Organotin(IV) complexes with epigenetic modulator ligands: new promising candidates in cancer therapy

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Abstract

Organotin(IV) compounds have found their place among a class of non-platinum chemotherapeutic metallo-pharmaceuticals exhibiting good antitumor activity. The cytotoxicity induced by the different kinds of organotins has been related to several mechanism.

In this review we report our last decade research activity concerning the synthesis and chemical characterization of new organotin(IV) derivatives containing epigenetic modulator ligand by using, FT-IR, Mössbauer spectroscopy, $^{119}Sn\{^{1}H\}$ cross polarization magic angle spinning, electrospray ionization mass spectroscopy, UV–Vis, ^{1}H, $^{13}C\{^{1}H\}$ and $^{119}Sn\{^{1}H\}$ NMR spectroscopy and density functional theory calculations.

The first aim of the biological studies was to evaluate the effects exerted by organotin(IV) complexes in comparison with corresponding free ligands on a panel of different cancer cell lines, including human HepG2 hepatoma cells, HCT116 colorectal carcinoma cells and MDA-MB-231 breast cancer cells. We provide evidence that the compounds, generally, exert significant cytotoxic activity in cancer cells even at nanomolar (nM) or micromolar (μM) range concentrations. We also evaluated the biochemical pathways underlining these effects and the epigenetic activity of the compounds in the coordinated form compared to their parental constituents.

Keywords: Organotin(IV); butyric acid; valproic acid; caffeic acid; apoptosis; epigenetic modification.

1. Introduction

For a long time we have been involved in organotin(IV) complexes synthesis, characterization and biological studies to test their potential anti-tumor efficacy. The leitmotiv has been the modulation of the intrinsic organometallic moiety cytotoxicity by means of biologically related molecules (synthetic or natural). Such a modulation is usually achieved by two (often overlapping) routes: dampen the damaging effects of the metal core and serving as a carrier for specific tissue districts [1–6].

Cancer cells have aberrant gene expression, which has multiple causes: genetic (gene mutations, homozygous deletions, loss of heterozygosity, etc.), cytogenetic (monosomies, trisomies, homogenous staining regions, double minutes, etc.) and epigenetic (Histone and DNA covalent modifications) [7].

The goal of combining different therapies in the tumour management is to increase and prolong the response rate as well as to decrease the toxicity associated with each treatment. Two different approach can be utilized to achieve these objectives. Treatments may be combined based on the absence of overlapping or synergistic toxicities leading to empiric combinations, otherwise the combination of treatments with known convergent molecular mechanisms.

Thus, in this review, we focused our discussion on the recent advances in the studies on organotin(IV) derivatives of short chain fatty acids and hydroxamic acid derivatives, that behaves as epigenetic modulators and exerts antitumoral effects at low concentrations.

This candidates organotin complexes with epigenetic modulator ligands as new promising antitumoral agents.

No doubt exists that combining traditional cancer therapy with epigenetic modulators and reversing DNA methylation and histone modification patterns holds a huge potential for successful treatment of some malignancies, even if there are a number of important steps that need to be accomplished on the path towards efficient epigenetic therapy.

2. Cell signaling and epigenetic

Cell signaling is a complex system of communication that coordinates basic cellular activities. Cells perceive and correctly respond to microenvironment via this complex system engaging in cellular processes such as, tissue repair, immunity, as well as normal tissue homeostasis.

The human genome is epigenetically organized through a series of modifications to the histone proteins that interact with the DNA.

Histone modifications, together with factors responsible for adding, interpreting, and removing epigenetic marks, regulate specific responses of the eukaryote genome, and this represents the basis of the "histone code hypothesis" [8,9].

Epigenetic modifications rely on re-building of chromatin structure resulting in an open or closed configuration and thereby expressing or repressing genes which control basic cellular processes such as differentiation, proliferation and apoptosis, and as a consequence, cell functions. Many post-translational modifications are responsible for epigenetic modifications of chromatin structure, including phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation, but the best characterised are DNA methylation and nucleosomal histone tail acetylation. Both these modifications can significantly influence the epigenetic regulation of the gene expression pattern [10].

The epigenetic etiology of many human diseases has led to the development of "epigenetic" therapies. For instance, the acetylation balance of chromatin regulates cell determination and cell fate, suggesting that epigenetic drugs could prove useful for the treatment of muscle diseases, neurodegenerative disorders, and cancer [11,12].

Epigenetic modifications are potentially reversible, unlike genetic mutations, thus representing an attractive target for cancer therapy.

In cancer, many of the proteins that regulate these modifications can be altered in both function and expression. The involvement of several enzymatic activities in cell transformation has stimulated the development of combinatory therapies.

Deregulation of the equilibrium between histone acetyl transferases (HATs) and histone deacetylases (HDACs) has also been detected in several cancer types [13]. Similarly, errors in cell signalling which involve aberrant DNA methyltransferase (DNMT) activity play a role in the development as well as in the progression of cancer.

Some kind of acquired or intrinsic chemoresistance may be epigenetic in nature and operate at DNA mismatch repair level [14,15].

As mentioned before, histone modifications provide an important mechanism of epigenetic regulation [16] and many organotin compounds may influence epigenetics as well. For instance, evidence has been provided that organotin compounds are capable of affecting HAT activity [17] or modifying DNA methylation pattern. Organotin exposure has in fact been shown to affect the level of DNA methylation in Marble trouts (S. marmoratus) treated with tributyltin (TBT) or triphenyltin (TPT) derivatives at increasing dosages for 48 days [18].

Recently a research provides new evidence of the epigenetic action caused by organotin(IV). Tributyltin induces epigenetic changes such as hypermethylation that is related to nuclear respiratory factor-1 decrease and epigenetic changes [19].

3. Epigenetic modifications: acetylation and methylation status of histones

The balance between acetylation and deacetylation is an important factor in regulating gene expression and is thus linked to the control of eukaryotic cell fate.

The acetylation status of histones is regulated by two types of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), which are responsible for the addition or the hydrolysis of acetyl group on lysines residues in N-terminal tails of histone [20]. In general, though not exclusively, histone acetylation is associated with a positive regulation of transcription, whereas histone deacetylation is correlated with transcriptional silencing.

Today, it is known that histone acetyltransferases transfer the acetyl group from acetyl-CoA forming ε-N-acetyl lysine on conserved lysines of the N-terminal tails of histones H3 and H4 (and to a lesser extent H2A and H2B), resulting in an open nucleosomal structure. This can be reversed by different HDACs that have been divided into four classes in mammals, based on cellular localization and function [21,22].

The introduction of acetyl groups by histone acetyltransferases (HATs) reduces the affinity of the histone cores to the DNA strands. As a result a relaxed chromatin structure is formed, enabling initiation of transcription.

Histone deacetylation enhances the electrostatic interaction between the histone core of nucleosomes and DNA, leading to chromatin condensation [23].

Apart from altering the physical properties of chromatin, removal of acetyl groups modifies the array of post-translational modifications of amino acids in histones amino termini. Addition or removal of a functional group on one amino acid residue may influence the status of neighbouring amino acid residues, not only in the same histone tail but also in tails of other histones, or even in other nucleosomes. As a result, the context of the encrypted message may be changed [24].

The role of HDACs in cellular proliferation was emphasized by the discovery that their anomalous action constitutes a cornerstone of carcinogenesis, a process which results from a disrupted balance between cell proliferation and apoptosis, in favour of unlimited growth.

HDACs have increased expression in cancer and are also believed to promote carcinogenesis through the acetylation and interaction with key transcriptional regulators [25,26].

In addition, a number of cytoplasmic and nuclear proteins, including transcription factors, DNA repair enzymes, cell-cycle regulators, molecular chaperones as well as structural proteins were found to be reversibly acetylated. Acetylation/deacetylation may modify the stability of the protein involved, its localization, DNA-binding ability and its interactions with other proteins [27].

The identification of a large number of acetylated targets has uncovered new players involved in the acetylation balance.

The elucidation of unequivocal links between aberrant action of HDACs and tumorigenesis lies at the base of key scientific importance of these enzymes. In particular, the potential benefit of HDAC inhibition has been confirmed in various tumour cell lines, demonstrating antiproliferative, differentiating and pro-apoptotic effects.

The development of HDAC inhibitors (HDACIs) has become the subject of intense interest, and many of these agents have now entered the clinical arena. Small molecules with histone deacetylases inhibitory effects have been identified and developed. They not only inhibit HDACs, but can also lead to growth arrest, differentiation, and/or apoptosis in tumors both in vitro and in vivo [28].

The use of HDACIs, probably in association with classical chemotherapy drugs or in combination with DNA-demethylating agents, could be promising for cancer patients. Further evaluation is needed to establish the clinical activity of combination therapy using HDACIs with cytotoxic drugs or differentiation-inducing agents [29].

Effective inhibitors have been found among the derivatives of hydroxamic acid. These agents are generally active in the low-to-intermediate nanomolar (nM) concentration range; other classes of HDACIs include carboxylic acids, benzamide and cyclic peptides. Furthermore, short-chain fatty acids such as butyrate, phenylbutyrate or valproic acid are active as HDAC inhibitors, even if at higher concentrations. They are likely to act in a noncompetitive manner and they are the least potent of the HDACIs, requiring millimolar (mM) concentrations to achieve their effects. The potency of HDACIs in cancer therapy has been reviewed, e.g., by Kouraklis and Theocharis. So far, promising findings have been obtained with butyrate-releasing pro-drugs and butyrate derivatives rather than with butyrate itself [30].

Furthermore, HDACIs were suggested to be involved in chemoprevention since they may keep genetically modified putative preneoplastic cells in a more differentiated state and/ or enhance their removal via apoptosis [31,32].

DNA methyltransferase (DNMT) inhibitors also represent a promising class of epigenetic modulators.

In cancer, epigenetic silencing through methylation occurs just as frequently as mutations or deletions and leads to aberrant silencing of genes with tumor-suppressor functions [33].

DNA methylation is the most characterized epigenetic phenomenon described as a stable epigenetic marker and its biological role is to maintain DNA transcriptionally quiescent, resulting in gene silencing.

This process involves enzymes belonging to the DNA methyltransferase family (DNMTs).

DNA methylation involves the covalent addition of a methyl group to the carbon-5 position of cytosine (C) to form the fifth base, 5-methylcytosine (5-mC), in cytosineguanine (CpG) dinucleotides. Methylation is catalyzed by three major DNA methyltransferases (DNMTs: DNMT1, DNMT3a, DNMT3b), and the methyl group donor is S-adenosylmethionine (SAM) [34].

Nevertheless, DNA methylation and histone modifications are closely related. Methylated CpG sites in gene promoter regions are easily recognized by specific methyl CpG binding proteins (MBPs) which act as adapters between methylated DNA and chromatin modifying factors. MBPs can recruit co-repressors such as HDAC, methyltransferase and chromatin remodeling factors, creating the protein complex which regulates gene expression [35].

If the promoter region is methylated, the corresponding gene is repressed due to its poor recognition by transcription factors [36]. Indeed, DNMTs affect protein-DNA interactions by chromatin remodeling, determine the accessibility of DNA to transcription factors, and are associated with under- or overexpression of certain proteins, ultimately leading to diverse pathologies, among which cancer [37].

The use of HDACIs, probably in association with classical chemotherapy drugs or in combination with DNA-demethylating agents, could be promising for cancer patients.

Further evaluation is needed to establish the clinical activity of combination therapy using HDACIs with cytotoxic drugs or differentiation induced agents [29].

4. Organotin(IV) complexes with epigenetic modulator ligands

4.1. Tributyltin(IV) derivative of butyric acid

We recently showed that tributyltin(IV) butyrate act as a novel epigenetic modifier with ER Stress- and apoptosis-inducing properties in colon cancer cells [38].

Short-chain fatty acid (SCFA) are naturally occurring fatty acids and exhibit various pharmacological applications [39–42].

Since 1860, complexes of organotins with short chain fatty acids have been produced and chemically characterised [43,44].

Butyric acid (BTA, Fig. 1A) belongs to short-chain fatty acid class produced by anaerobic fermentation of dietary fibres in the human colon. Beyond its metabolic function sustaining normal colonocytes, it has been shown to inhibit growth and to induce differentiation of colon cancer cells as well as a variety of other tumor cell types [45].

Although its precise mechanisms of action are not well understood, it has long being known that butyrate inhibits histone deacetylases, resulting in a relative hyperacetylation of core histone proteins

(H3 and H4) [46] that, disrupting ionic interactions with the adjacent DNA backbone, creates less densely packed chromatin and allows transcription factors to activate specific genes [47].

This action of butyrate is likely to occur in vivo because rats fed a high fiber diet had high luminal butyrate levels, and this was associated with histone hyperacetylation and growth inhibition in colonic epithelial cells [48].

Butyrate has also been shown to induce apoptosis in tumor cells, an effect which is usually displayed at mM range concentration and which is related with histone hyperacetylation due to HDAC inhibition. However, other mechanisms including butyrate-induced DNA methylation inhibition and post-translational modifications of specific proteins have been reported [49].

Butyrate has also been shown to potentiate the effects of traditional chemotherapeutics such as 5-fluorouracil (5-FU), vincristine, adryamycine, methotrexate and cisplatin [50–53].

However, these potentiating effects have also been observed using relatively high concentrations of butyrate.

Promising results have been obtained with butyrate-releasing pro-drugs and butyrate derivatives rather than with butyrate itself [54–56].

Considering the relatively low efficacy of butyrate compared to that of new generation HDACIs that have impacted in epigenetic tumor targeted therapy [57], we focused on butyrate organotin conjugates to improve its efficacy and to understand whether it maintains epigenetic properties in colon cancer cells [38]. Specifically, tributyltin(IV) butyrate was chosen as the most promising compound and its chemical synthesis and structural characterization as well as biological activity are summarized.

4.1.1. Chemical synthesis and structural characterization

Tributyltin(IV) butyrate was prepared according to the previously reported neutralization method [58], by refluxing methanolic solution of the free butyric acid and of bis(tributyltin) oxide [38].

The analytical data revealed the formation of compounds with a 1:1 metal-to-ligand ratio, leading to formula [Bu_3SnBT]. The structure of the synthesized compound was confirmed by Fourier transform infrared (FT-IR) and electrospray ionization mass (ESI-MS) spectroscopy in solid state and ^{1}H, $^{13}C\{^{1}H\}$ and $^{119}Sn\{^{1}H\}$ nuclear magnetic resonance (NMR) in solution phase.

The coordinating mode of butyric acid towards the tributyltin(IV) moiety can be inferred by comparing the infrared spectroscopy spectra of free and coordinated ligand (Table 1A).

In the free ligand, the C=O stretching of the carboxylic group, observed at 1712 cm^{-1}, disappears upon coordination, indicating deprotonation of the carboxylic group. The tributyltin(IV) butyrate IR spectrum showed, in the range 1600–1300 cm^{-1}, two bands attributable to asymmetric and symmetric stretchings of the carboxylate group with $\Delta\nu$ (= $\nu_{as}COO^{-} - \nu_{s}COO^{-}$) characteristic for coordinated

ester-type carboxylate groups [59,60]. Characteristics bands of ν(Sn–C) and ν(Sn–O) are present near 500 cm^{-1} in the complex spectrum [61].

The solid-state configuration of the tributyltin(IV) complex determined by IR spectroscopy suggested that the tin atom is tetra-coordinated with the ligand acting as a monoanionic monodentate ligand, through an ester-like carboxylate anion (Fig. 1B).

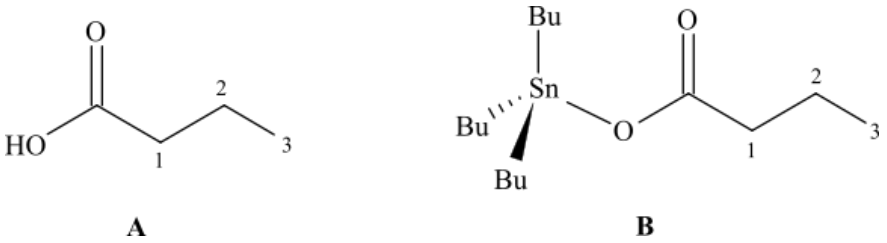

Fig. 1. (A) Butyric acid, BTA and **(B)** Bu_3SnBT proposed structure, with the numbering scheme referred to the NMR assignments (see Table 1B).

The ^1H, $^{13}C\{^1H\}$ spectra of the butyric acid and the studied complex were recorded in $CDCl_3$ (Table 1B). The absence of ^1H NMR signal corresponding to acidic hydrogen (COOH) in the spectrum of complex suggests the coordination of tributyltin(IV) moiety with the carboxylate group. The $^{119}Sn\{^1H\}$ of Bu_3SnBT showed a single signal at 100.94 ppm, typical value of four coordinated tin centers, in agreement with what was inferred from FTIR findings.

The electrospray ionization-mass spectrometry (ESI-MS) spectrum of Bu_3SnBT showed very complicated fragmentation patterns because of the presence of several adducts and a wide range of fragment ions in the first-order mass spectra. The spectrum revealed the peaks relative to the monomeric adducts that Bu_3SnBT form with $SnBu_3$ and with alkali metal ions such as Na^+ (Table 1C) [62]. The assignments of the individual ions were based on the combination of positive-ion, and tandem mass spectrometric experiments, supported by a comparison between theoretical and experimental isotopic distributions of monomeric.

Table 1. Spectroscopic data of butyric acid (BTA) and Bu_3SnBT complex.

(A)

The wavenumbers (cm^{-1}) and assignments of more relevant bands occurring in the experimental FT-IR[a]

Assignment[b]	**BTA**	**Bu_3SnBT**
ν(C=O)	1712 s	-
$\nu_{as}(COO^-)$	-	1574 s
$\nu_s(COO^-)$	-	1338 m
ν(Sn–C)	-	508 w
ν(Sn–O)	-	488 w
Δν (cm^{-1})	-	236

(B)

Selected ^{1}H NMR, $^{13}C\{^{1}H\}$ and $^{119}Sn\{^{1}H\}$ NMR chemical shifts (ppm) in $CDCl_3$ at 298 K. Integration values are given in square brackets, coupling constants in parentheses (absolute values in Hz).[c]

δ(^{1}H)	**BTA**	**Bu_3SnBT**
COOH	11.85	-
H_α	-	1.60 [6] (70)[d]
H_β, H_γ[e]	-	1.31 [6], 1.23 [6]
CH_3	-	0.89 [9]
δ(^{13}C)		
COOH	183.31	181.85
$CH_{2\alpha}$	-	18.99
$CH_{2\beta}$	-	30.43
$CH_{2\gamma}$	-	29.57
CH_3	-	n.o.[f]
δ(^{119}Sn)		100.94

(C)

ESI-MS data

Positive-ion MS (*m/z*)

777 $[2M+Na]^+$, 720 $[2M\text{-butyl}+Na]^+$, 668 $[M+SnBu_3]^+$, 600 $[2M\text{-SnBu}+Na]^+$, 320 $[M\text{-butyl}]^+$, 291 $[SnBu_3]^+$, 234 $[SnBu_2]^+$, 177 $[SnBu]^+$

[a] s, strong; m, medium; w, weak.
[b] *ν*, stands for stretching vibrations.
[c] Numbering of butyl group in di- and tributyltin moiety: CH_3–$CH_{2\gamma}$–$CH_{2\beta}$–$CH_{2\alpha}$–Sn.
[d] $^{2}J(^{119}Sn,^{1}H)$.
[e] It was not possible to discriminate the separate resonances for each carbon.
[f] n.o., not observed.

4.1.2. Biological Study

As previously mentioned, butyric acid is a short-chain fatty acid naturally produced by bacteria in the human colon and metabolized by healthy colonocytes. Therefore, its therapeutic antitumor potential is somehow limited by rapid metabolic use from the intestine and by its efficacy at relatively high doses (mM range). For these reasons, the antitumor efficacy of its organotin derivative tributyltin(IV) butyrate (Bu_3SnBT) was tested in colon cancer cells in comparison with butyric acid (BTA) and parental organotin compound (Bu_3SnCl). Interestingly, Bu_3SnBT exerted significant cytotoxic effects at low concentrations (0.5 μM) while butyric acid was not effective in the same concentration range, but showed cytotoxicity at mM concentration. The parent compound Bu_3SnCl reduced cell viability to a much lower extent compared to the same concentrations of Bu_3SnBT (Fig. 2).

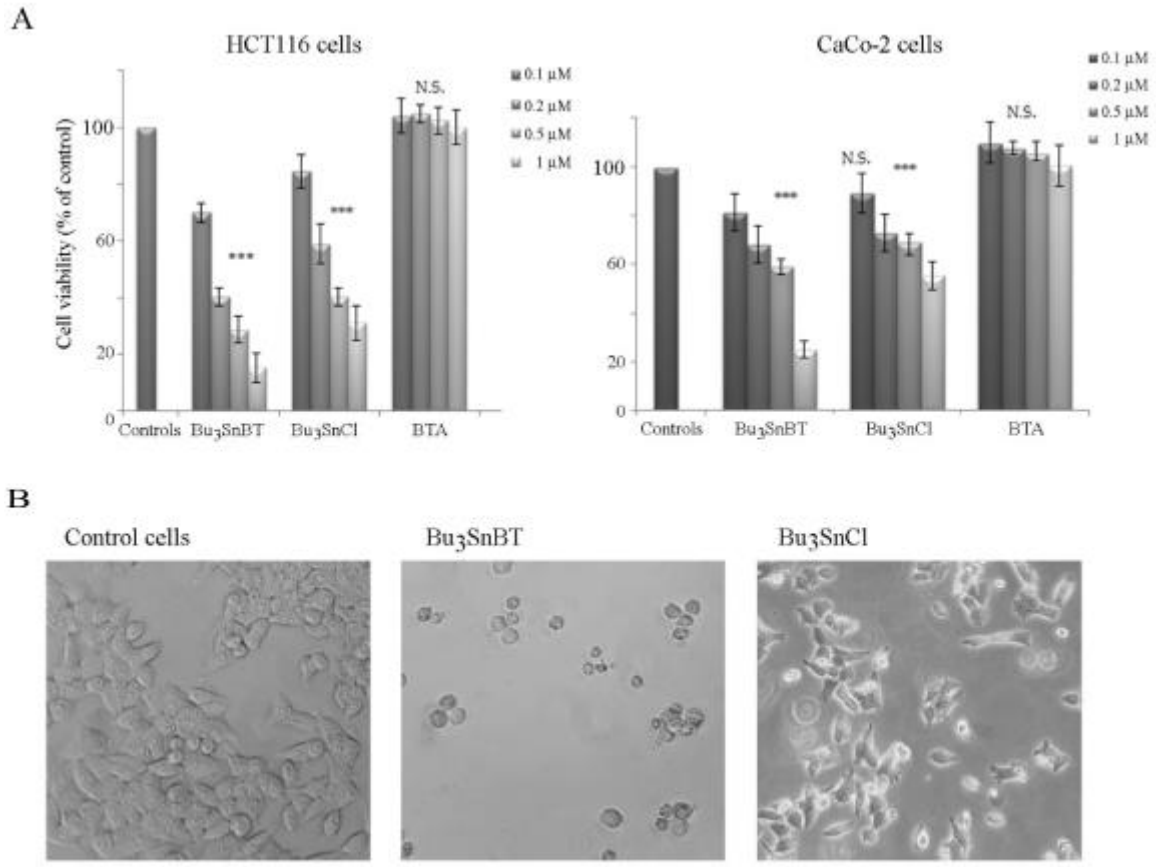

Fig. 2. Tributyltin(IV) butyrate (Bu_3SnBT) induces cytotoxic effects in colon cancer cells: (**A**) MTT assay was used to measure cell viability in HCT116 and CaCo-2 colon cancer cell lines. Cells were incubated for 48 h in the presence of the conjugate Bu_3SnBT and the corresponding parent compound Bu_3SnCl at the indicated concentrations. The effects of butyric acid (BTA) were also evaluated. The results reported in the histograms are representative of three separate experiments. (***) *p*-value < 0.001 compared with untreated cells; N.S. = not significant; (**B**) Morphological analysis of HCT116 cells treated with 0.5 µM Bu_3SnBT and Bu_3SnCl for 48 h. The cells were visualized under a light microscope at 200× magnification and the pictures were acquired by IM50 Leica Software (Leika Microsystems, Wetzlar, Germany). [*modified from [38]*]

The antitumor action of Bu_3SnBT was correlated with G2/M arrest of the cell cycle (Fig. 3A), endoplasmic reticulum (ER) stress and consequent apoptosis. To assess whether butyrate in the coordinated form maintains its epigenetic effects, histone acetylation was evaluated. Surprisingly, a dramatic decrease in acetyl-H3 and -H4 histones was found following Bu_3SnBT treatment. In contrast, butyrate alone stimulated histone acetylation at a higher concentration (5 mM) (Fig. 3B). Therefore, it is interesting to note that the compound also exerted an epigenetic effect (consisting in histone deacetylation) but this was opposite to that of the parental butyrate, that instead determined histone acetylation due to HDAC inhibition. Concerning the epigenetic mechanism of Bu_3SnBT action, although different mechanisms, such as histone acetyl transferase (HAT) inhibition or a reduced acetyl CoA availability, can account for histone deacetylation, a suggestive hypothesis is that Bu_3SnBT acquires a new property in the conjugated form, most likely consisting in direct HDAC activation.

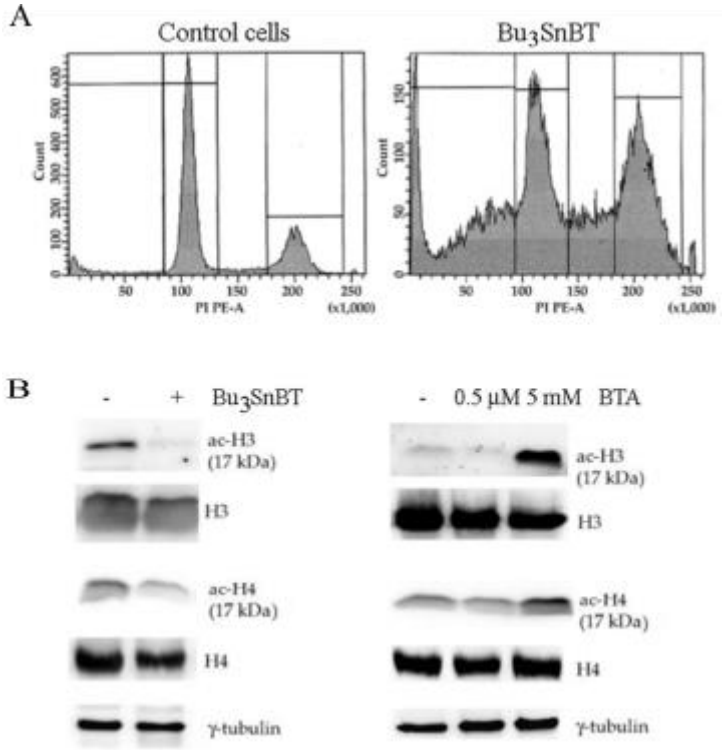

Fig. 3. Tributyltin(IV) butyrate (Bu_3SnBT) induces cell cycle arrest accompanied by DNA fragmentation. (**A**) The cell cycle phase distribution of HCT116 cells was evaluated by flow cytometry analysis. Cells were incubated for 48 h in the presence of 0.5 µM Bu_3SnBT. Then, the DNA content was evaluated after incubating the cells in a hypotonic propidium iodide solution. Fluorescence was estimated by FacsDiva Software. (**B**) Bu_3SnBT remarkably reduces histone acetylation. Western blot analysis of acetylated-H3 and H4 histones after treatment for 48 h with 0.5 µM Bu_3SnBT or 0.5 µM and 5 mM butyric acid (BTA) are shown. [*modified from [38]*]

To confirm this hypothesis, suberoylanilide hydroxamic acid (SAHA), another HDAC inhibitor was used. Notably, Bu_3SnBT was capable of preventing histone acetylation induced by SAHA, thus suggesting that it may activate HDACs. Ongoing studies aim to elucidate the precise epigenetic mechanism activated by the compound and its relationship with the antitumor effects exerted.

The results obtained so far support a potential use of Bu_3SnBT colon cancer treatment as a novel epigenetic modulator.

4.2 Tributyltin(IV) derivative of valproic acid

Valproic acid (VPA, Fig. 4A) a well-established therapy for seizures and bipolar disorder, has recently been shown to inhibit histone deacetylases (HDACs). Similar to more widely studied HDAC inhibitors, VPA can cause growth arrest and induce differentiation of transformed cultured cells [63,64]. VPA relieves HDAC-dependent transcriptional repression and causes hyperacetylation of the N-terminal tails of histones H3 and H4 both in vitro and in vivo [65,66]. VPA also inhibits tumor growth and metastasis in animal experiments [67].

Furthermore, considering the toxicity of some HDAC inhibitors (e.g. trapoxin) in mammals, VPA shows surprisingly mild adverse effects in the adult even if serum levels exceed the normal therapeutic range during anti-epileptic therapy [68].

Combination anticancer therapies including VPA with other drugs, especially non-toxic drugs, may offer a substantial advantage over VPA monotherapy in a clinical setting. Novel experiments suggest that induction of differentiation by inhibition of HDAC combined with the activation of the peroxisome proliferator-activated receptor gamma (PPAR-γ) may be attractive in the treatment of multiple malignancies [69].PPAR-γ is a member of the nuclear receptor superfamily which regulates the expression of genes by heterodimerizing with RXR. PPAR-γ is activated by a variety of fatty acids and a class of synthetic antidiabetic agents, the thiazolidinediones [17,70]. It has been demonstrated that some organotins(IV) also function as nM agonists for PPAR-γ [71,72].

Based on these data and existing clinical experience with VPA, we designed a new organotin(IV) complex with valproic acid in order to evaluate if combining both this SCFA and organotin(IV) moiety it increase each other's effectiveness.

4.2.1. Chemical synthesis and structural characterization

In 2013 we reported the synthesis and chemical characterization of the newly synthesized potential anti-tumor tributyltin(IV) valproate and the individuation of a possible specific cytotoxic action on tumor cells [3].

Analytical data clearly indicated that tributyltin(IV) moiety reacted with VPA in 1:1 ratio, leading to formula [Bu_3SnVP]. The coordination environment at the tin center was investigated spectroscopically through FTIR, Mössbauer, ESI-MS, in solid-state and ^{119}Sn, ^{1}H, ^{13}C NMR spectroscopy, in solution phase.

The biggest changes in the IR spectra on coordination compared with free valproic acid occurred in the regions 1600–1400 cm^{-1} and 600–500 cm^{-1} (Table 2A), ranges in which two new bands were observed, the asymmetric and symmetric stretching of the carboxylate group with Δν value characteristic for coordinated ester-type carboxylate groups [59,60]. In the latter range, variations occurred also for the presence of Sn–C vibrations. Moreover, in the free ligand, the C=O stretching of the carboxylic group, observed at 1707 cm^{-1}, disappears upon coordination, indicating deprotonation of the carboxylic group.

The experimental Mössbauer parameters, isomer shift, δ (mm s^{-1}), and quadrupole splittings, Δ (mm s^{-1}) provide charge-density and molecular geometry data around the tin atoms. Collected Mössbauer data showed two Sn environments in a ratio 1:1 (Table 2B). The Γ value for Bu_3SnVP is consistent with the number of observed absorbing Mössbauer species [73–77]. By means of point charge

formalism [73,75,77–79], the Δ values were rationalized and a partial geometry attribution was made; since the δ observed values fall in the range of tetra-coordinated triorganotin derivatives, applying the Parish equation for distorted structures [77], an average C-Sn-X angle was calculated (X being the non-carbon atom). Obtained value (97 ± 12)° for Bu_3SnVP is typical of the distortion naturally occurring in tetra-coordinated triorganotin derivatives (Fig. 4B).

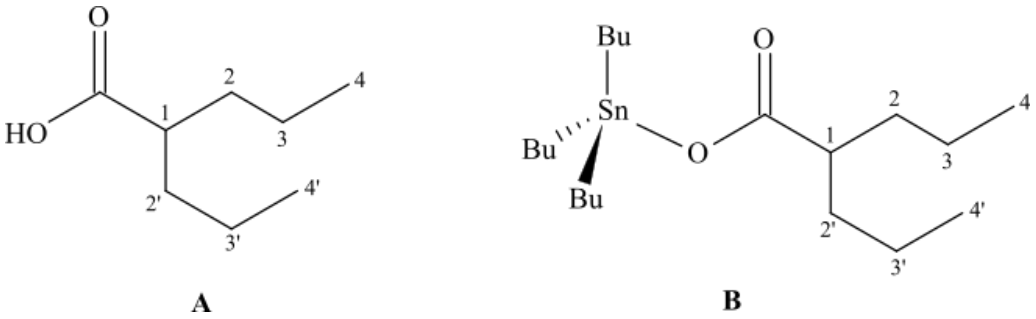

Fig. 4. (A) Valproic acid, VPA and **(B)** Bu_3SnVP proposed structure, with the numbering scheme referred to the NMR assignments (see Table 2C).

The ^{1}H and $^{13}C\{^{1}H\}$ NMR measurements were carried out in $CDCl_3$ and measured chemical shift of VPA and Bu_3SnVP, together with relevant $^{n}J(^{119}Sn,^{13}C)$ (n = 1,2,3) are summarized in Table 2C. The disappearance of the carboxylic proton signal in the spectrum of the complex was diagnostic of the coordination of the ligand upon the tributyltin(IV) moiety. From the $^{1}J(^{119}Sn,^{13}C)$ satellites, it was possible to calculate a C-Sn-C angle of (111 ± 2)° [80]. This value suggests a local tetrahedral Sn geometry, with valproate binding the Sn atom conceivably via an ester-like carboxylate. $^{n}J(^{119}Sn,^{13}C)$ values (n = 2,3) of Bu_3SnVP also confirm the occurrence of a local tetrahedral geometry at the Sn atom [81].

The ESI-MS spectrum of Bu_3SnVP showed very complicated fragmentation patterns, because of the presence of several adducts and a wide range of fragment ions in the first-order mass spectra (Table 2D). The spectrum revealed the peak relative to the monomeric adduct that Bu_3SnVP forms with $SnBu_3$ group and with alkali metal ions like Na^+ or K^+. Such a complexity is typical of organotin compounds, as previously reported [62,82]. A dimeric ion with lower relative abundances is also present. The assignments of the individual ions are based on the combination of positive-ion, negative-ion and tandem mass spectrometric experiments, supported by comparison between theoretical and experimental isotopic distributions of monomeric and dimeric ions. The ions observed in negative mode mainly form the adducts with valproate or Cl^- ions.

Table 2. Spectroscopic data of valproic acid (VPA) and Bu_3SnVP complex.

(A)

The wavenumbers (cm^{-1}) and assignments of more relevant bands occurring in the experimental FT-IR[a]

Assignment[b]	**VPA**	**Bu_3SnVP**
ν(C=O)	1707 s	-
$\nu_{as}(COO^-)$	-	1574 s
$\nu_{s}(COO^-)$	-	1338 m
ν(Sn–C)	-	510 w
ν(Sn–O)	-	477 w
Δν (cm^{-1})	-	209

(B)

Experimental Mössbauer parameter, isomer shift, δ mm s^{-1}, and nuclear quadrupole splittings, Δ (mm s^{-1}), measured at liquid N_2 temperature.

δ_1	Δ_1	δ_2	Δ_2	Γ_{avg}[c]
1.40	4.11	1.40	2.97	0.85

(C)

Selected ^1H NMR, $^{13}C\{^1H\}$ and $^{119}Sn\{^1H\}$ NMR chemical shifts (ppm) in $CDCl_3$ at 298 K. Integration values are given in square brackets, coupling constants in parentheses (absolute values in Hz).[d]

δ(^{1}H)	**VPA**	**Bu_3SnVP**
COOH	11.88	-
H_α	-	0.90 [6]
H_β, H_γ[e]	-	1.56 [6], 1.22 [12]
CH_3	-	0.88 [9]
δ(^{13}C)		
COOH	183.6	182.3
$CH_{2\alpha}$	-	16.5 (360.7)[f]
$CH_{2\beta}$	-	28.0 (20.9)[g]
$CH_{2\gamma}$	-	27.2 (65.0)[h]
CH_3	-	13.8
δ(^{119}Sn)		100.94

(D)

ESI-MS data of Bu_3SnVP

Positive-ion MS (*m/z*)

889 $[2M+Na]^+$, 724 $[M+SnBu_3]^+$, 599 $[(OSnBu_2)_3$-butane-2butene$+H_2O+H]^+$, 376 [M-butane+H]$^+$, 291 $[SnBu_3]^+$

Negative-ion MS; *m/z*

576 [M+valproate]$^-$, 468 $[M+Cl]^-$, 392 $[M$-butane-$H_2O+Cl]^-$, 352 $[M$-2butane$+Cl]^-$, 143 [valproate]$^-$

[a] s, strong; m, medium; w, weak.

[b] *v*, stands for stretching vibrations.

[c] Γ_{avg} value is the average full width at half height of the resonant peaks, respectively at greater and lower velocity with respect to the centroid of the Mössbauer spectra.
[d] Numbering of butyl group in di- and tributyltin moiety: CH_3–$CH_{2\gamma}$–$CH_{2\beta}$–$CH_{2\alpha}$–Sn.
[e] It was not possible to discriminate the separate resonances for each carbon.
[f] $^1J(^{119}Sn,^{13}C)$ in Hz.
[g] $^2J(^{119}Sn,^{13}C)$ in Hz.
[h] $^3J(^{119}Sn,^{13}C)$ in Hz.

4.2.2. Biological Study

Recent literature indicates that valproic acid is particularly effective in overcoming sorafenib resistance in hepatocellular carcinoma [83,84]. Considering valproic derivatives, we have recently focused on hepatocellular carcinoma models to improve the therapeutic potential [3]. Biological evaluations evidenced a clear cytotoxic action of the complexes in hepatocellular carcinoma cells. This effect was due to apoptosis induction as evidenced by DNA fragmentation, positivity to annexin V and caspase activation. Interestingly, these effects were not observed in non tumor Chang liver cells that were considered as a normal counterpart, highlighting the targeted therapeutic potential of these compounds (Fig. 5). It is also noteworthy that valproic acid alone was completely ineffective either in hepatocarcinoma or Chang liver cells at the same treatment conditions.

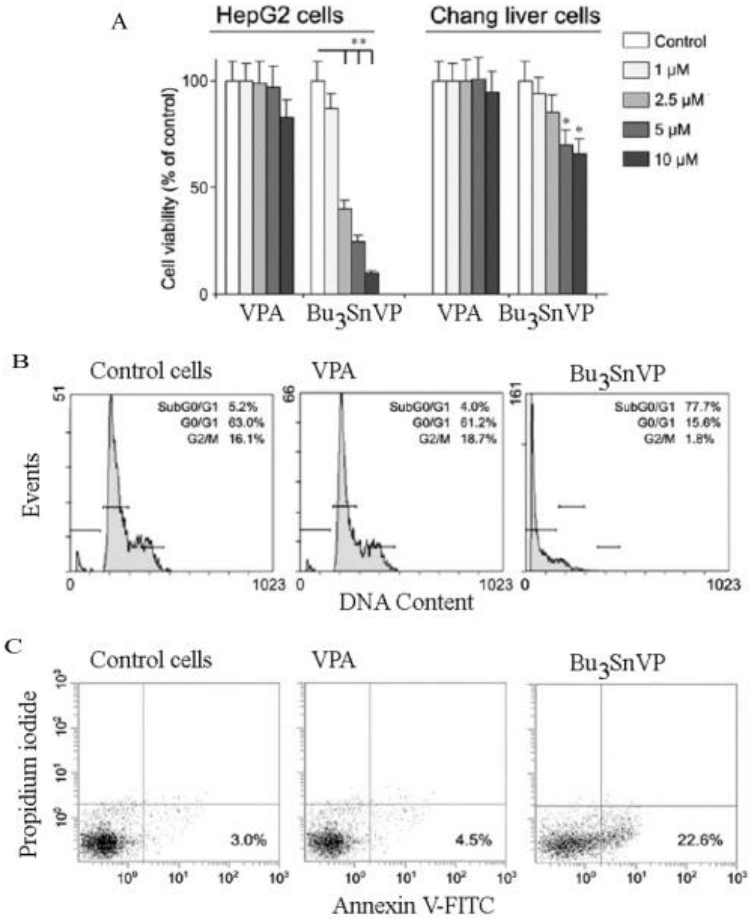

Fig. 5. Study on apoptotic effect induced by tributyltin(IV) valproate (Bu_3SnVP) complex in human hepatic liver cells. **A**. Cells treated with vehicle (control) or with the indicated concentrations of valproate (VPA) or Bu_3SnVP. Cell viability is indicated as percentage with respect to control. Data are represented as mean ± S.D. from six independent experiments performed in triplicate. *p<0.05,**p<0.01 vs. untreated cells. **B**. Representative flow cytometric analysis of cell cycle distribution after incubation with 2.5 μM VPA or Bu_3SnVP for 24 h. Cells were stained with propidium iodide and the percentage of cells in the different phases of the cycle was calculated using Expo32 software. **C**. Flow cytometric analysis of annexin V/propidium iodide labelled HepG2 cells treated for 8 h with 2.5 μM VPA or Bu_3SnVP. Flow cytometry using Annexin V/PI staining permits to discriminate the percentage of live cells (Annexin V negative/ PI negative, lower left quadrant), early apoptotic cells (Annexin V positive/PI negative, lower right quadrant), necrosis or late apoptotic cells (Annexin V positive/PI positive, upper right quadrant), and deadcells (Annexin V negative/PI positive, upper left quadrant). In B and C the diagrams are representative of four independent experiments with similar results. [*modified from [3]*]

To detect epigenetic effects, preliminary evaluations on the acetylation state of histone H3 in tributyltin-valproate treated HepG2 cells revealed a significant increase in Ac-H3 (histone H3 acetylated at lys-9 and lys-14), suggesting that the compound maintains the deacetylase inhibition activity of its ligand valproate (Fig. 6).

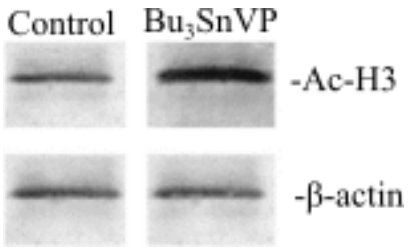

Fig. 6. Histone deacetylase inhibition investigation in HepG2 cells treated for 24 h with Bu_3SnVP. Cells lysates were analyzed by Western blotting analysis using a specific antibody. The results are representative of three independent experiments with similar results. [*modified from [3]*]

Taken together, these results open a new perspective to further characterise the epigenetic potential of these compounds and to correlate it to their antitumor activity.

4.3 Dibutyltin(IV) derivative of caffeic acid

Aberrant methylation patterns are associated with certain human tumors and developmental abnormalities. It is known that caffeic acid (H_3CAF, Fig. 7A) can modulate DNMT1-mediated DNA methylation.

Caffeic acid is a widespread phenolic acid that occurs naturally in many agricultural products such as fruits, vegetables, wine, olive oil, and coffee [85].

Besides its well-known antioxidant activity [86,87] H_3CAF inhibits certain enzyme activities such as lipoxygenases, cyclooxygenase, glutathione S-transferase, and xanthine oxidase [88–92]. H_3CAF has been reported also to have antitumor activity [93,94].

A study by Fang et al. [95] showed that tea catechins are effective inhibitors of human DNMT-mediated DNA methylation in cultured cancer cells and that caffeic acid and chlorogenic acid (two

common catechol-containing coffee polyphenols) are strong inhibitors of DNA methylation in vitro, with apparent IC50 values of 3.0 and 0.75 mM, respectively, for inhibition of a bacterial homologue of human DNA methyl transferase, and 2.3 and 0.9 mM, respectively, for inhibition of DNMT1-mediated DNA methylation.

In 2015 the aim of our study was to develop potential anti-cancer drugs with tumor regression capacity selecting caffeic acid, catechol-containing coffee polyphenol as a ligand for coordinating dibutyltin(IV) moiety [5]. Both the moieties, caffeic acid and organotin(IV) acted as cytotoxic compounds, interfering with apoptosis pathways and epigenetic regulation in cancer cells.

4.3.1. Chemical synthesis and structural characterization

Dibutyltin(IV) complex of caffeic acid was obtained by refluxing methanolic solution of the free acid and dibutyltin oxide in 1:1 mole ratio, resulting in complex with general formula [$Bu_2SnHCAF$] [5]. The coordination environment at the tin center was investigated spectroscopically through a series of techniques, both in solid and solution phases. Density functional theory (DFT) study confirmed the proposed structures in solution phase as well as the determination of the most probable stable conformation.

Caffeic acid possesses two possible chelating sites in competition: the catechol (ortho-dihydroxybenzene) and the carboxylic acid functions (Fig. 7A).

The FTIR data revealed that the complex is monomer and that the free ligand caffeic binds the metal as dianionic bidentate ligand through the catecholic functions. The most relevant absorption bands and their assignment, both for the free and the coordinated caffeic acid, are reported in Table 3A. Disappearance of vibrations was observed in the spectrum of complex for stretching $\nu(OH)_{ar}$, and bending $(OH)_{ar}$ in and out of plane. Persistence of 3058 cm^{-1} ascribed to the stretching $\nu(OH)_{COOH}$ of carboxylic function allowed to rule out the involvement of –COOH in coordination around the organotin(IV)moiety [96–99].

The $^{119}Sn\{^1H\}$ cross polarization magic angle spinning ($^{119}Sn\{^1H\}$ CP-MAS) spectra value shows a single sharp signal at −15.47 ppm, typical of four coordinated tin centers, in a tetrahedral structure (Fig. 7B), and in agreement with the monomeric structure suggested for solid state [100–103].

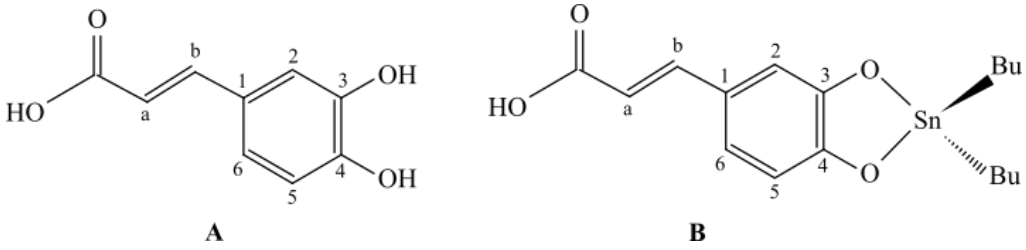

Fig. 7. (A) Caffeic acid, H_3CAF and **(B)** $Bu_2Sn(IV)HCAF$ proposed structure, with the numbering scheme referred to the NMR assignments (see Table 1C).

Electrospray ionization mass (ESI-MS) spectrum revealed the peaks relative to the monomeric adducts that $Bu_2SnHCAF$ forms with the $SnBu_2$ group and with alkali metal ions like Na^+ (Table 3B). In solution phase, spectroscopic UV–vis and fluorescence results support that chelation occurs at the o-dihydroxy sites in complex $Bu_2SnHCAF$.

The experimental ^{119}Sn, ^{1}H, $^{13}C\{^{1}H\}$ NMR data (Table 3C) indicated that tin is pentacoordinated, where the fifth coordination is attained by the presence of the coordinated solvent DMSO-d_6 molecule in the tin coordination sphere [104–106].

Density functional theory study confirmed the proposed structures in solution phase and indicated the most probably stable conformation [5].

Table 3. Spectroscopic data of butyric acid (H_3CAF) and $Bu_2SnHCAF$ complex.

(A)

The wavenumbers (cm^{-1}) and assignments of more relevant bands occurring in the experimental FT-IR[a]

Assignment[b]	**H_3CAF**	**$Bu_2SnHCAF$**
$\nu(OH)_{ar}$	3434 s 3231 s	- -
$\nu(OH)_{COOH}$	3058 m	3058 m
$\nu(OH)_{ar}$	2834 2572	-
$\nu(C=O)$	1645 s	1633 s
$\beta(OH)_{ar}$	1296	-
$\nu(C\text{-}OH)$	1220	-
$\gamma(OH)_{ar}$	596	-
$\nu(Sn–C)$	-	551 w
$\nu(Sn–O)$	-	n.o.[c]

(B)

ESI-MS data

Positive-ion MS (*m/z*)

412 $[M+H]^+$, 310 $[M–COOH–butyl+H]^+$, 275 $[M–COOH–2butyl+Na]^+$, 253 $[M–COOH–2butyl+H]^+$

(C)

Selected ^{1}H NMR, $^{13}C\{^{1}H\}$ and $^{119}Sn\{^{1}H\}$ NMR chemical shifts (ppm) in $CDCl_3$ at 298 K. Integration values are given in square brackets, coupling constants in parentheses (absolute values in Hz).[d]

$\delta(^{1}H)$	**H_3CAF**	**$Bu_2SnHCAF$**
COOH	12.07	11.78
OH(3)	9.08	-
OH(4)	9.48	-
H_α	-	1.41, 1.51, 1.65
H_β, H_γ[e]	-	1.29
CH_3	-	11.78
$\delta(^{13}C)$		
COOH	167.83	n.o.[c]

C(3)	145.52	153.94
C(4)	148.09	158.10
$CH_{2\alpha}$	-	26.50, 26.72
$CH_{2\beta}$	-	25.78
$CH_{2\gamma}$	-	24.43
CH_3	-	13.50
δ(^{119}Sn)		−129.00

[a] s, strong; m, medium; w, weak.
[b] *ν*, stands for stretching vibrations, β and γ, stand for bending vibrations, ar = aromatic.
[c] n.o., not observed.
[d] Numbering of butyl group in di- and tributyltin moiety: CH_3–$CH_{2\gamma}$–$CH_{2\beta}$–$CH_{2\alpha}$–Sn.
[e] It was not possible to discriminate the separate resonances for each carbon.

4.3.2. Biological Study

The antitumor potential of caffeic acid has been well established in different tumor cell types [107]. For this reason, we chose different tumor cell lines to test the effectiveness of caffeic organotin derivatives. The data obtained by biological evaluations showed the potential anti-tumor activity of Bu_2SnHCAF on MDA-MB 231 breast cancer, HCT116 colon cancer, HpG2 hepatocarcinoma cells. Bu_2SnHCAF complex induced a time and dose dependent reduction of cell number in tumor cell tested, while in non-tumor Chang liver cells, its effects were very modest (Fig. 8A).

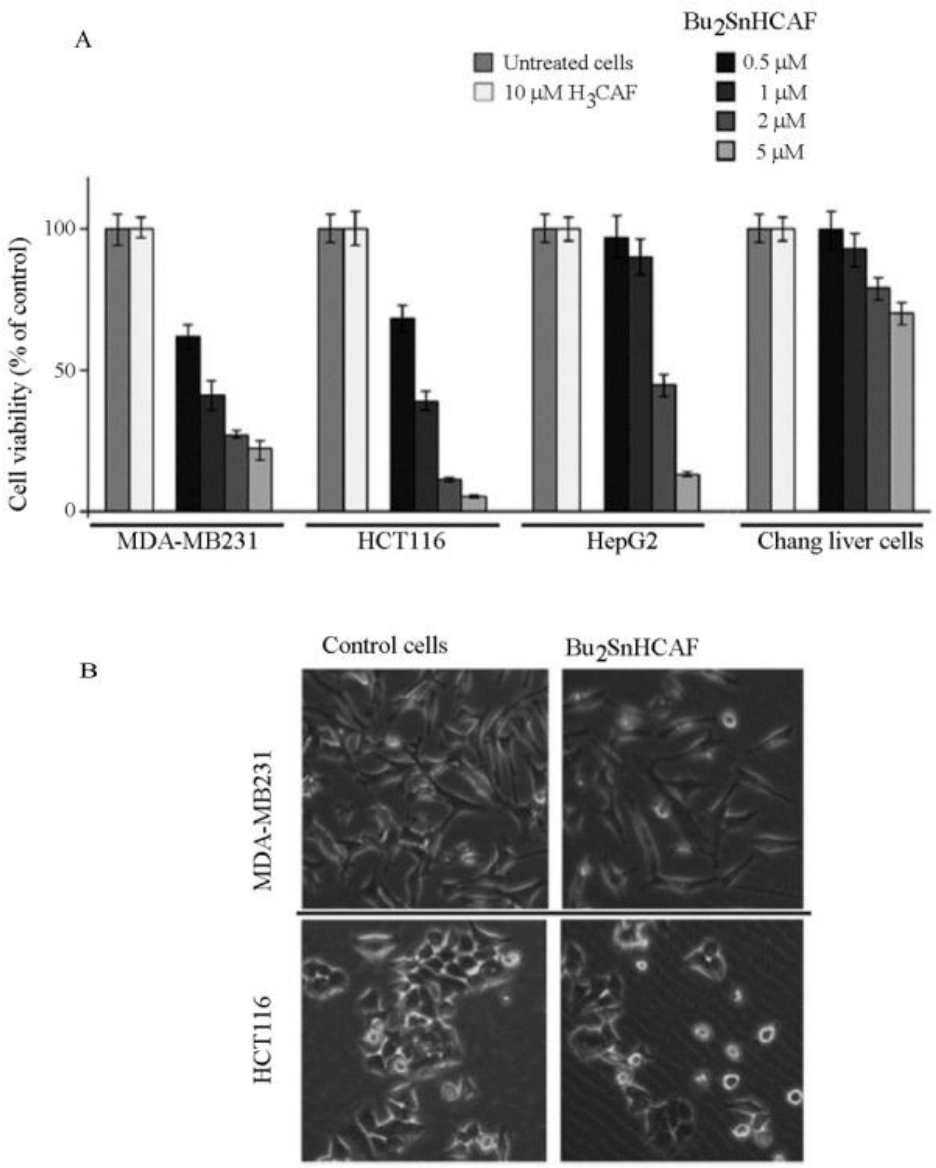

Fig. 8. $Bu_2SnHCAF$ treatment induces reduction of cell number in MDA-MB 231, HCT 116 and HepG2 cells. **A**. MDA-MB 231, HCT 116, HepG2 and Chang liver cells were incubated for the indicated times with 10 μM H_3CAF or different doses (0.5–5μM) of $Bu_2SnHCAF$. Cell viability was estimated by MTT assay and expressed as the percentage of the control value. **B**. Effects of 1 μM $Bu_2SnHCAF$ for 24 h on cell morphology. Cell morphology was evaluated using light microscopy. [*modified from [5]*]

$Bu_2SnHCAF$ was active when employed at low concentration (1 μM) while only high doses of H_3CAF generated biological effects. Moreover, $Bu_2SnHCAF$ was able to modulate its parental organotin toxicity as demonstrated by morphological analysis that showed typical features of necrosis induced by the parental compound Bu_2SnCl_2 (not shown).

The antitumor effects of $Bu_2SnHCAF$ was mainly related with both cell cycle arrest and pro-apoptotic inducing properties as evidenced by analysis of the cell cycle distribution, decrease in Cdc2 levels as well as propidium iodide positivity and loss of mitochondrial transmembrane potential. The induction

of apoptotic cell death was also confirmed by caspase-3 activation and concomitant caspase dependent cleavage of PARP (not shown).

As concerns epigenetic effects, it is well known that caffeic acid can modulate DNMT1-mediated DNA methylation [95]. Aberrant methylation represent another important epigenetic modification that is associated with a number human tumors [12]. Our findings indicate that $Bu_2SnHCAF$ treatment was able to induce a time-dependent reduction of global DNA methylated status (Fig. 9A). This effect was also confirmed by a concomitant reduction in DNA (cytosine-5)-methyltransferase 1 expression level (Fig. 9B). The effect induced by $Bu_2SnHCAF$ was more evident compared to that induced by H_3CAF, thus sustaining its therapeutic potential.

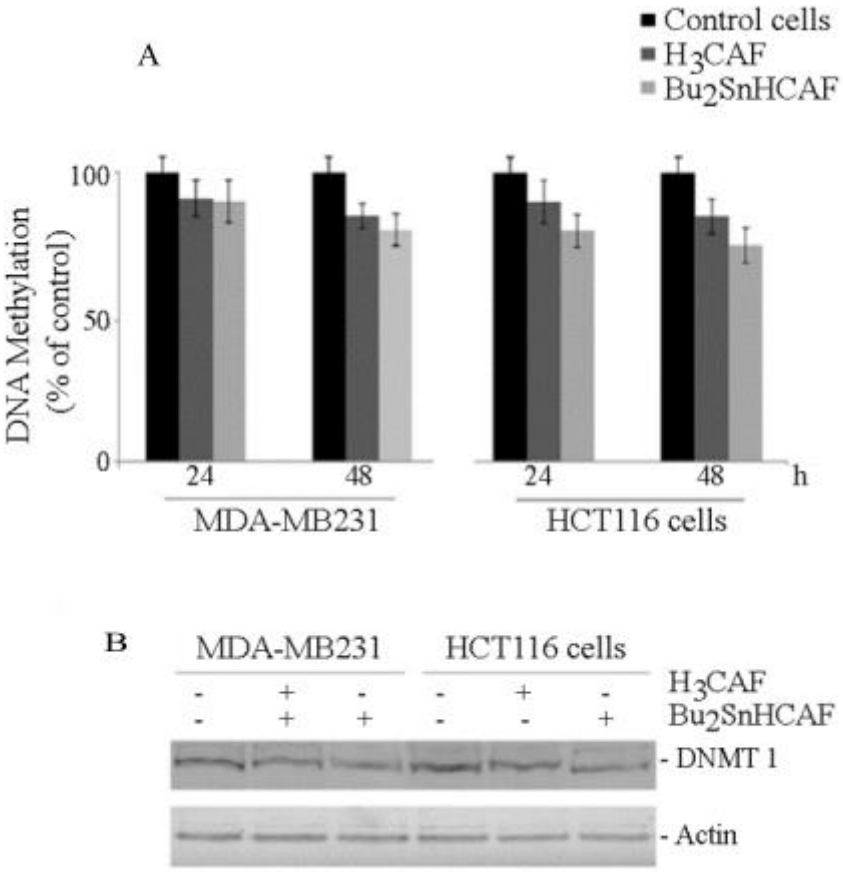

Fig. 9. $Bu_2SnHCAF$ induced a reduction of global DNA methylation. **A**. Cells were treated for 24 and 48 h with 1 μM $Bu_2SnHCAF$ or 10 μM H_3CAF and then DNA was extracted. Imprint DNA Methylation Kit was used to evaluate global DNA methylation. Methylated DNA control was included. Percent methylation relative to the global DNA methylation from control cells was reported. **B**. Representative western blots of DNMT1 levels in MDA-MB 231 and HCT116 cells. After treatment for 24 h with 10 μM H_3CAF and 1 μM $Bu_2SnHCAF$. Cell lysates were analyzed by immunoblotting using specific antibodies. Actin blots were included as a loading control. [*modified from [5]*]

5. Conclusions: questions and limits on epigenetic therapies

HDAC inhibitors represent a promising new group of anticancer agents, even though the mechanisms of HDAC inhibitor-induced tumor cell death require further elucidation: a substantial number of questions remain to be answered.

Critical questions remaining to be resolved are whether HDACI isoform specificity offers therapeutic advantages, or whether more broadly acting HDACIs (that is, pan-HDACis) will prove to be superior in the clinic.

The development of selective HDACIs with anticancer activities remains challenging in part because of the difficulty of probing the interaction of small molecules with mega-dalton protein complexes.

It is still not completely understood why HDACIs are relatively more toxic to transformed cells than to normal cells. This phenomenon may reflect the genetic dysregulation characteristic of malignant cells and/or an impaired capacity of such cells to respond to noxious stimuli as compared with their normal counterparts.

More recently, it has been shown that following HDACI treatment, transformed cells show reductions in DNA-repair proteins (for example, RAD50, MRE) and are selectively impaired in their ability to repair DNA damage [108]. It is therefore possible that the selectivity HDACIs exert toward transformed cells represents the combined consequences of increased ROS generation in neoplastic cells in conjunction with disruption of DNA-repair processes. Whether these events are responsible for or contribute to the therapeutic index of HDACIs remains to be determined.

Conflicting evidence exist in literature regarding the effect of DNMT inhibitors on normal cells.

Additionally, the understanding of different mechanisms as well as the long term safety of DNMT inhibitors on normal cells alone or in combination with standard treatments remains very limited and requires further research efforts.

Also another future goal is to identify compounds able to enhance the therapeutic index and protect the non-malignant tissues from side effects.

Although the efficacy of the first-generation epigenetic drugs (epi-drugs) in tumor management has been disappointing, there is an increasing evidence showing that epigenome modulation, in synergy with other therapeutic approaches, could play an important role in cancer treatment, reversing acquired therapy resistance.

Among the options that could contribute to successful clinical applications of epidrugs are new technologies for safer and more efficient cancer cell epigenome modulation.

One future perspective could be to design more precise and safer nanoscale organic and inorganic nanomaterials for drug delivery (e.g., dendrimers, micelles, liposomes, gels, metal- and carbon-based

nanomaterials). Several nanomaterials have been successfully studied and introduced in cancer treatment, and many others are undergoing clinical trials. Encapsulation by intelligent nanocarriers of antitumor drugs, conventional chemotherapeutics, epi-drugs, or both, can improve their solubility and stability by protecting the drugs from fast clearance and degradation, thus prolonging their half-life in the systemic circulation [109].

Acknowledgments

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