SPANDIDOS PUBLICATIONS

Effects of two organotin(IV)(sulfonatophenyl)porphinates on MAPKs and on the growth of A375 human melanoma cells

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Abstract. Previously we showed apoptotic induction in A375 human melanoma cells using two complexes of the meso-tetra(4-sulfonatophenyl)porphinate (TPPS), $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$TPPS. To understand how these compounds activate apoptosis in melanoma cells we studied MAPKs and the $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$TPPS cellular uptake. Western blotting experiments showed activated protein kinases ERK 1/2, JNK and p38 in 10 μM $(Bu_2Sn)_2$TPPS- and 1 μM $(Bu_3Sn)_4$TPPS-treated melanoma cells, which suggests that the three MAP kinases are involved in the apoptotic death of A375-treated cells. By taking advantage of the porphyrin fluorescence, we found a fast concentration of $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$TPPS in the nucleus and in the nucleoli compared to TPPS. A significantly reduced growth of A375 human melanoma cells was also observed after only 48 h treatment by using 500 nM of $(Bu_2Sn)_2$TPPS or 80 nM of $(Bu_3Sn)_4$TPPS. A strong slowdown of cell growth and loss of cell-cell interactions were visible by *in vitro* wound repair assay.

Introduction

Malignant melanoma is the most aggressive and lethal type of cutaneous cancer. The incidence of melanoma is rising steadily in western populations, with the number of cases having doubled in the past 20 years. If melanoma is diagnosed early it can be cured by surgical resection, but once it has progressed to the metastatic stage it is largely refractory to existing therapies and has a very poor prognosis (1,2). Melanoma cells have low levels of spontaneous apoptosis *in vivo* compared with other tumor cell types, and are relatively resistant to drug-induced apoptosis *in vitro*. Most chemotherapeutic drugs function by inducing apoptosis in malignant cells. Thus, resistance to apoptosis is likely to underlie drug resistance in melanoma, and this notable resistance to chemotherapy, radiotherapy and immunotherapy is a significant barrier to successful treatment of melanoma (1). Therefore, studies in the field of melanoma are required to identify new molecular targets for effective anti-cancer agents suitable for overcoming melanoma drug resistance. Further knowledge of the intrinsic survival features acquired by the melanoma cells, promoting proliferation, invasion and blocking of the apoptotic cell death pathways, is being exploited to provide targeted drugs and new therapeutic approaches.

In this context, we regarded a class of porphyrin derivatives as potential anti-tumor drugs for their cytotoxicity and for the property of the porphyrins themselves to be accumulated in large amounts and retained for prolonged periods of time by the malignant lesions as noteworthy (3-5). We showed that two complexes of the meso-tetra(4-sulfonatophenyl) porphinate (TPPS), $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$TPPS, induce the activation of extrinsic and intrinsic apoptotic pathways in melanoma cells (6). Based on these results, we aimed to identify the molecular targets of the compounds involved in apoptotic death of melanoma cells. In particular, we investigated the MAPK family and FAK.

MAPKs play key roles in oncogenic and tumor-suppressing activities. Prior to the discovery of the JNK and p38 kinases, the MAPK family included the extracellular signal-regulated kinases ERK1 and ERK2. ERKs were known to become active in response to mitogenic signals and proliferative cytokines (7), while initially JNKs and later p38s were considered to be stress-activated protein kinases (SAPKs) (8,9). It was considered that ERKs, p38s and JNKs have distinct physiological properties, and it was generally accepted that ERKs are pro-oncogenic, while p38s and JNKs inhibit proliferation. Investigations have challenged these ideas, as several MAP kinases may phosphorylate the same substrates and affect each other via cross-talk reactions and feedback mechanisms

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(10). In human melanoma it has been demonstrated that ERK is constitutively active (11). ERK activity is important for imposing the cancerous phenotype characterized by cell growth, invasion and metastasis. ERK overexpression or constitutive activation of this pathway has been shown to play an important role in the pathogenesis and progression of breast and other cancer types, making the components of this signaling cascade potentially important as therapeutic targets (12).

FAK, a cytoplasmic protein kinase that co-localizes with integrins at structures called focal adhesions, heavily enriched in structural and signaling molecules (13), promotes cell survival and migration when it is activated (14,15). FAK is expressed at higher levels in invasive tumors than in benign pre-neoplastic tumors (16), and in human malignant melanoma it is constitutively active and essential for maintaining adhesiveness in melanoma cells (17).

Concomitant to these studies, we identified concentrations of $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$TPPS which were less toxic but sufficient to affect A375 melanoma cell growth.

Invasion and metastasis of tumors are highly complex and multistep processes. Tumor cells modulate their ability to adhere, degrade the surrounding extracellular matrix (ECM), migrate and proliferate (18). Cell movement is controlled by various factors, including growth and motility factors, cytokines, matrix proteins and cell-adhesion molecules (19). Knowledge of this process has increased in recent times and the identification of an even larger number of the involved molecules is necessary to provide additional targets for therapies.

In this study, we show that the three MAPKs, ERK 1/2, JNK and p38, are involved in A375 melanoma cell apoptosis induced by 10 μM $(Bu_2Sn)_2$TPPS or 1 μM $(Bu_3Sn)_4$TPPS, and that FAK does not activate this pathway. We show a rapid cell uptake of the two compounds, including the nucleus and nucleoli, as well as a considerable slowdown of cell growth by using lower concentrations of $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$ TPPS at 500 and 80 nM, respectively.

Materials and methods

Cell cultures. The A375 human melanoma cell line (ATCC-CRL-1619) (20) was provided by Professor Catherine Alcaide-Loridan and Dr Reem Al-Daccak. The cells were cultured in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin-streptomycin in 5% CO_2 at 37˚C.

Compounds and antibodies. $(Bu_2Sn)_2$TPPS, and $(Bu_3Sn)_4$TPPS were prepared according to previously reported procedures (3) and dissolved as previously reported (6).

Antibodies that recognize the phosphorylated form of ERK 1/2, JNK and p38 were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-FAK (clone 77) and anti-pFAK (pTyr-397 motif clone 14) monoclonal antibodies were purchased from BD Transduction Laboratories (Lexington, KY). Anti-ß-actin is a mouse monoclonal antibody purchased from Sigma (St. Louis, MO). HRP-conjugated anti-rabbit and anti-mouse antibodies were purchased from GE Healthcare, Biosciences (Buckinghamshire, UK).

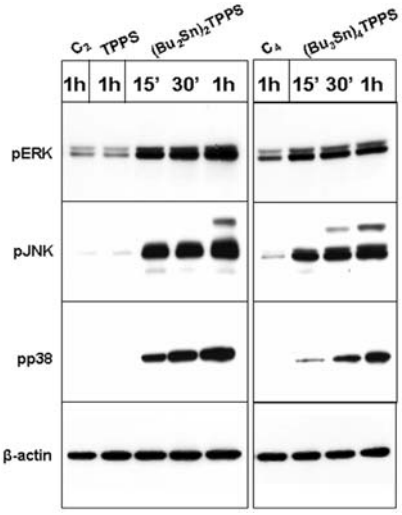

Figure 1. Effects of $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$TPPS on MAPK phosphorylation in A375 melanoma cells. Cells were grown in 100-mm culture dishes ($3x10^6$ cells/dish) for 24 h and then treated with 10 μM $(Bu_2Sn)_2$ TPPS or 1 μM $(Bu_3Sn)_4$TPPS for 15 min, 30 min and 1 h. Equal amounts of protein (50 μg) from each sample were subjected to 10% SDS-PAGE and analyzed by immunoblotting using anti-phospho-ERK 1/2, -JNK and -p38 at the dilution described in Materials and methods. C_2, untreated cells as the control for $(Bu_2Sn)_2$TPPS and C_4, 0.1% DMSO-treated cells as the control for $(Bu_3Sn)_4$TPPS. The reaction with ß-actin antibody confirms the equal protein loading. The results are representative of three independent experiments.

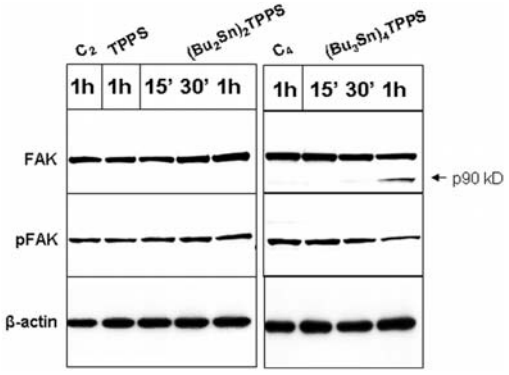

Figure 2. FAK in $(Bu_2Sn)_2$TPPS- or $(Bu_3Sn)_4$TPPS-treated A375 cells. Cells were grown and treated as described in Fig. 1. Protein (50 μg) per lane was separated by 10% SDS-PAGE and analyzed by immunoblotting using anti-FAK and anti-phospho-FAK at the dilution described in Materials and methods. C_2 and C_4 are the controls of $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$TPPS, respectively, as described in Fig. 1. Equal protein loading was confirmed by reaction with ß-actin antibody. The results are representative of three independent experiments.

Western blotting. Cell lysates, protein determination, and Western blotting were performed as previously described (6). The membranes were incubated overnight with anti-phospho-ERK 1/2, -JNK and -p38 (1:1000), and anti-FAK and anti-phospho-FAK (1:500). After several washes, the membranes were incubated with either HRP-conjugated anti-rabbit or anti-mouse antibodies (diluted at 1:5000) for 1 h. Bound antibodies were detected using an enhanced chemiluminescence kit (SuperSignal West Pico Chemiluminescent Substrate, Pierce).

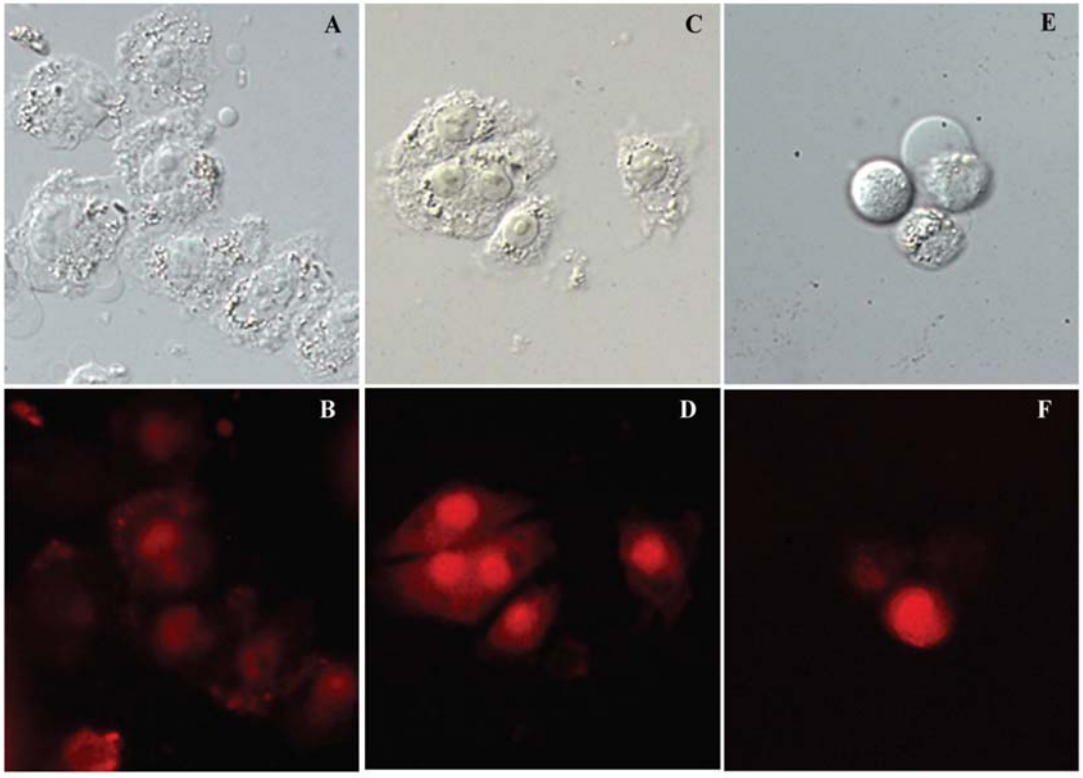

Figure 3. Localization of TPPS, $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$TPPS in A375 cells. Normaski (A, C and E) and fluorescence (B, D and F) images of living A375 cells treated with TPPS (A and B), $(Bu_3Sn)_4$TPPS (C and D) and $(Bu_2Sn)_2$TPPS (E and F). Cells were grown, treated and processed as described in Materials and methods.

Fluorescence microscope digital imaging. Cells ($4x10^4$ cells/well in 400 μl growth medium) were plated on an 8-well Lab-Tek II chamber slide, cultured for 24 h and then treated with 80 μg/ml of TPPS, or $(Bu_2Sn)_2$TPPS, or $(Bu_3Sn)_4$TPPS for 1 h. We removed the medium from every chamber and washed twice with 400 μl of medium without serum and twice more with 200 μl. Then we removed the medium leaving 50 μl and put coverslips on the slides. Fluorescence images (excitation, 550 nm and emission, 580 nm) were obtained using a Zeiss Axioskop 2 Plus microscope, equipped for epi-fluorescence and recorded by a digital camera system.

Cell growth assay. A375 cells ($1.5x10^5$ cells/well) were grown in 12-well plates for 24 h and then treated with 500 nM $(Bu_2Sn)_2$TPPS or with 80 nM $(Bu_3Sn)_4$TPPS in complete medium for 24, 48, or 72 h. The treatment was renewed daily. At the end of drug challenge, cells were recovered and counted using trypan blue exclusion test. We used trypsin 0.05% and EDTA 0.02% in PBS to harvest cells.

In vitro wound repair assay. Cells ($3x10^6$ cells/dish) were grown in 100-mm culture dishes for 24 h. Cell dishes were scraped with a sterile micropipette tip, and the culture medium was replaced with fresh medium to which 500 nM $(Bu_2Sn)_2$ TPPS or 80 nM $(Bu_3Sn)_4$TPPS was added. The treatment and culture medium were renewed daily. Photographs were taken at 0, 24, 48, and 72 h after treatment. Pictures were taken by inverted microscope with X4 objective.

Results

Phosphorylation of ERK 1/2, JNK and p38 MAPKs in response to $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$TPPS does not require FAK involvement. In order to determine whether MAPKs are involved in A375 human melanoma cell apoptosis induced by 10 μM $(Bu_2Sn)_2$TPPS or 1 μM $(Bu_3Sn)_4$TPPS (6), we examined the three members of the MAPK family, ERK1/2, JNK and p38, by using Western blotting with antibodies that recognize the activated phosphorylated forms of the three kinases. We analyzed initial treatment and found the activation of the three kinases to be at 15 min for the two compounds (Fig. 1). FAK was analyzed concomitantly. We did not find any difference in FAK activity between the $(Bu_2Sn)_2$-TPPS-treated cells and the untreated cells by Western blotting. We found FAK dephosphorylation and cleavage (p90 kD) in $(Bu_3Sn)_4$TPPS-treated A375 cells, but only after 1 h of treatment (Fig. 2) when the caspases had already been activated (6). Accordingly, FAK is not involved in activation of the apoptotic pathway, but is one of the final targets of the pathway (21).

Cell localization of $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$TPPS. The cell localization of $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$TPPS in A375 human melanoma cells was characterized by using a conventional fluorescence microscope (Fig. 3). By exploiting porphyrin fluorescence, living cells were observed after 1 h of incubation with 80 μg/ml of TPPS, or $(Bu_2Sn)_2$TPPS, or

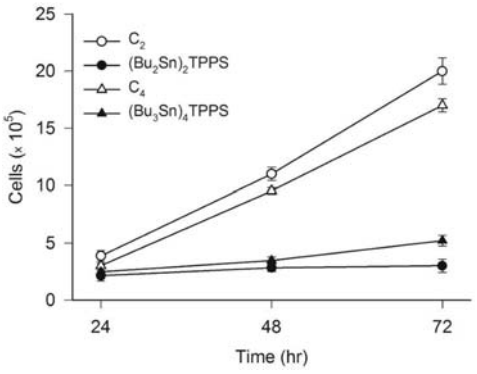

Figure 4. Effects of $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$TPPS on A375 melanoma cell growth. Cells, grown in 12-well plates 24 h before, were challenged with 500 nM $(Bu_2Sn)_2$TPPS or with 80 nM $(Bu_3Sn)_4$TPPS for 24, 48, or 72 h. The incubation medium containing the drug was changed daily. C_2, $(Bu_2Sn)_2$TPPS control for untreated cells and C_4, $(Bu_3Sn)_4$TPPS control for cells treated with 0.1% DMSO. The cell number was evaluated by trypan blue exclusion test at the end of treatment. Error bars represent the standard deviation of three separate experiments.

$(Bu_3Sn)_4$TPPS. As shown in Fig. 3, in $(Bu_3Sn)_4$TPPS-treated A375 cells a minor concentration of the compound remains in the cytoplasm while the majority of the concentration enters the nucleus and concentrates in the nucleoli compared with the TPPS-treated cells. In $(Bu_2Sn)_2$TPPS-treated cells this distribution is poorly evaluated since cells get detached and round immediately after treatment.

Cell growth kinetics. Fig. 4 shows the effects of $(Bu_2Sn)_2$ TPPS or $(Bu_3Sn)_4$TPPS on the proliferation of A375 cells, calculated by variation of the number of cells recovered from multiwell plates after 24, 48 and 72 h treatment. We used lower concentrations than those inducing apoptosis, specifically 500 nM of $(Bu_2Sn)_2$TPPS and 80 nM of $(Bu_3Sn)_4$ TPPS. No significant increase in cell number was observed over these treatment periods for the two compounds, as determined by the cell count harvested from the plates. Moreover, after 72 h and without any more treatment the $(Bu_3Sn)_4$TPPS-treated cells remained alive and did not

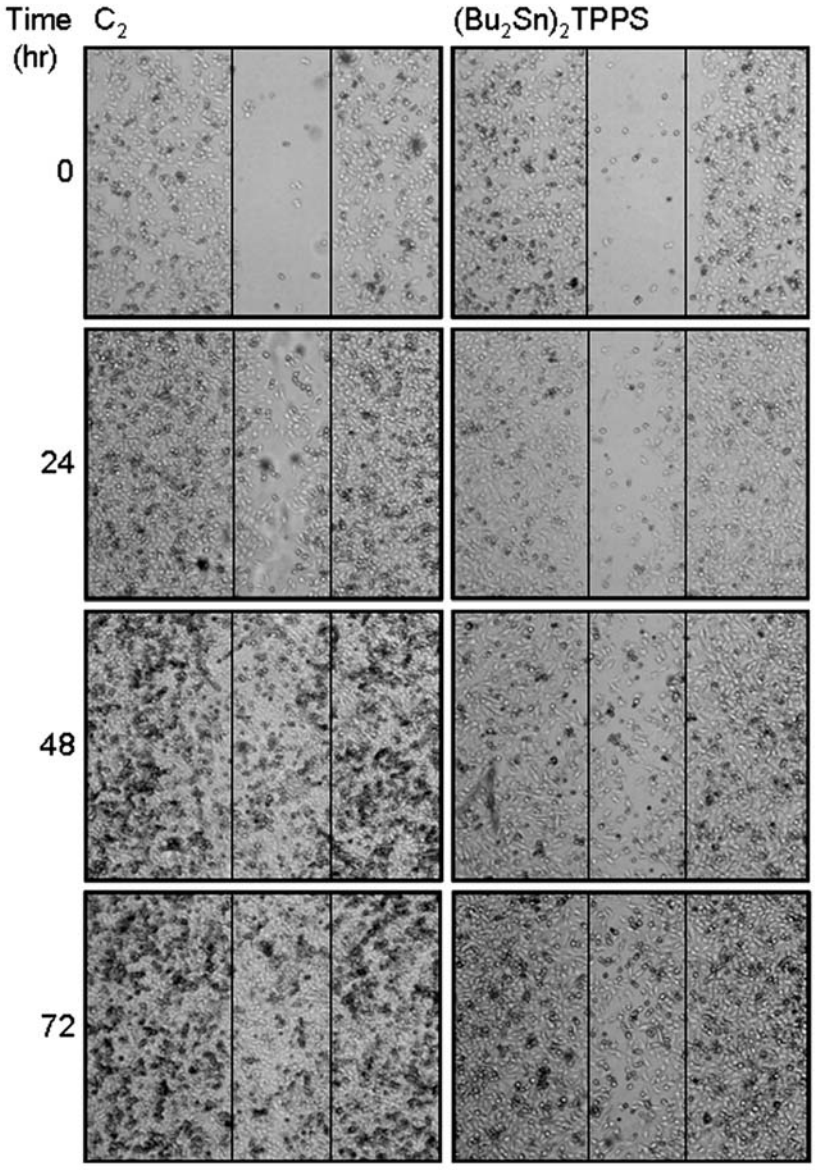

Figure 5. Wound repair assay in A375 cells treated with 500 nM of $(Bu_2Sn)_2$TPPS. Cells were grown and treated as described in Materials and methods, an artificial wound was introduced with a micropipette tip into cultures and wound closure was followed for 3 days. C_2, $(Bu_2Sn)_2$TPPS control for untreated cells. Pictures were taken every 24 h with X4 objective.

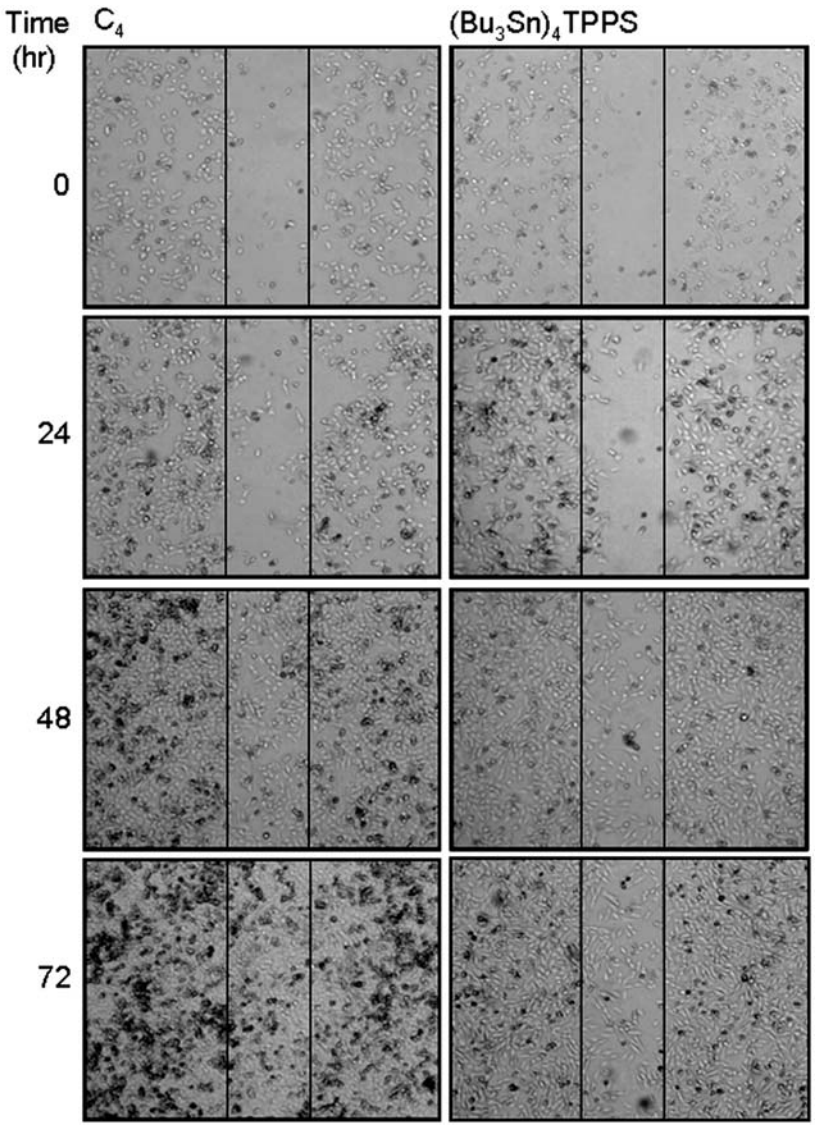

Figure 6. Wound repair assay in A375 cells treated with 80 nM of $(Bu_3Sn)_4TPPS$. Assay was performed as described in Fig. 5. C_4, $(Bu_3Sn)_4TPPS$ control for cells treated with 0.1% DMSO.

proliferate for another 3 days. In contrast, the $(Bu_2Sn)_2TPPS$-treated cells detached and died after this time (data not shown).

Scratch wound healing assay. The effects of $(Bu_2Sn)_2TPPS$ 500 nM and $(Bu_3Sn)_4TPPS$ 80 nM on A375 cell proliferation were investigated by *in vitro* wound repair assay. After scraping, the untreated cells invaded the denuded area more quickly than the treated cells, as shown in Figs. 5 and 6. A cell morphological transformation can already be observed in the treated cells at 24 h, which becomes greatly evident at 72 h of treatment. The cells appear to lose cell-cell interactions and to become spindle-shaped, as shown in Fig. 7. Specifically the $(Bu_3Sn)_4TPPS$-treated cells showed this spindle and elongated shape, as well as a strong adhesiveness to the plate. The $(Bu_3Sn)_4TPPS$-treated cells were more resistant to trypsin than the untreated and $(Bu_2Sn)_2$ TPPS-treated cells. Usually, we detach A375 melanoma cells from plates by trypsin/EDTA solution for 5 min at room temperature. For the $(Bu_3Sn)_4TPPS$-treated cells we had to use longer periods of time and incubation at 37˚C to detach cells.

Discussion

Cutaneous melanoma has been one of the fastest-rising malignancies for several decades. In contrast to many other cancer types, melanoma affects a relatively younger population and is notorious for its propensity to metastasize and for its poor response to current therapeutic regimens (22,23). At present, the available therapies contribute little to patient survival. Thus, the identification of molecules involved in the signaling pathways, leading to melanoma drug resistance and progression, provides the opportunity to develop targeted therapies.

Two porphyrin derivatives, $(Bu_2Sn)_2TPPS$ and $(Bu_3Sn)_4$ TPPS, proved effective in countering melanoma cell drug resistance *in vitro*. The two compounds induce apoptosis in A375 human melanoma cells, as we showed previously (6). It was important to understand how these compounds activate apoptosis in melanoma cells. Therefore, we focused our attention on the identification of targets of the $(Bu_2Sn)_2TPPS$ and $(Bu_3Sn)_4TPPS$ complexes and their cellular uptake. We analyzed MAPKs, and their analysis showed the activation of

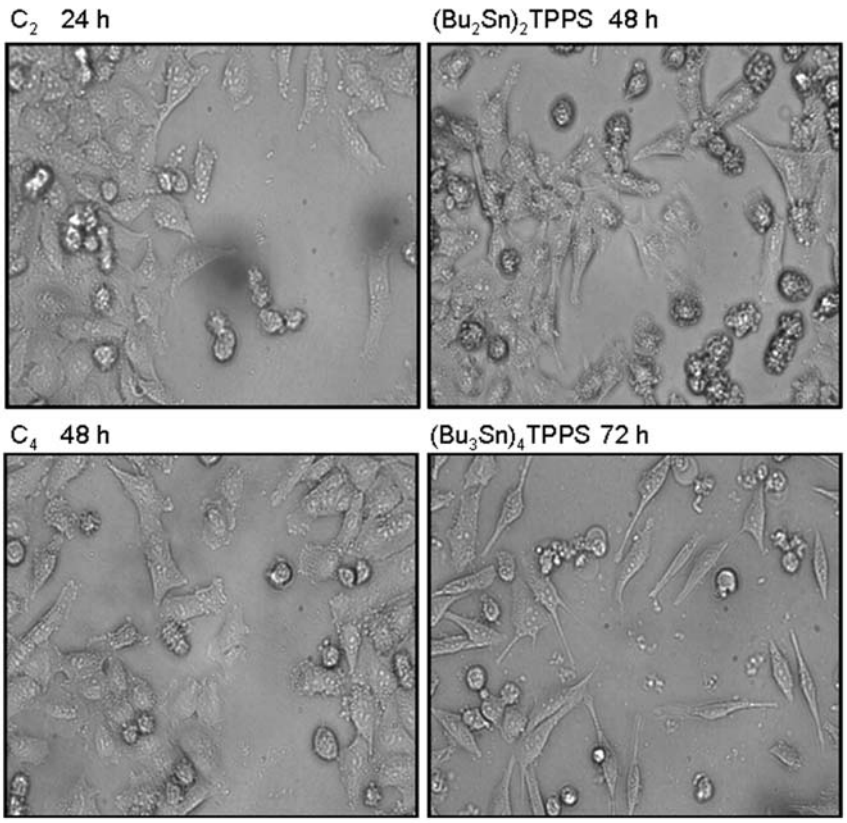

Figure 7. Magnification of some regions in Figs. 5 and 6. Pictures were taken with X20 objective. Different times were chosen in order to highlight better the cell morphology.

the three kinases, ERK 1/2, JNK and p38. These results surprised us initially, because we expected JNK and p38 activation but not a hyperphosphorylation of ERKs. JNK and p38 MAPK are involved in the regulation of apoptosis (24-26), whereas ERK activation results in cell survival and proliferation (27,28). Nevertheless, increasing studies now assign contrasting physiological roles to the same MAPK (10). Therefore we state that the $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$ TPPS complexes induce apoptotic death of A375 human melanoma cells through activation of the three MAPKs. We aimed to investigate what triggers MAPK activation and, since the two compounds contain butyl, our hypothesis was that they would be able to affect certain proteins associated with plasma membrane. Thus, we analyzed FAK that is constitutively activated in melanoma cells and whose activation has also been shown to promote cell survival and migration (14,15). Moreover, FAK expression attenuation induces apoptosis in several human cell lines including melanoma (29), and its dephosphorylation is correlated with apoptosis (30). We did not find any difference in FAK phosphorylation between the $(Bu_2Sn)_2$TPPS-treated and untreated cells at different treatment times. We observed FAK dephosphorylation and cleavage only in $(Bu_3Sn)_4$TPPS-treated A375 cells, but after 1 h of treatment when the caspases had already been activated (6), which is in agreement with what has been reported (21). Therefore, FAK is not a direct target of these compounds but rather a downstream target of the apoptotic pathway. However, by investigating the $(Bu_2Sn)_2$TPPS and $(Bu_3Sn)_4$TPPS cellular uptake, we found a rapid concentration of the two compounds within cells. In particular, after only 1 h of treatment a minor concentration remained in the cytoplasm while the majority of the concentration entered the nucleus and concentrated in the nucleoli compared to the TPPS-treated cells. It is likely that the targets are in the cytoplasm and/or in the nucleus, since the butyl-TPPS derivatives enter the cells quickly.

During these studies, we aimed to identify the lowest concentrations of the two compounds that were less toxic but sufficient to kill the melanoma cells. Interestingly, we found a significantly reduced growth of tumor cells after only 48 h treatment by using 500 nM of $(Bu_2Sn)_2$TPPS or 80 nM of $(Bu_3Sn)_4$TPPS, concentrations that were non-toxic even after 72 h of treatment, at least for the $(Bu_3Sn)_4$TPPS compound. At 24 h of treatment, cell morphology changed and the cell proliferation slowed down for the two compounds. With time the cells treated with $(Bu_3Sn)_4$TPPS appeared to stretch and lose cell contact. We analyzed MAPKs and FAK in A375 cells treated with these low concentrations, but found no difference in comparison with the untreated cells (data not shown).

These results indicate that investigation of the Wnt/ß-catenin signaling pathway is very important. Many melanomas exhibit constitutively elevated levels of nuclear ß-catenin (31,32). ß-catenin plays a key role in several developmental processes (33,34), and the activation of Wnt/ß-catenin signaling pathway has been linked to a metastatic or migratory phenotype (35). It is likely that our compounds affect this pathway and the identification of possible targets are a contribution to the development of newly targeted therapies which will challenge melanoma progression.

nclusion, we demonstrated that $(Bu_2Sn)_2$TPPS and TPPS induce A375 melanoma cell apoptosis by MAPK activation, and that low concentrations of the two compounds substantially slow down the growth of A375 cells *in vitro*.

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