30) to the treatment with Panobinostat.

Panobinostat hinders cell proliferation and induces apoptosis in oesophageal cell lines

In vitro assays were performed to evaluate the functional effects of Panobinostat treatment. All oesophageal cell lines exposed to Panobinostat exhibited lower
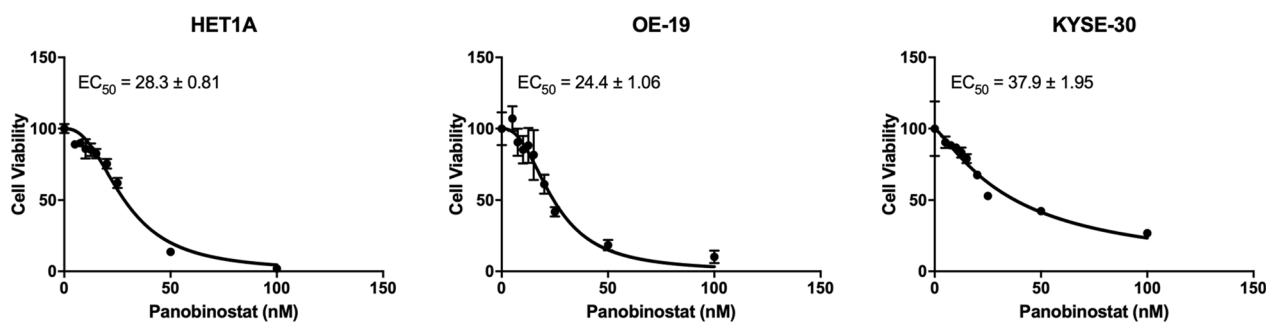

Fig. 1 Dose–response curves of oesophageal cell lines upon Panobinostat treatment. EC_{50} values after 3 days of treatment are indicated in each graph
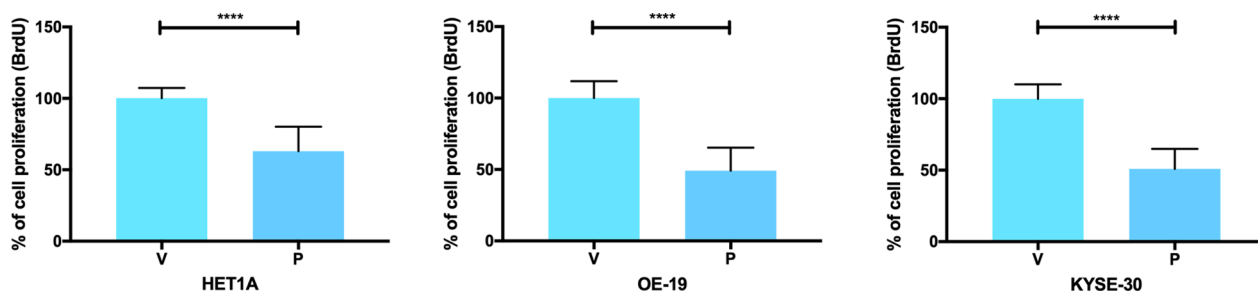

Fig. 2 Effect of Panobinostat treatment in the proliferation rates of oesophageal cell lines. Values were normalised to the vehicle condition. (V—vehicle, P—Panobinostat; drug concentration: HET1A—28 nM, OE-19—24 nM and KYSE-30—38 nM)
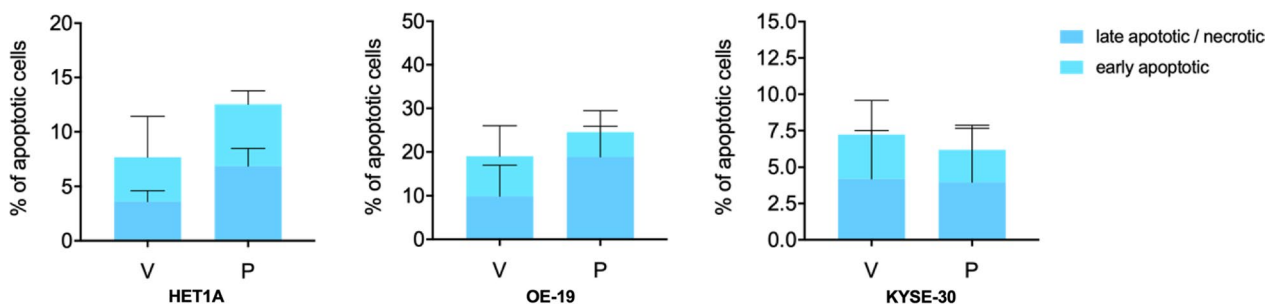
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Fig. 3 Effect of Panobinostat treatment in the apoptosis of oesophageal cell lines. (V—vehicle, P—Panobinostat; drug concentration: HET1A—28 nM, OE-19—24 nM and KYSE-30—38 nM)
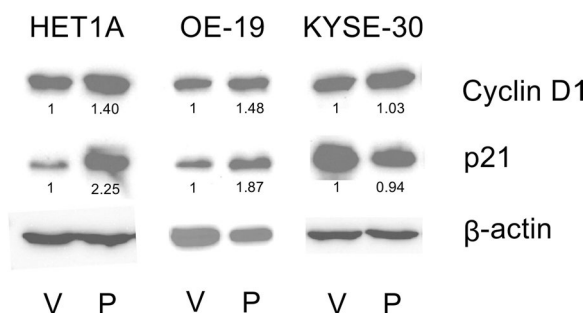

Fig. 4 – Effect of Panobinostat treatment in the expression of p21 and Cyclin D1 in oesophageal cell lines. Images were analysed using the ImageJ software. (V—vehicle, P—Panobinostat; drug concentration: HET1A—28 nM, OE-19—24 nM and KYSE-30—38 nM)

cells. In SCC, it was demonstrated that cell cycle arrest was a consequence of p21 expression increase as well as the reduction in cyclin D1 expression (18). Similarly, upon Panobinostat treatment, HET1A and OE-19 exhibited p21 expression upregulation, whereas a marginal decrease was observed in KYSE-30 cells (Fig. 4 and Additional file 1: Fig. S1). Accordingly, Cyclin D1 expression levels were also increased following drug administration in all oesophageal cell lines. Furthermore, the effect of Panobinostat treatment on class I HDAC members protein levels was evaluated (Additional file 2: Fig. S2, Additional file 3: Fig. S3, Additional file 4: Fig. S4, Additional file 5: Fig. S5). Collectively, the expression levels of HDAC1, 2, 3 and 8 were decreased following drug treatment and this effect was stronger in oesophageal cancer cell lines (OE-19 and KYSE-30) than in the normal oesophageal cell line HET1A.

Panobinostat reduces colony formation and inhibits cell invasion in oesophageal cancer cells

The clonogenic and invasion assays were only performed in OE-19 and KYSE-30 cell lines, since they assess features that are typical of tumour cells.
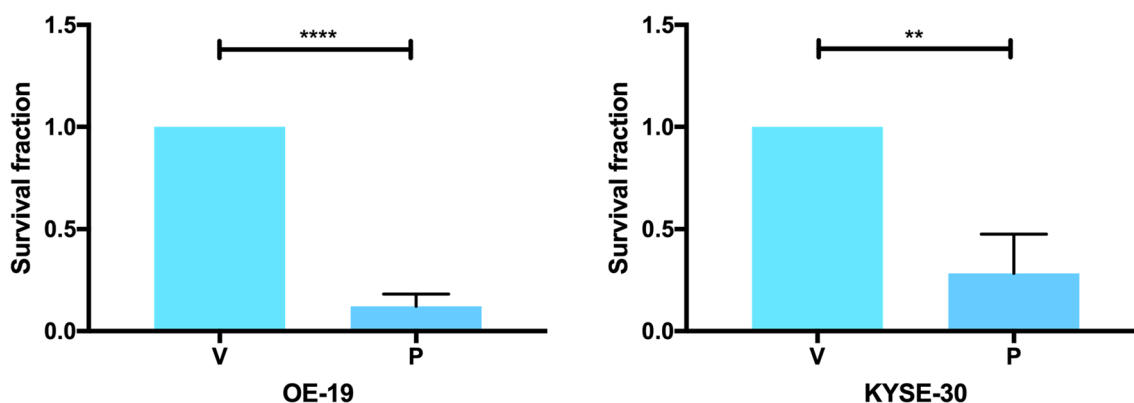
proliferation rates than vehicle-treated cells and differences were statistically significant (Fig. 2).

Treatment with Panobinostat induced an increase in the percentage of apoptotic cells in HET1A and OE-19 oesophageal cells (Fig. 3). In contrast, KYSE-30 cells only displayed a small decline in the apoptotic response after drug exposure when compared to vehicle-treated

Fig. 5 Effect of Panobinostat treatment in the colony formation ability of oesophageal cancer cells. Values were normalised to the vehicle condition. (V—vehicle, P—Panobinostat; drug concentration: HET1A—28 nM, OE-19—24 nM and KYSE-30—38 nM)

Regarding colony formation, the treatment with Panobinostat significantly decreased the number of colonies in both oesophageal cancer cell lines when compared to control cells (Fig. 5).

OE-19 and KYSE-30 treated with Panobinostat exhibited a statistically significant lower percentage of invasive cells than vehicle-treated cells (Fig. 6).

Expression of HDAC class I members in normal and cancerous oesophageal tissue samples

The expression of HDAC class I members (HDAC1, HDAC2, HDAC3 and HDAC8) was evaluated by immunohistochemistry in oesophageal samples arranged in TMAs comprising both normal mucosa and cancer tissue. All proteins were expressed in the cell nucleus (Fig. 7). In normal tissue, expression was observed predominantly in the basal and parabasal cells with decreasing levels of expression towards the surface of the epithelium.

HDAC1 expression levels were significantly lower in oesophageal cancer compared with normal tissue (Fig. 8). Considering HDAC2, the expression levels were similar in normal and tumour tissue. HDAC3 was upregulated in oesophageal carcinomas in comparison with non-malignant mucosa and this difference was statistically significant ($p < 0.01$). The same trend was observed for HDAC8 expression ($p = 0.58$).

Concerning histological subtypes, differences were disclosed between normal oesophagus and SCC, as well as between AC and SCC for HDAC1 expression (Fig. 9). Regarding HDAC2, AC samples presented significantly higher levels of expression than SCC. HDAC3 was upregulated in AC comparatively to normal tissue and SCC. Considering HDAC8, AC samples exhibited significantly higher expression levels than SCC.

Discussion

Oesophageal cancer is a challenging disease, with poor survival rates and lacking effective targeted therapies [1, 4]. With the only treatment modalities being surgical resection and radiochemotherapy [19, 20], alternative options are urgently needed and have been the focus of intensive research.

Epigenetic alterations, due to their plastic nature, are good candidates for therapeutic targets. Here, we evaluated the functional effects of Panobinostat, a pan-HDAC inhibitor already approved for the treatment of multiple myeloma [11], in oesophageal cell lines. Additionally, the expression of HDAC class I members (HDAC1, HDAC2, HDAC3 and HDAC8) was assessed in oesophageal SCC, AC and non-malignant mucosa to evaluate their potential therapeutic actionability.

Using oesophageal cell lines of both tumour and non-malignant origin, phenotypic assays have been performed to evaluate the effect of Panobinostat treatment in various cell features. After drug exposure, a reduction in cell viability was detected in all the tested oesophageal cell lines. Although they display comparable EC_{50} values, OE-19 tumour cells were slightly more sensitive to Panobinostat treatment than the normal HET1A cells, an effect that has been observed in prostate cells in a previous study by our group [21]. We believe that some degree of toxicity upon Panobinostat treatment of normal oesophageal cells would be expected, since these cells also present epigenetic machinery, namely HDAC proteins, and are therefore susceptible of being affected by this drug. This effect may be reflected in decreased cell viability and proliferation, as well as in apoptosis induction. Our data also confirmed other reports which described an inhibitory effect on cell viability upon drug exposure [21–28]. In agreement with this, Panobinostat significantly suppressed proliferation of non-malignant
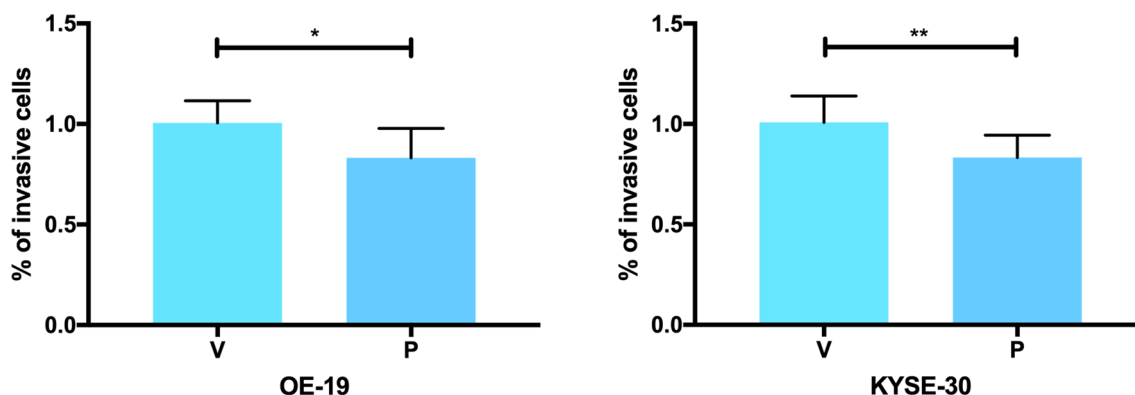

Fig. 6 Effect of Panobinostat treatment in the invasive capacity of oesophageal cancer cells. Values were normalised to the vehicle condition. (V—vehicle, P—Panobinostat; drug concentration: HET1A—28 nM, OE-19—24 nM and KYSE-30—38 nM)
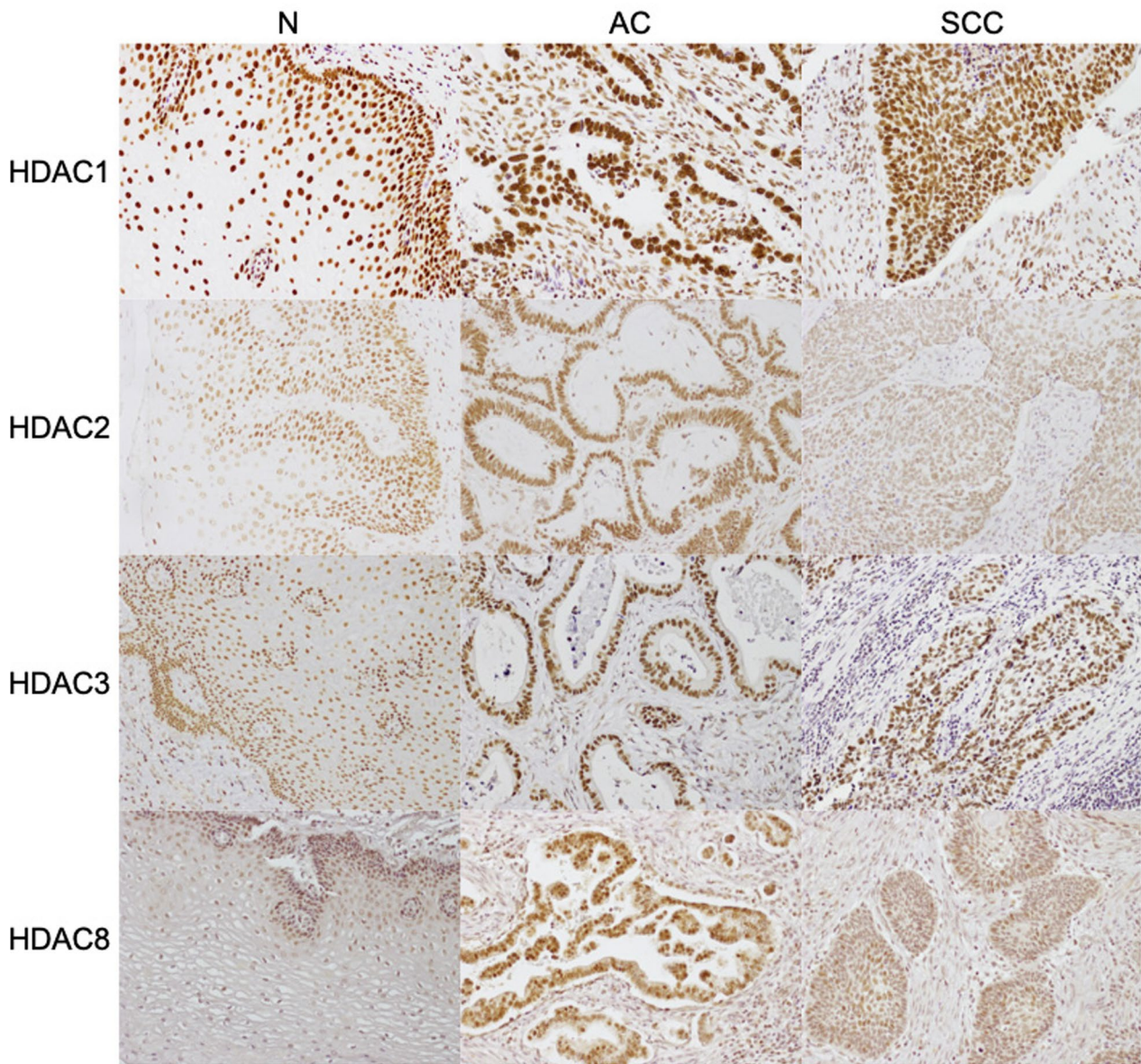
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Fig. 7 Immunohistochemical staining of HDAC1, HDAC2, HDAC3 and HDAC8 in the different types of oesophageal tissue. (N—normal, AC—adenocarcinoma, SCC—squamous cell carcinoma, magnification: 200x)
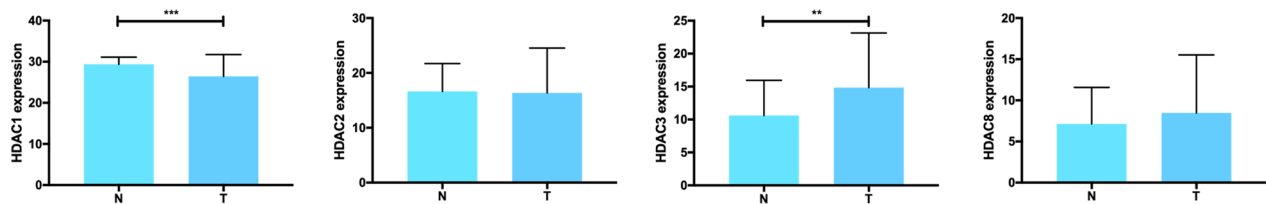

Fig. 8 HDAC1, HDAC2, HDAC3 and HDAC8 expression levels in normal oesophagus and tumour tissue. (N—normal, T—tumour)

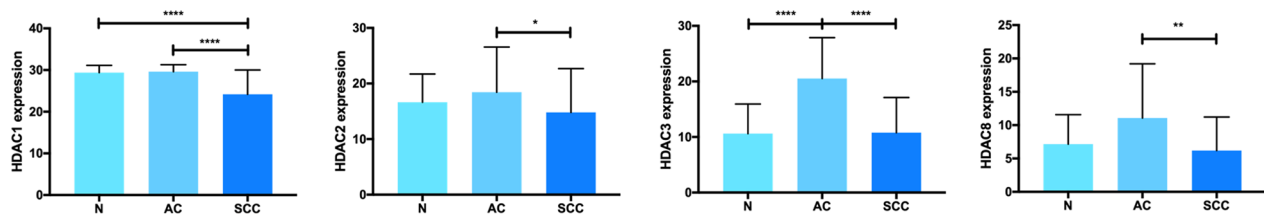Lopes *et al. Clinical Epigenetics*   (2024) 16:102

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Fig. 9 HDAC1, HDAC2, HDAC3 and HDAC8 expression levels in normal oesophageal tissue, AC and SCC. (AC—adenocarcinoma, SCC—squamous cell carcinoma)

and tumour oesophageal cells, further validating the growth inhibitory effects of this drug. Importantly, similar results on cell proliferation have been observed and reported in other cancer types, such as prostate and testicular tumours, as well as melanomas [21, 29, 30].

Interestingly, the exposure of oesophageal cell lines to Panobinostat produced discrepant results in apoptosis, with a dual outcome: it promoted cell death in HET1A and OE-19, whereas the opposite effect was observed in KYSE-30 cells. This result is probably a consequence of the different sensitivity to this drug exhibited by the cell lines, which is reflected in their EC_{50} values. Numerous studies have reported apoptosis induction after Panobinostat treatment [22–26, 28, 31–34], thus lending support to the results we have obtained in HET1A and OE-19 cell lines. Furthermore, in SCC, cell cycle arrest was accomplished by simultaneous upregulation of p21 and cyclin D1 expression decline [18]. A similar result was observed in thyroid cancer [28]. Here, we demonstrated an increment in p21 expression following Panobinostat treatment in HET1A (normal) and OE-19 (AC) cells, making ours the first study to evaluate the molecular mechanisms of action of Panobinostat in these types of oesophageal samples. Also in SCC cells, MS-275 (entinostat), a specific inhibitor of class I HDACs [35], induced apoptosis with a concomitant decrease of cyclin D1 and cyclin A expression (36). Upon Panobinostat exposure, KYSE-30 cells (SCC) showed a reduction in the percentage of apoptotic cells, which is in line with the slight increase in Cyclin D1 expression levels observed, as well as with the minimal p21 expression decrease. The other tested cell lines also exhibited Cyclin D1 expression upregulation, although to a lesser extent compared to the pronounced increase observed for p21 expression.

Upon Panobinostat treatment, there was also a significant decline in the invasive capacity of oesophageal cancer cell lines. Panobinostat has been previously shown by our group to decrease the invasive capacity of prostate cancer cells [21] and similar results were obtained in other tumour models, such as breast, thyroid and liver cancer [22, 32, 37–39]. Concomitantly, Panobinostat exposure or HDAC-silencing has reduced migration of malignant cells, namely in gastric, thyroid, breast and

bladder cancer [33, 37–40]. Moreover, a reduction of the number of colonies of oesophageal cancer cell lines upon drug exposure was observed, confirming previous results in SCC cells [18] and, to the best of our knowledge, describing the same effect in AC cells for the first time. Furthermore, the effect of Panobinostat treatment in the downregulation of the clonogenic potential of cancer cells is well described in prostate, gastric, renal, colorectal, glioblastoma, ovary and thyroid tumours [21, 23, 24, 26–28, 33, 34]. Thus, Panobinostat demonstrated an effective ability to counteract these important malignant traits, foreseeing relevant anti-tumour activity in vivo.

Finally, the expression of HDAC class I members was assessed in normal and malignant (both SCC and AC) oesophageal tissue. Lower HDAC1 expression levels have been detected in oesophageal cancer in comparison with non-malignant tissue. Published data concerning HDAC1 expression in oesophageal cancer, however, are conflicting. Unlike our findings, it has been reported that HDAC1 is more highly expressed in oesophageal tumours than normal mucosa [36, 41, 42], but only SCC samples have been evaluated, whereas our data include both AC and SCC histological subtypes. Nonetheless, among our samples (normal, AC and SCC), the lowest HDAC1 levels were detected in SCC. Another study compared the expression in SCC and the corresponding normal epithelium and found a marginal increase in HDAC1 expression in the non-malignant mucosa in comparison with the tumour tissue (100% *versus* 95% of cases) [43]. However, only 20 samples were evaluated and the tumour-adjacent epithelium was considered as normal tissue, whereas we have used tissue from the oesophageal margin of gastrectomy specimens as normal mucosa, thus excluding potential carcinogenic field effects. A similar amount of HDAC1 expression in SCC and the corresponding non-malignant mucosa has also been reported [44], whereas, in AC, one report found that the majority of tumour cases presented low or negative expression (45).

Concerning HDAC2, no differences were detected between normal and tumour tissue, although AC samples presented higher levels of expression than SCC. In contrast to our data, HDAC2 expression was shown to be

upregulated in SCC compared with non-cancerous tissue [36, 43], whereas in AC 68% of cases exhibited moderate or high expression (45).

As for HDAC3 expression, significant differences were observed, with non-malignant oesophageal mucosa displaying significantly lower levels than tumour tissue. Among tumour subtypes, HDAC3 expression was higher in AC than in SCC. HDAC3 has been described as more frequently expressed in SCC than in normal tissue (95% *versus* 80% of cases) [43], but, in that study, blocks that contained both normal and tumour tissue were used. Another study also reported higher HDAC3 expression in SCC in comparison with adjacent epithelium along with lower patients' overall survival [46]. Ahrens and colleagues [47] observed lower HDAC1, HDAC2 and HDAC3 expression in 10–20% of SCC and AC compared with normal mucosa, but they have only assessed 10 samples of each histological subtype and the respective case-matched oesophageal non-malignant epithelium. HDAC3 deregulation has been implicated in various types of diseases, with its inhibitors being suggested as therapeutic options [48, 49]. In SCC, HDAC3 silencing promoted apoptosis and inhibited proliferation, migration and invasion [46].

Considering HDAC8, malignant tissue displayed higher expression levels than normal oesophagus, but this difference failed to reach statistical significance. AC samples exhibited higher expression levels than normal oesophageal tissue and SCC. Only one study reported on HDAC8 expression, showing that it was more frequently expressed in SCC than in non-malignant mucosa (43), but, to the best of our knowledge, no report has been published concerning oesophageal AC. As far as we know, this is the first study reporting on the expression of all class I HDACs in a comprehensive series of oesophageal samples comprising SCC, AC and normal epithelium. Variations in results of immunohistochemical analysis may derive from differences in populations under study, technical protocols (e.g. antibody clones) and origin of the control (normal) tissue. Nonetheless, it should be emphasised that most previously published studies used small tissue series, which may also impact the results as it might not capture the full range of expression across oesophageal tumours and even normal mucosa.

Conclusions

Altogether, our results demonstrate that Panobinostat treatment exerts anti-tumour effects in oesophageal cell lines, endorsing HDACs as potential therapeutic targets in oesophageal cancer. To confirm this, further studies will be required to identify which members of the HDAC family are responsible for the observed outcomes,

thus enabling the use of more specific targeted therapies. Nonetheless, immunoexpression analysis suggests HDAC3 as the most likely candidate for therapeutic actionability, considering its overexpression in cancerous tissues. Further validation in an in vivo oesophageal cancer context is now required to further test the efficacy of Panobinostat and eventually consider testing it in a clinical trial.

Abbreviations

AC	Adenocarcinoma
DMSO	Dimethyl sulphoxide
EC$_{50}$	Half maximal effective concentration
HDAC	Histone deacetylase
MTT	3-(4, 5Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide
ON	Overnight
PBS	Phosphate-buffered saline
PE	Plating efficiency
SCC	Squamous cell carcinoma
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SF	Survival fraction
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-tween
TMA	Tissue microarray

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-024-01700-3.

Additional file 1: Fig. S1. Effect of Panobinostat treatment on the expression of Cyclin D1 and p21 in oesophageal cell lines HET1A, OE-19 and KYSE-30 (V – vehicle, P – Panobinostat).

Additional file 2: Fig. S2. Effect of Panobinostat treatment on the expression of HDAC1 in oesophageal cell lines HET1A, OE-19 and KYSE-30 (V—vehicle, P—Panobinostat).

Additional file 3: Fig. S3. Effect of Panobinostat treatment on the expression of HDAC2 in oesophageal cell lines HET1A, OE-19 and KYSE-30 (V—vehicle, P—Panobinostat).

Additional file 4: Fig. S4. Effect of Panobinostat treatment on the expression of HDAC3 in oesophageal cell lines HET1A, OE-19 and KYSE-30 (V—vehicle, P—Panobinostat).

Additional file 5: Fig. S5. Effect of Panobinostat treatment on the expression of HDAC8 in oesophageal cell lines HET1A, OE-19 and KYSE-30 (V—vehicle, P—Panobinostat).

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Availability of data and materials
All data generated or analysed during this study are included in this article.

Declarations

Ethics approval and consent to participate
This study has been approved by the Institutional Review Board (Comissão de Ética para a Saúde) of IPO Porto (CES 202/017). All the procedures involving the use of human samples were in accordance with the national ethical standards and following the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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