Protein expression and modulation of Carnitine palmitoyltransferase I in human carcinomas: a novel role in histone deacetylation?

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Financial support: This work was supported in part by grant from Sigma Tau Pharmaceutical S.p.A.

Keywords: Carnitine palmitoyltransferase I (CPT I), Fatty acid synthase (FAS), Histone deacetylase activity (HDAC), acetyl-histone H4, cancer.

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ABSTRACT

CPT I catalyzes the transport of long-chain fatty acids into mitochondria for β-oxidation. A link between CPT I and apoptosis has been suggested on the basis of several experimental data. On the other hand, Fatty acid synthase (FAS), the major enzyme converting dietary carbohydrates to fatty acids, is highly increased in human tumours and its block *in vitro* seems to induce apoptosis.

Here, we studied the protein expression of FAS and CPT I in human carcinomas (n=30) and neoplastic cells (MCF-7). FAS significantly increased ($p \leq 0.04$), whereas CPT I decreased in the cytoplasm of the tumoural samples ($p \leq 0.04$). Furthermore, an unusual nuclear localization of CPT I was evident in the human tumours ($p \leq 0.04$) and in neoplastic cells.

Attempting to clarify the role of nuclear CPT I, we studied the histonic and not histonic acetylation status in the human carcinomas. The tumoural tissues showed hypoacetylation of histone H4, compared with controls ($p=0.02$). MCF-7 cells, markedly expressing CPT I in the nucleus, showed significantly higher levels of HDAC activity, compared with MCF-12F non neoplastic cells, that exhibit no CPT I nuclear staining. HDAC1 and CPT I proteins co-immunoprecipitated in nuclear extracts from neoplastic cells and the treatment with Sodium butyrate (a known inhibitor of HDAC activity) significantly decreased nuclear expression of CPT I and its bond to HDAC1.

The peculiar localization of CPT I in the nuclei of human carcinomas and the functional interaction demonstrated between nuclear CPT I and HDAC1 could point out CPT I as a new target for anticancer treatments able to modulate the transcription of genes related to cancer development by histonic acetylation at specific gene promoters.

INTRODUCTION

Biochemical studies have demonstrated that one of the multiple differences between tumoural cells and healthy counterparts consists in the metabolic pathway, the uptake of glucose and the glycolitic process. The altered metabolism of neoplasia would favor an increased cell survival, promoting the increase of tumour size and cancer aggressiveness. In particular, during apoptosis and neoplastic transformation, functional alteration of enzymes involved in the mitochondrial oxidative metabolism has been found (1-3).

Normal cells in most human tissues use dietary lipids. In contrast, neoplastic cells can also use endogenously synthetic fatty acids to satisfy their metabolic necessities; therefore they express high levels of fatty-acids-synthesizing enzymes. Several publications report an altered expression and/or activity of Fatty acid synthase (FAS) in tumours (4, 5). FAS is the major enzyme required for the anabolic conversion of dietary carbohydrate to fatty acids. In particular, colon carcinomas display high levels of FAS expression and elevated fatty acids synthesis when compared with normal colonic mucosa (6, 7). Furthermore, inhibition of FAS triggers apoptosis in human cancer cells. The apoptosis triggered by FAS inhibition would be dependent on high levels of malonyl-CoA, substrate of FAS (8). Malonyl-CoA acts as competitive inhibitor of CPT I, reducing oxidation of newly synthesized fatty acids. CPT I in fact resides at the outer mitochondrial membrane and together with CPT II and Carnitine/acyl-Carnitine Translocase (CaCT), transports long-chain fatty acids into mitochondria for β-oxidation. CPT I catalyzes the rate-limiting step in this process and is a site for intracellular regulation of fatty acid metabolism. Two isoforms of CPT I have been characterized, known as L-CPT1 (CPT1A) and M-CPT1 (CPT1B) in liver (L-) and muscle (M-) respectively, where the expression of each was initially described, showing overlapping tissue specific expression (9). While CPT1B is expressed in skeletal muscle, heart, testis and adipose tissue, CPT1A has a more widespread distribution.

Mitochondria have long been postulated to be involved in apoptosis (10,11). It was demonstrated that CPT I interacts with Bcl-2 protein, that regulates programmed cell death in several systems and it is also expressed at the outer mitochondrial membrane (12-14). Bcl-2 binding to CPT I may modulate sphingolipid metabolism in a yet to be defined way and it would control a cell death specific activity of CPT I at the mitochondrial membrane.

Taking into account the re-modulation of the metabolic pathway in the cancerous cells and the supposed role of CPT I as regulator of programmed cell death together with Bcl-2, we studied the protein expression of CPT I and FAS in human tumour tissues (n=30) and healthy counterparts (n=25). CPT I protein expression was also evaluated in breast epithelial neoplastic (MCF-7) and non neoplastic (MCF-12F) cell lines. Here we show that FAS expression increases in the cytoplasm of tumoural tissues, whereas CPT I decreases in the cytoplasm of human tumours and neoplastic cells. In contrast, a nuclear localization of CPT I is evident both in tumoural tissues and in neoplastic cells (MCF-7). CPT I was previously described in the nucleus (15), but its specific function in this cell compartment has not been clarified yet. However, in the nuclear environment, the protein would modulate the levels of acyl-CoA and carnitine, both of which have been implicated in the regulation of gene transcription (16). To better clarify the role of CPT I in the nucleus of tumours we studied the histonic and non histonic acetylation status in the same samples. The acetylation of H4 histone is much lower in the tumours analyzed, compared with controls. The expression of nuclear CPT I in MCF-7 correlates with an histone deacetylase (HDAC) activity significantly higher than in nuclear extracts from MCF-12F cells. Moreover, HDAC1 and CPT I proteins co-immunoprecipitate in nuclear extracts from neoplastic cells.

Sodium butyrate, a known inhibitor of histone deacetylase activity, is able to induce multiple effects on cultured mammalian cells that include inhibition of proliferation, induction of differentiation and induction or repression of gene expression (17,18). Treatment with

sodium-butyrate induces a significant decrease of CPT I nuclear expression. The binding between HDAC1 and CPT I also decreases, following Na-butyrate treatment.

Here we hypothesize a new role of CPT I in cancerogenesis, further than the physiological role in the transport of long-chain fatty acids into mitochondria for β-oxidation. In fact, the presence of CPT I in the nuclei of cancerous tissues and neoplastic cells, the association with histonic hypo-acetylation in human tumours and the disclosed functional link between CPT I and HDAC1, show that CPT I play a role in the hypoacetylation status of the tumours. As a consequence, it could be involved in the transcriptional regulation of specific genes related to cancer development.

Hence, the nuclear form of CPT I might represent a new target for anti-cancer treatments that, acting alike HDAC inhibitors, maintain histones in an acetylated state and through the resulting alterations in gene regulation, induce apoptosis.

RESULTS

Fatty acid synthase expression in human tumours by IHC. FAS protein was significantly increased in the cytoplasm of the malignant tumours analyzed (tumours *vs* controls: p=0.003) (Table 1). Only 13% of tumours (4/30) displayed absence or weak staining in the cytoplasm of neoplastic cells, versus 83% of controls (25/30). Further, strong (2+) specific staining was detected in the 54% of positive tumours (14/26) (Table 1). In particular, colon carcinomas displayed 45% of strongly positive cells, compared with almost the 100% of weakly stained cells in normal mucosa (Fig. 1C-F).

Expression of CPT I in human tumours by IHC. The same tumours were also evaluated for CPT I protein expression. Corresponding sections of distant normal mucosa (n=11) were used as controls for the colorectal patients, whereas adjacent-to-tumour normal tissue (ANNT, n=9) and non neoplastic breast samples (NB, n=12) was examined for the other cases.

The 67% of the tumoural tissues analyzed (20/30) displayed absent or weak cytoplasmic staining for CPT I, whereas normal samples displayed moderate(1)-to-strong(2) staining in 80% of cases (tumours *vs* controls: $p \leq 0.04$) (Table 1). The cytoplasmic staining showed the typical granular pattern due to the mitochondrial localization of CPT I. Moreover, 8/11 colorectal cancers (73%), 11/12 breast cancers (92%) and 6/7 other tumours (86%) showed a speckled nuclear staining, with percentage >20% of positive cells in 5/25 cases. In particular, the colorectal carcinomas showed a significant decrease of cytoplasmic expression compared with both healthy colonic mucosae and adenomas analyzed (CRC *vs* NM: p=0.001 and *vs* ALGD: p=0.04) (Fig. 1G-L). Colon adenomas displayed a protein localization and expression comparable to their normal counterparts (ALGD *vs* NM: p=0.06). In Figure 2A and 2B, human hepato-carcinoma and ductal infiltrating breast carcinoma were compared to adjacent

non neoplastic tissue. A decreased expression of cytoplasmic CPT I and a specific nuclear staining in the cancerous cells were evident.

Expression of acetyl-lysine and acetyl-histone H4 in human tissues. Colorectal tumours (n=11) and human carcinomas from other tissues (OT n=7), together with corresponding normal tissues (distant normal mucosae for the colorectal cases and adjacent normal tissues for the others) were analyzed for expression of acetyl-histone H4 and whole cell acetylation status, by IHC. A significant decrease of acetylated-histone H4 was evident in nuclei of CRC, compared with the normal counterparts (NM *vs* CRC: p=0.02; OT *vs* ANNT p=0.3, Table 2). The hypoacetylation at histone level could represent a known mechanism for transcriptional repression of *tumour suppressors genes* during carcinogenesis (25).

The staining with the anti-acetyl-lysine antibody (able to recognize the whole cell acetylation status) confirmed a decreased signal in the nuclei on the same samples (NM vs CRC p=0.03; ANNT *vs* OT p=0.05), but also showed an increased cytoplasmic staining in tumours, as compared with controls (ANNT *vs* OT p= 0.03) (Fig. 2C).

***In vitro* experiments. CPT I protein expression.** Detection of CPT I by immunocytochemistry (ICC) shows the nuclear staining markedly increased in MCF-7 compared with MCF-12F normal cells, which displayed a low staining intensity, both in the nucleus and in the cytoplasm (Fig. 3A). CPT I nuclear staining varied in intensity through the cell cycle, showing the highest level in condensing chromosomes, as cells progress through mitosis (not shown).

Nuclear protein extracts from tumoural (MCF-7) and not tumoural (MCF-12F) cell lines were analyzed for CPT IA expression with specific rabbit polyclonal antibody by western blot analysis. We were able to identify both 86kDa and 92kDa bands in the breast epithelial cells (Fig. 3B). CPT I was evident in the cytoplasmic extracts from both MCF-7 (lane 1) and MCF-12F (lane 2). The expression of nuclear CPT I was specific of neoplastic cells (lane 3).

Histone deacetylase (HDAC) activity. We tested HDAC activity on nuclear extracts from neoplastic (MCF-7) and normal cells (MCF-12F). The nuclear activity of histone deacetylase was significantly higher in neoplastic cells, than in normal cells (Fig.4). These results suggest a role for CPT I in the acetylation status of neoplastic cells.

Butyrate treatment was effective to strongly inhibit HDAC activity both on MCF-7 and MCF-12F cells (Fig. 4). The expression of CPT I nuclear protein was strongly decreased following treatment with Na-butyrate, on MCF-7 cells (Fig. 5A).

Co-immunoprecipitation experiment. Tumoural cells (MCF-7) were treated or not with sodium butyrate at the concentration 5mM, able to inhibit HDAC1 activity (Fig 4). The nuclear proteins were extracted and immunoprecipitated with anti-HDAC1 antibody. Untreated MCF-7 cells showed the co-immunoprecipitation of CPT I nuclear protein. This specific band was strongly reduced after Na-butyrate treatment (Fig. 5B).

DISCUSSION

This study shows, for the first time, the expression of CPT I in nuclei of human carcinomas and cancerous cells. The nuclear expression of CPT I is linked to histone hypoacetylation in the tumours analyzed. Further, a functional interaction is demonstrated between HDAC1 and nuclear CPT I. Conversely, the enzyme is significantly decreased in the cytoplasm of the tumours compared with control tissues and non-transformed cells, whereas the fatty acids synthase (FAS) expression is highly increased in the cytoplasm. The downregulation of mitochondrial CPT I would prevent oxidation of newly synthesized fatty acids in human carcinomas. The altered metabolism in tumours can provide a chance to chemotherapy that does not affect normal cycling cells (8).

Several studies provide data about a potential link between CPT I and regulation of apoptosis. Nevertheless, results are contradictory about the effective role of CPT I in cell survival control and cancer development (8,12,26,27). All these works take into account the physiological mitochondrial enzyme, without considering the peculiar distribution of CPT I that we have found in cancerous tissues. Moreover, previous *in vitro* experiments used etomoxir to irreversibly inhibit CPT I activity. Nevertheless, it is known that etomoxir blocks mitochondrial fatty acid oxidation, but does not affect extra-mitochondrial CPT I activity (8). Further, it does not appear that etomoxir has cytotoxic effect on MCF-7 cells (8,26) and induces apoptosis when added *in vitro* in neoplastic cell lines (manuscript in preparation, Pucci S. *et al.*).

Therefore, we have tried to clarify the role of cytoplasmic and nuclear CPT I modulation seen in the tumoural samples. In the tumours analyzed, nuclear expression of CPT I was related to an hypoacetylation status at histone level. *In vitro*, we further demonstrated that HDAC activity was much higher in nuclear extracts from neoplastic cells, than from normal counterparts. Concomitantly CPT I nuclear protein co-precipitates with HDAC1 protein, as seen in neoplastic breast epithelium.

Histone deacetylation is associated with transcriptional repression of genes, as the removal of acetyl groups from lysine residues limits accessibility of the DNA for transcription (28). Hypoacetylation at specific promoters has been correlated with recruitment of HDAC complexes to repressed genes. The discovery that certain HDAC complexes contained known transcriptional repressors further strengthened the correlations between hypoacetylation and repression (28). HDAC inhibitors from a number of chemical classes (e.g. short fatty acids like butyrate and valproic acid) have shown promise as anti-cancer agents in animal studies and early clinical trials (29-32). In fact, the HDAC inhibitors maintain histones in an acetylated state, and through the resulting alterations in gene regulation, inhibit cell cycle progression, differentiation and in some cases induce apoptosis (32,33). Exposure to HDAC inhibitors may also allow reactivation of tumour suppressor genes by hypoacetylation during tumourigenesis (33).

The use of Na-butyrate to specifically inhibit HDAC1 activity, surprisingly induced, in our model, a decrease of both CPT I nuclear expression and HDAC1 protein bond to CPTI.

Thus, CPT I in the nucleus of neoplastic cell could be responsible in the recruitment of HDAC complexes at specific promoters, to silence genes involved in the control of cancer development, such as tumour suppressor genes.

On the other hand, our experiments performed with anti-acetyl-lysine antibody showed an hyper-acetylation (most likely of non histonic proteins) in the cytoplasm of human tumours, beside a nuclear histone hypo-acetylation. We can speculate that different mechanisms of post-translational regulation of target proteins could be involved in cell growth/death regulation (34,35). Nonetheless, nuclear CPT I could represent an highly specific target for new, more selective and more effective anti-cancer therapies. In fact, drugs suppressing specifically the nuclear activity of CPT I might allow reactivation of *tumour suppressors* silenced by hypoacetylation, alike molecules who directly inhibit HDAC activity.

METHODS

Patient characteristics and tissue samples

Eleven colorectal adeno-carcinomas (CRC), twelve breast invasive ductal carcinomas (IDC) and seven tumours from other tissues (OT: stomach n=2, ovary, lung, liver, prostate and bladder) were collected in the authors' department, irrespectively of the clinical staging. Informed consent was obtained from all the patients included in the study. Ethical approval was obtained by the authors' institutional review board. The mean age of the patients at the time of surgery was 69±5 and the male/female ratio was 2,6. Adjacent-to-tumour normal tissue (ANNT, n=7) at a distance of 1 cm from neoplasia was examined for tumours from other tissues (n=7) and were used as controls. Distant normal mucosa (NM, n=11) and adenomas with low grade dysplasia (ALGD, n=6) were collected and analyzed for the colorectal cases. Histological classification was carried out on H&E-stained slides (19-21).

Tissue microarrays

Tissue microarrays (TMA) were performed for breast cancer cases. Three 0.6 mm^2 cores of breast cancer tissue were removed from representative tumour areas on each block identified by the pathologist (EB). These cores were used to construct recipient array blocks in triplicate (24 cores per block). Each TMA was composed of 12 infiltrating ductal carcinomas (IDC) (3G1; 6G2; 3G3), and non neoplastic breast tissues (NB) (n=12: 5 fibroadenomas, 4 usual ductal hyperplasia or UDH, 3 adenosis) as controls. Adjacent normal breast were occasionally present and evaluated in the IDC cores. If one or more core was uninformative because of loss or lack of tumour in core then the scoring results were taken from the remaining core(s).

The tissue array were sectioned, stained with hematoxylin-eosin and received a final pathology review. For immunohistochemistry, the tissue array was cut in 5µm sections and stained with appropriate antibodies and controls.

Immunohistochemistry (IHC) and Immunocytochemistry (ICC)

Serial four micron sections from formalin-fixed and paraffin embedded specimens were immunostained for CPT IA, FAS, acetyl-lysine and acetyl-histone-H4 following the streptoavidin-biotin method, as previously described (22). In brief, sections were deparaffinezed and rehydrated in decreasing alcohol. Tissue antigen retrieval was performed in citrate buffer by microwave. Endogenous peroxidase activity was quenched with 0.03% hydrogen peroxide in absolute methanol, for 30 min at room temperature. The primary antibodies used were rabbit polyclonal anti-CPT IA (kindly provided by Sigma Tau S.p.A.) and anti-FAS antibodies (H300, Santa Cruz Biotechnology Inc. CA 95060, USA) validated for immunohistochemical analysis, in 1:650 and 1:50 dilution, respectively. Anti-acetyl-lysine rabbit serum and rabbit polyclonal anti-acetyl-histone H4 (Upstate, NY 12946) were diluted 1:400 and 1:75 respectively. Biotinylated goat anti-rabbit IgG (Dako A/S, Denmark) were used as secondary antibody. After washing, sections were treated with streptoavidin-peroxidase reagent (Dakopatts A\S, Denmark), incubated with diaminobenzidine (DAB) and lightly counterstained with hematoxylin. Slides were examined by two pathologists, unaware of the clinical data and molecular results. Tissue staining was semi-quantitatively graded for intensity as negative/weak: 0, moderate: 1 and strong: 2. FAS and cytoplasmic CPT I was regarded as positive when moderate-to-strong granular cytoplasmic staining was detected (6, 7). Acetyl-lysine and acetyl-histone-H4 were deemed positive when moderate(1)-to-strong(2) immunostaining was present in cytoplasm and/or nuclei. For nuclear CPT I, positively stained cells were quantified as a percentage and assigned to one of three categories: 0-10% (Neg); 11-20% and >20% (Pos) (Tables 1 and 2).

For immunocytochemistry, MCF-7 and MCF-12F cells were plated in 4 wells/chamberslides at a concentration of 60.000 x cm^2. After overnight culture, cells were treated or not with sodium butyrate (Sigma-Aldrich S.r.l., I-20151 MI, Italy) 5mM for 24h, to inhibit cellular HDAC activity. At the end, growth medium was removed and cells were rinsed in phosphate-buffered saline (PBS) and fixed in formalin 10% solution (Sigma-

Aldrich) for 5 min. Then, cells were permeablized with 0,5% Triton X-100 and 0.05% Tween-20 (Bio-Rad Laboratories, Munchen) in PBS. Incubation and dilution times of primary antibody (rabbit polyclonal anti-CPT IA), secondary antibody (biotinylated goat anti-rabbit IgG) and following reagents (streptoavidin-peroxidase) were the same chosen for IHC experiments. After washing, slides were incubated with diaminobenzidine (DAB) and counterstained with haematoxylin.

Statistical analysis

All values provided in the text and figures are means of three independent experiments ± standard deviations (SD). Mean values were compared using the two-tailed Student *t*-test, for independent samples. For FAS and cytoplasmic CPT I immunostaining, statistical significance was calculated comparing intensity values (from 0 to 2) between different groups, e.g. FAS staining values in normal mucosae (NM) *versus* adenocarcinomas (CRC). Percent values of positively stained cells were compared between different groups, for CPT I nuclear staining (see Results). Differences were considered statistically significant for $p < 0.05$.

Cell culture, chemicals and protein extracts preparation

MCF-7 (HTB-22) and MCF-12F (CRL-10783) were purchased from American Type Culture Collection (ATCC) and grown in complete culture medium, according to conditions suggested from ATCC, at 37°C temperature with 95% humidified air, 5% CO_2. For HDAC experiments, 60.000 x cm^2 cells were plated in 25 cm^2 flasks. After overnight culture, cells (treated or not with sodium butyrate, 5mM for 24hours) were trypsinized, washed in PBS and pelleted for protein extraction according to Dignam method (23), modified as described by Pucci et al. (24). Briefly, nuclear and cytoplasmic fractions were separated by centrifugation in a microfuge at 10,000 rpm and stored at –80°C. Protein content in nuclear and cytoplasmic extracts was determined in triplicate by Bradford assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Munchen).

Western Blotting and Immunoprecipitation

Nuclear extracts (10µg) were boiled in SDS-PAGE loading buffer and separated by 10% SDS-PAGE. Proteins were transferred to a PVDF membrane (Hybond-P, Amersham-Pharmacia Biotech, England HP7 9NA) using an electroblotting apparatus. Membranes were stained with Poinceau S dye, to check for equal loading and homogeneous transfer and incubated for 1h at RT with 3% skim milk (Difco Lab. Detroit, MI, USA) and 0.5%Tween 20 (USB, Cleveland, Ohio, USA). Primary antibody (rabbit polyclonal anti-CPT IA, Sigma Tau S.p.A.) was diluted 1:1000 in 1% BSA and incubated for 1h at room temperature; samples were washed extensively with 0.5% Tween-20 in Tris buffered saline and diluted 1:8000 secondary antibody (anti-rabbit-HRP IgG, Santa Cruz Biotechnologies, Inc.) was added in 1%BSA, 1%milk, 0.5% Tween-20, for 45' at room temperature. Filters were reprobed with (Sigma-Aldrich, Saint Louis, Missouri 63103 USA) anti-Sp1 mouse IgG_1 monoclonal antibody, to test purity of the nuclear extracts. Filters were washed and developed using an enhanced chemiluminescence system (ECL, Amersham-Pharmacia Biotech, Pharmacia Biotech, England HP7 9NA).

Immunoprecipitation assay (IP) was performed on nuclear extracts from tumoural cells (MCF-7) treated and not with inhibitor of HDAC activity, sodium butyrate. Fifty micrograms of nuclear extracts were immunoprecipitated with anti-HDAC1. In brief, protein extracts were pre-cleared incubating them with Protein A-Agarose (50% slurry) for 30 minutes at 4°C with agitation. The Agarose was removed, then 4µg of anti-HDAC1 mouse monoclonal antibody (Upstate, NY12946) was added overnight at 4°C with rotation. IP negative control was performed without HDAC1 antibody. After incubation, Protein A-Agarose was added for 1 hour at 4°C with rotation. At the end, supernatants containing unbound proteins were carefully removed and the IP complexes were washed in wash buffer (PBS, NaCl 0.5M, and after in PBS alone). Each immunoprecipitates was electrophoresed on a 10% poliacrylamide gel. Gel was stained with Ponceau S-dye, blotted onto PVDF membrane, probed with anti-

CPT I and anti-HDAC1 and detected with ECL. Supernatant from HDAC1-IP samples, containing unbound protein fraction (UB) was also loaded on poliacrylamide gel.

Histone deacetylase (HDAC) activity

Nuclear protein extracts obtained from MCF-7 and MCF-12F cells were used to detect HDAC activity with a colorimetric assay kit (BioVision Research Products, Mountain View, CA 94043 USA). In brief, HDAC colorimetric substrate, which comprise an acetylated lysine side chain, was added with 50µg of nuclear protein extracts to each well of a 96-well plate. A 2-µl volume of 1 mg/ml of the HDAC inhibitor trichostatin A was added to control well. Deacetylation of the substrate sensitized it, so that, following treatment with Lysine Developer produced a chromophore. Samples were analyzed using an ELISA plate reader at 405 nm.

REFERENCES

1. Helmlinger G, Yuan F, Dellian M, Jain RK. 1997. Interstitial pH and pO2 gradients in solid tumours in vivo: high-resolution measurements reveal a lack of correlation. *Nat Med*. **3**: 177-82.
2. Sutherland RM. 1988. Cell and environment interactions in tumour microregions: the multicell spheroid model. *Science* **240**(4849):177-84.
3. Wang GL, Jiang BH, Rue EA, Semenza GL. 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *PNAS USA*, **92**: 5510.
4. Pizer ES, Lax SF, Kuhajda FP, Pasternack GR, Kurman RJ. 1998. Fatty acid synthase expression in endometrial carcinoma: correlation with cell proliferation and hormone receptors. *Cancer* **83**(3): 528-37.
5. Alo' PL, Visca P, Marci A, Mangoni A, Botti C, Di Tondo U. 1996. Expression of fatty acid synthase (FAS) as a predictor of recurrence in stage I breast carcinoma patients. *Cancer*, **77**(3), 474-82.
6. Visca P, Alo PL, Del Nonno F, Botti C, Trombetta G, Marandino F, Filippi S, Di Tondo U, Donnorso RP. 1999. Expression of fatty acid synthase (FAS) as a predictor of recurrence in stage I breast carcinoma patients. *Clin Cancer Res*. **5**(12): 4111-8.
7. Rashid A, Pizer ES, Moga M, Milgraum LZ, Zahurak M, Pasternack GR, Kuhajda FP, Hamilton SR. 1997. Elevated expression of fatty acid synthase and fatty acid synthetic activity in colorectal neoplasia. *Am J Pathol.* **150**(1): 201-8.
8. Zhou W, Simpson PJ, McFadden JM, Townsend CA, Medghalchi SM, Vadlamudi A, Pinn ML, Ronnett GV, Kuhajda FP. 2003. Fatty acid synthase inhibition triggers apoptosis during S phase in human cancer cells. *Cancer Res.* **63**: 7330-7.

9. Weis BC, Esser V, Foster DW, McGarry JD. 1994. Rat heart expresses two forms of mitochondrial carnitine palmitoyltransferase I. The minor component is identical to the liver enzyme. *J Biol Chem*. **269**(29): 18712-5.
10. Griffin JL and Shockcor JP. 2004. Metabolic profiles of cancer cells. *Nat Rev Cancer* **4**(7): 551-61.
11. Mazurek S, Eigenbrodt E. 2003. The tumour metabolome. *Anticancer Res*. **23**(2A): 1149-54.
12. Paumen MB, Ishida Y, Han H, Muramatsu M, Eguchi Y, Tsujimoto Y, Honjo T. 1997. Direct interaction of the mitochondrial membrane protein carnitine palmitoyltransferase I with Bcl-2. *Biochem Biophys Res Commun.* **231**(3): 523-5.
13. Reed JC. 1994. Bcl-2 and the regulation of programmed cell death. *J Cell Biol.* **124**(1-2): 1-6.
14. Nunez G, Clarke MF. 1994. The Bcl-2 family of proteins: regulators of cell death and survival. *Trends Cell Biol*. **4**(11): 399-403.
15. Bremer J. 1997. The role of carnitine in cell metabolism. In *Carnitine today.* C. De Simone, G. Famularo, editors. Springer-Verlag Press: Heidelberg. 1-37.
16. Murthy MS, Pande SV. 1994. A stress-regulated protein, GRP58, a member of thioredoxin superfamily, is a carnitine palmitoyltransferase isoenzyme. *Biochem J.* **304** (Pt 1): 31-4.
17. Davie JR. 2003. Inhibition of histone deacetylase activity by butyrate. *J Nutr.* **133**(7 Suppl): 2485S-2493S.
18. Tracey Davis. Histone Deacetylase Inhibitors Decrease Proliferation and Modulate Cell Cycle Gene Expression in Normal Mammary Epithelial Cells.
19. O'Brien MJ, Winawer SJ, Zauber AG, Gottlieb LS, Sternberg SS, Diaz B, Dickersin GR, Ewing S, Geller S, Kasimian D, *et al*. 1990. The National Polyp Study. Patient and

polyp characteristics associated with high-grade dysplasia in colorectal adenomas. *Gastroenterology* **98**: 371-379.

20. Jass JR and Sobin LH. 1989. *Histological typing of intestinal tumours: World Health Organization International Histological Classification of Tumours*. Springer-Verlag Press: Berlin.
21. Rampaul R.S., Pinder S.E., Elston C.W and Ellis I.O. on behalf of the Nottingham Breast Team 2001. Prognostic and predictive factors in primary breast cancer and their role in patient management: The Nottingham Breast Team. *EJSO* **27**: 229–238.
22. Hsu SM, Raine L, Fanger H. 1981. The use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase technique: a comparison between ABC and unlabelled antibody (PAP) procedures. *J Histochem Cytochem* **29**: 577-580.
23. Dignam JD, Lebovitz RM, Roeder RG. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res*. **1**: 1475-1489.
24. Pucci S, Mazzarelli P, Rabitti C, Giai M, Gallucci M, Flammia G, Alcini A, Altomare V, Fazio VM. 2001. Tumour specific modulation of Ku70/80 DNA binding activity in breast and bladder human tumour biopsies. *Oncogene* **20:** 739-747.
25. Kim DH, Kim M, Kwon HJ. 2003. Histone deacetylase in carcinogenesis and its inhibitors as anti-cancer agents. *J Biochem Mol Biol.* **36**(1): 110-9.
26. Paumen MB, Ishida Y, Muramatsu M, Yamamoto M, and Honjo T. 1997. Inhibition of carnitine Palmitoyltransferase I (by etomoxir) augments Sphingolipid Synthesis and Palmitate-induced Apoptosis. *J Biol Chem.* **272**(6): 3324–3329.
27. Tronstad KJ, Berge K, Berge RK, Bruserud O. 2003. Modified fatty acids and their possible therapeutic targets in malignant diseases. *Expert Opin Ther Targets* **7**: 663-677.

28. Khochbin S, Verdel A, Lemercier C, Seigneurin-Berny D. 2001. Functional significance of histone deacetylase diversity. *Curr Opin Genet Dev.* **11**(2): 162-6.

29. Moe-Behrens GH, Pandolfi PP. 2003. Targeting aberrant transcriptional repression in acute myeloid leukemia. *Rev Clin Exp Hematol*. **7**: 139-59.

30. Kelly WK, O'Connor OA, Krug LM, Chiao JH, Heaney M, Curley T, MacGregore-Cortelli B, Tong W, Secrist JP, Schwartz L, *et al*. 2005. Phase I study of an oral histone deacetylase inhibitor, suberoylanilide hydroxamic acid, in patients with advanced cancer. *J Clin Oncol.* **23**(17): 3923-31.

31. Ryan QC, Headlee D, Acharya M, Sparreboom A, Trepel JB, Ye J, Figg WD, Hwang K, Chung EJ, Murgo A, *et al*. 2005. Phase I and pharmacokinetic study of MS-275, a histone deacetylase inhibitor, in patients with advanced and refractory solid tumours or lymphoma. *J Clin Oncol.* **23**(17): 3912-22.

32. Romanski A, Bacic B, Bug G, Pfeifer H, Gul H, Remiszewski S, Hoelzer D, Atadja P, Ruthardt M, Ottmann OG. 2004. Use of a novel histone deacetylase inhibitor to induce apoptosis in cell lines of acute lymphoblastic leukaemia. *Haematologica* **89**: 419-426.

33. Esteller M, Villar-Garea A. 2004. Histone deacetylase inhibitors: understanding a new wave of anticancer agents. *Int J Cancer* **112**: 171–178.

34. Das C, Kundu TK. 2005. Transcriptional regulation by the acetylation of nonhistone proteins in humans-a new target for therapeutics. *IUBMB Life* **57**(3): 137-49.

35. Ito A, Kawaguchi Y, Lai CH, Kovacs JJ, Higashimoto Y, Appella E, Yao TP. 2002. MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. EMBO J. **21**(22): 6236-45.

FIGURE LEGENDS

Figure 1: Panels A-F show immunohistochemical FAS expression in a representative human breast tissue (panels A,B) and in two representative colon cancer cases (panels C,D,E,F). Panel **A** shows the H&E staining in a ductal carcinoma with 'lobular cancerization'. FAS protein is strongly (2+) increased in the cancerous part (see arrows), compared with the weak/moderate (0, 1+) expression evident in the non-transformed section (panel **B**). FAS expression increases also in two colon carcinomas (panels **D,F**) compared with the corresponding distant normal mucosae (panels **C,E**) (20X original magnification). **Panels G-L** show CPT I protein expression detected by immunohistochemistry in two representative colon cancer cases. The decrease of CPT I cytoplasmic staining is mostly evident in these colon carcinomas (panels H,L) compared with the corresponding distant normal mucosae (panels G,I) (40X original magnification).

Figure 2: Panel A shows CPT I protein expression on cirrhotic human liver (N, upper panel) and hepatocarcinoma (K, lower panel) from the same patient. The magnification 40X allows to discriminate the cytoplasmic localization of CPT I in the non neoplastic tissue and the specific nuclear staining on the tumoral section. Squared areas on the right upper side (20X magnification) better display the decrease of CPT I cytoplasmic expression in the tumoural section. **Panel B** shows CPT I protein expression in non neoplastic breast (N, upper panel) (20X) and in breast infiltrating ductal carcinoma (K, lower panel) (40X) from the same patient. Area in the square displays the entire breast tissue with both the normal and neoplastic area (20X magnification). **Panel C** shows the immunohistochemical expression of acetyl-lysine (informative for the global acetylation status of the tissue) on normal mucosa (N, upper panel) and colorectal cancer tissue (K, lower panel). The absence of nuclear staining is evident in the colon cancer tissue, compared with the normal control (40X). Right upper areas (20X) better show the increase of cytoplasmic staining in the tumoural section.

Table 1. FAS and CPT I protein expression detected by immunohistochemistry, in human tumours and controls.

		CRC % (n=11)	ALGD % (n=6)	NM[A] % (n=11)	OT % (n=7)	ANNT[B] % (n=7)	IDC % (n=12)	NB[C] %(n=12)
FAS								
Neg	Neg/weak	9 (1)	83 (5)	91 (10)	14 (1)	86 (6)	17(2)	75(9)
Pos	Moderate	46 (5)	17 (1)	9 (1)	14 (1)	14 (1)	50(6)	25(3)
	Strong	45 (5)	0 (0)	0 (0)	71 (5)	0 (0)	33(4)	(0)
CPT I *cyt*								
Neg	Neg/weak	73 (8)	17 (1)	0 (0)	71 (5)	14 (1)	58(7)	42(5)
Pos	Moderate	27 (3)	83 (5)	27 (3)	29 (2)	86 (6)	33(4)	33(4)
	Strong	0 (0)	0 (0)	73 (8)	0 (0)	0(0)	8(1)	25(3)
CPT I *n*								
Neg	0-10%	27 (3)	83 (5)	82 (9)	14 (1)	100 (7)	8(1)	83(10)
Pos	11-20%	64 (7)	17 (1)	18 (2)	71 (5)	0 (0)	67(8)	17(2)
	>20%	9 (1)	0 (0)	0 (0)	14 (1)	0 (0)	25(3)	0

Number of cases was shown as a percentage. In parentheses, absolute numbers were reported. CRC: colorectal adenocarcinomas; ALGD: adenomas with low-grade dysplasia (19). IDC: Breast invasive ductal carcinomas.
[A]Distant normal mucosa (NM) was tested for the colon samples. [B]Adjacent-to-tumor normal tissue (ANNT) was analyzed for tumours from other tissues (OT) (see Mat. and Methods). [C]NB: Non neoplastic breast tissues (fibroadenomas n=5, UDH n=4, adenosis n=3) were used as controls for the breast cancer cases.

FAS. NM *vs* CRC: p=0.003; ANNT vs OT: p=0.0; NB vs IDC: p=0.04 (see Statistical analysis in 'Mat. and Methods')

CPT I cyt. CRC *vs* NM: *p*=0.001; CRC *vs* ALGD: *p*=0.04; NB *vs* BDIC: p=0.04.

CPT I n. NM *vs* CRC: p=0.04; ANNT vs OT: p=0.0; NB vs IDC: p=0.03.

Table 2. Acetyl-histone H4 and acetyl-lysine protein expression detected by IHC, in human tumors and controls.

	CRC % (n=11)	NM % (n=11)	OT % (n=7)	ANNT % (n=7)
Acetyl-H4[A]				
Neg *n*	73 (8)	9 (1)	86(6)	57(4)
Pos *n*	27 (3)	91 (10)	14(1)	43(3)
Acetyl-lys[A]				
Neg *cyt*	36 (4)	45 (5)	0 (0)	86(6)
Pos *cyt*	64 (7)	55 (6)	100 (7)	14(1)
Neg *n*	64 (7)	27 (3)	86(6)	71(5)
Pos *n*	36 (4)	73 (8)	14(1)	29(2)

[A]Cases were deemed positive when moderate-to-strong (1-2) nuclear (*n*) and/or cytoplasmic (*cyt*) staining was present. Number of cases was shown as a percentage. In parentheses, absolute numbers were reported.
CRC: colorectal adenocarcinomas. NM: normal mucosa. OT: carcinomas from other tissues. ANNT: adjacent-to-tumor normal tissue (see Mat. and Methods).

AcH4. CRC *vs* NM: p=0.02; OT *vs* ANNT: p=0.3

Ac-Lys cyt. CRC *vs* NM: p=0.4; OT *vs* ANNT: p=0.03

Ac-Lys n. CRC *vs* NM: p=0.03; OT *vs* ANNT: p= 0.05 (see Statistical analysis in 'Mat. and Methods').

Figure 1

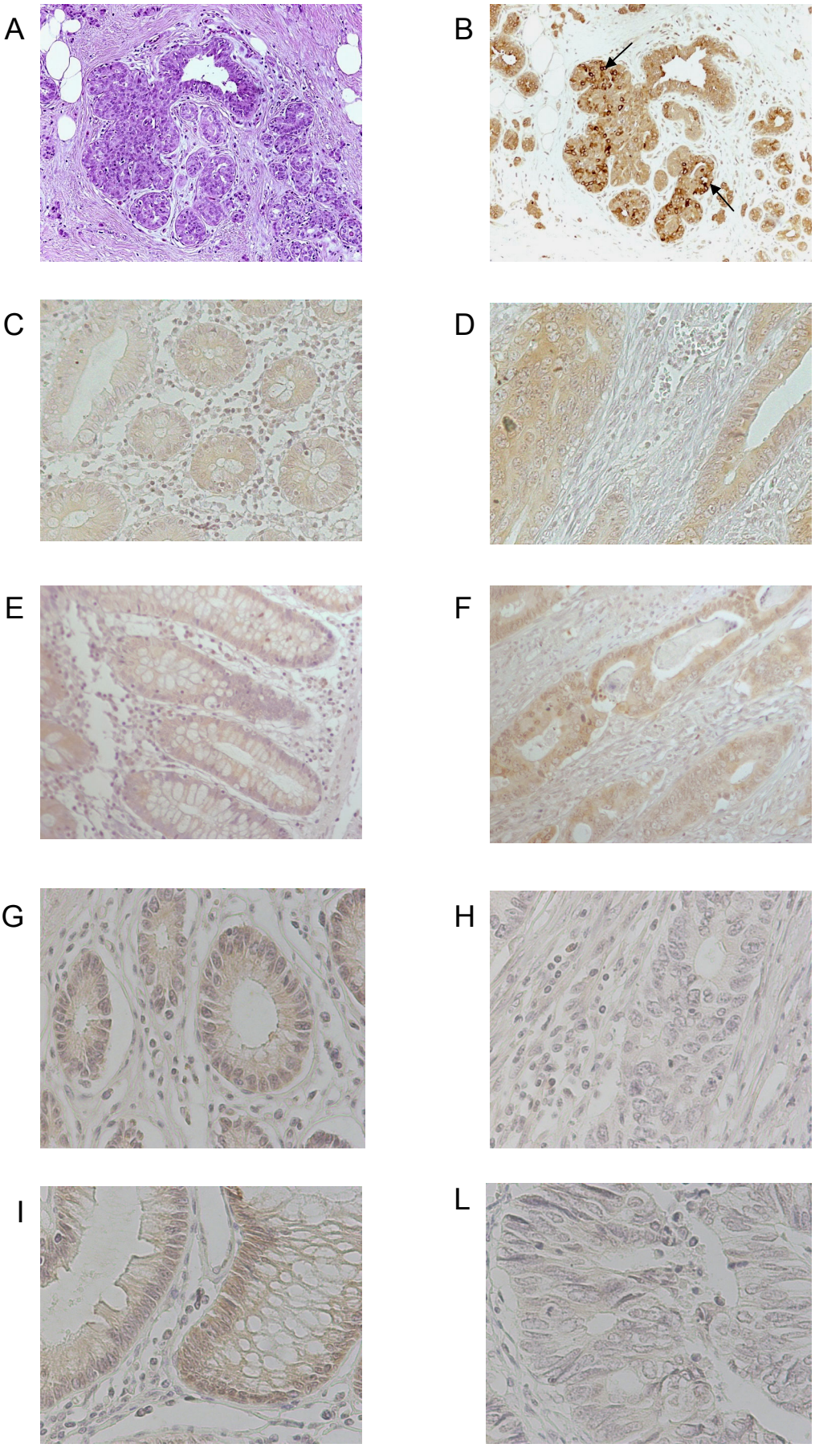


Figure 2

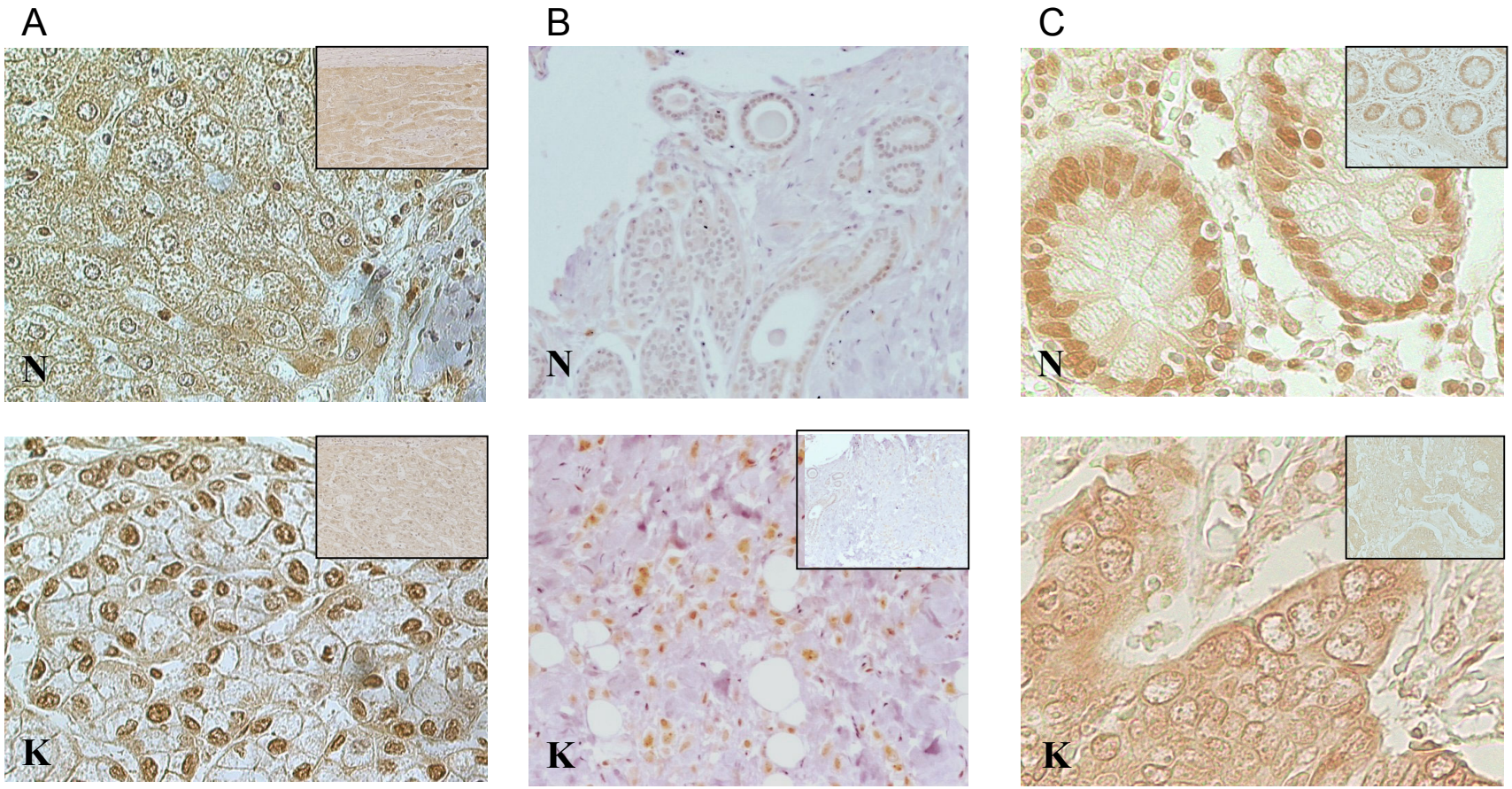
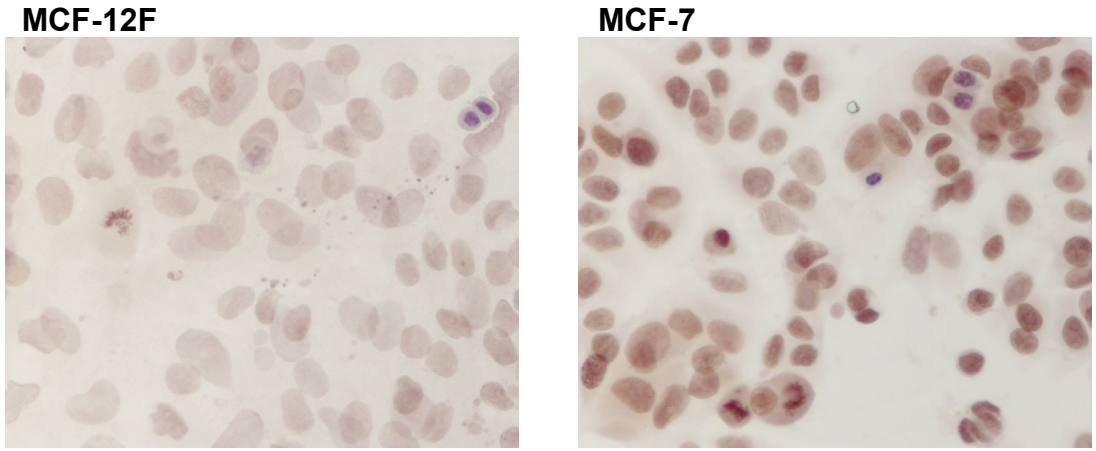


Figure 3

A



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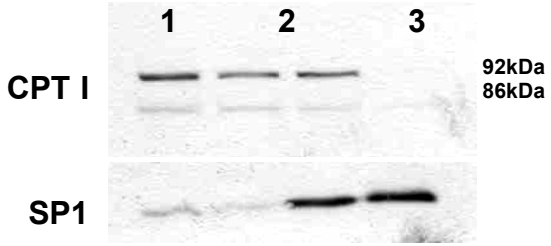


Figure 4

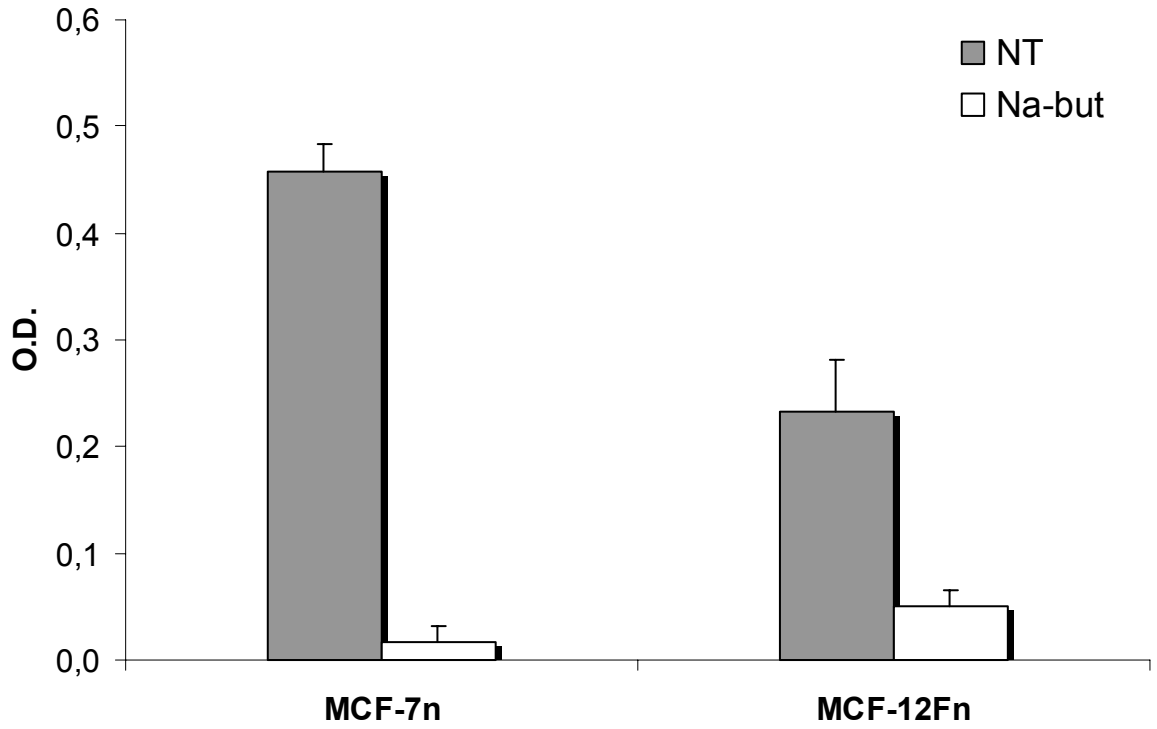
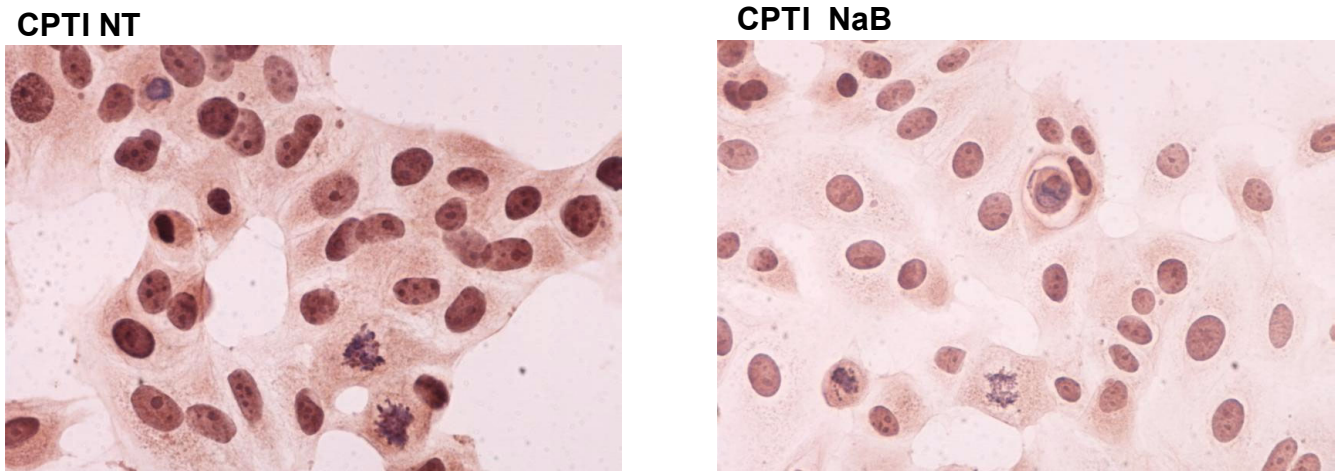


Figure 5

A



B