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Effects of the rare elements lanthanum and cerium on the growth of colorectal and hepatic cancer cell lines

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Abstract:

Human HT-29 and HepG2 cell lines were employed to test the effects of increasing concentrations of the REEs cerium (Ce) and lanthanum (La), alone or in combination. Effects on cell proliferation were measured using MTT colorimetric assay and confirmed by RealTime-Glo™ MT Cell Viability Assay and the evaluation of the proliferation marker PCNA, while cell mortality and type of cell death was determined by Annexin V binding to phosphatidyl serine at the cell surface of apoptotic cells using flow cytometry. The modulation of 84 genes, targeting pathways of oxidative stress was also studied using arrays based on RT-PCR. Major alterations in selected genes from basal expression levels of respective control groups were found in the 600 µM cerium exposed cells (48h). In HepG2 cells 51 out of 84 genes resulted significantly up/down regulated, while in HT-29 cells only 16 genes resulted significantly up/down regulated. The dosage of REEs seems to represent the pivotal factor for switching the biological effects from down to up-regulation of cell growth; thus, the low concentrations promote cell survival and proliferation, but when concentrations increased, REEs exert anti-proliferative and cytostatic/cytotoxic effects.

The molecular mechanisms underlying these effects are still not well defined and further analysis of the mechanisms which result in inhibition or induction of cell proliferation is crucially important.

Keywords: lanthanum; cerium; human cell lines; toxicity; cell growth.

1. Introduction

Lanthanum (La) and cerium (Ce) belong to the rare earth elements (REEs) that have many uses in industries throughout the world. As a group, rare earths constitute the fifteenth most abundant component of the earth's crust; cerium occurs more often in the earth's crust than lead, molybdenum or arsenic, and lanthanum occurs in similar quantities as lead, then they are not rare at all (Redling, 2006). In the last decade, REE has become one of the common xenobiotics in our surroundings (Zhao et al., 2004) as it is widely used in industry, stockbreeding and medicine, especially as trace fertilizers in agriculture, thus they can be concentrated by food chain.

China contains the largest mineral deposits of REEs in the world (Drew et al., 1990; Yang and A., 2006) and the application of rare earth elements as feed additives for livestock has been practiced in China for decades: the elements lanthanum, cerium and others are used as feed additives in animal production (Redling, 2006). Numerous reports in the Chinese literature described that a small amount of these REE mixtures in the diet can increase the body weight gain of pigs, cattle, sheep and chicken and it was reported that they increase milk and egg production (He and Xia, 1998; Rambeck and Wehr, 2000; Zhu et al., 1994).

Rare earth elements have several properties that make them attractive alternatives to antibiotics as growth promoter. Generally, absorption of orally applied rare earths is low with more than 95% being recovered in the feces of animals (Redling, 2006). As a result, the chances of residues being present in meat are low with studies reporting no higher levels of rare earth elements in the muscle tissue of supplemented animals than those fed commercial diets (Redling, 2006).

If only a very small amount of REE could be absorbed into body when they were supplemented orally (He and Rambeck, 2000), however, such small amount of REE in animal body may have effect on the metabolism through influence the hormones such as triiodothyronine (He et al., 2003) or growth hormone (Wang and Xu, 2003). Results indicate that the supplementation of REE to the media may affect adipogenesis and lipogenesis rates of 3T3-L1 mouse cells and that the effect may depend upon the dose or type of REE applied.

About a dozen of studies, especially in pigs, were performed under western animal production conditions (Rambeck et al., 2004). Most of these studies in piglets and in fattening pigs provided significant data, indicating that REE imported from China can improve weight gain and feed conversion (Rambeck et al., 2004). In addition, there have been no reports of the development of bacterial resistance in treated animals (Redling, 2006).

However, from October 2004 only that feed additives that passed a renewed European Food Safety Authority (EFSA) procedure can be put on the market in the European Union. Under these new rules feed additives will be categorized as technological additives, zoo technical additives and coccidiostats and histomostats (Khan, 2004). If REE have the character of trace elements, they belong to the nutritional additives, if they enhance the digestibility or stabilize the gut flora, they are zoo technical additives. In Switzerland, REE obtained a temporary registration under the trade name "Lancer" to be supplemented like other essential trace elements to the feed of piglets and pigs at a concentration between 150 and 300 mg REE per kg feed.

The European Food Safety Authority recently evaluated the safety and efficacy of Lancer as feed additive for weaned piglets (EFSA, 2013). Lancer is a feed additive mainly consisting of two rare earth elements, lanthanum and cerium, and although one study (Von Rosenberg et al., 2013) suggests that La and Ce are not deposited in tissues in piglets, this is apparently not consistent with data found in other species. e.g. cattle (Schwabe et al., 2011). Consequently, EFSA did not conclude that there is no potential for consumer exposure; in addition, in the absence of an established NOAEL (no observed adverse effect level) for the target species, EFSA was unable to relate any possible exposure to evidence of a safe dose.

In view of the increasing requests of application of lanthanides for improving animal growth, which may increase the possibility of human exposure, it is becoming necessary to obtain in-depth information on their environmental toxicity in impact on humans (Dai et al., 2002; Xue et al., 2009). It is well known that the biological effect spectrum of REE is wide and the dose-response relationship is complicated. Studies in rats have shown that REE could induce chromosome

damage of blood lymphocytes (Xu et al., 2000) and liver damage (Nakamura et al., 1997) depress learning and memory (Li et al., 2000) increase or suppress cell-mediated immunity of the spleen (Liu et al., 2000), change the expression levels of some kinds of genes (Zhao et al., 2004).

The safety evaluation of REE effects in animals and humans is difficult, and it is necessary to find sensitive biomarkers, utilizing different techniques to acquire a deeper understanding of their mechanism.

In this perspective, we explored the effects of different dosages of lanthanum and cerium on cell viability and proliferation in two human cancer cells lines, human colorectal (HT-29) and hepatocellular (HepG2), as well as the study of the modulation of 84 genes targeting pathways of oxidative stress in humans. In our knowledge, there are no previous studies considering all these aspects to elucidate the biological and molecular mechanism of REE toxicity in humans.

2. Material and Methods

2.1. Chemicals

Cerium (III) chloride heptahydrate, 99.9% purity grade and Lanthanum Chloride heptahydrate, 99.999% purity grade were purchased from Sigma–Aldrich (Milan, Italy). All other chemicals and reagents used were of analytical grade.

2.2. Cell culture

Human hepatoma HepG2 and Colon adenocarcinoma HT-29 cell lines were respectively cultured in DMEM and McCoy's 5A medium containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, USA), penicillin, 100 μg/mL streptomycin and amphotericin B (Sigma Chemical Co., St Luis, MO) at 37 °C in a 5% CO_2. Different batches of described media were prepared adding lanthanum and cerium, alone or in combination, in concentrations ranging from 0.1 μM to 10 mM and then filtered with filtropur 0.2 μm (SARSTEDT). Different biological replicates for each treatment condition, defined by exposure time and compound concentration, was then prepared for

the following analysis steps: quantification of effective exposure doses by ICP-MS, cell mortality and cell cycle progression by flow-cytometry, cell proliferation by MTT assay and gene expression profiling by quantitative RT-PCR. Negative control cell cultures for each time point and for both cell types were included in the experiment.

2.3. ICP-MS

The concentrations of all lanthanides solutions was checked by ICP-MS, in order to take in consideration possible effects of filtration and precipitation of studied compounds in medium used for cultured cells. Determination of Ce and La was performed after wet digestion with acids and oxidants (HNO_3 and H_2O_2) of the highest quality grade (Suprapure). About 0.5 g of material were subjected to microwave digestion (microwave oven ETHOS 1 from Milestone S.r.l., Sorisole (BG), Italy) with 7 ml of HNO_3 (70% v/v) and 1.5 ml of H_2O_2 (30% v/v). Samples were then bring at the final weight of 50 g with ultrapure water (Arium611VF system from Sartorius Stedim Italy S.p.A., Antella - Bagno a Ripoli, (FI), Italy). Ce and La determination was performed with ICP-MS (XseriesII, Thermo Scientific, Bremen, Germany) after daily optimization of instrumental parameters and external standard calibration curve; terbium was used as internal standard.

2.4. MTT assay

MTT assays were performed to determine cell proliferation (Mosmann, 1983). HepG2 and HT-29 cells were treated in 96 well plates with fixed concentrations of Cerium and Lanthanum (Table 1). After incubating cells for 24 h, 48 h and 72 h with lanthanum/cerium single and mixed compounds, cell proliferation was estimated by the MTT assay as follows: 5 mg mL^{-1} of MTT reagent (Sigma–Aldrich) was added and incubated for 3 h at 37 °C in humidified atmosphere (5% CO_2). After

incubation, the media was removed and 100 µL of DMSO was added to each well to dissolve the formazan (the metabolic product from MTT). Then, the absorbance at 590 nm was measured in a microplate reader (Spectramax, Gemini, EM). Results are expressed as percentage of means ± SD (Standard Deviation) of 3 experiments, each conducted in 6 replicates, calculated with respect to control considered as 100%.

Western blotting

HepG2 and HT-29 cells were plated in 6 well plates (10^5 cells/well) and treated for 24 and 72 h, respectively, with lanthanum/cerium single and mixed compounds. Total cell lysates, obtained as previously described, (Cannito et al., 2008) were subjected to sodium dodecyl sulfate-polyacrylamide gel-electrophoresis on 12% acrylamide gels, incubated with desired primary antibodies (PCNA sc-25280 ; primary antibody dilution was in agreement with the manufacturer's instructions), then with peroxidase-conjugated anti-mouse immunoglobulins in Tris-buffered saline-Tween containing 2% (w/v) non-fat dry milk and finally developed with the ECL reagents according to manufacturer's instructions. Sample loading was evaluated by reblotting the same membrane with β-actin antibody.

2.5. Flow Cytometry

Cell mortality and type of cell death was determined by Annexin V binding to phosphatidyl serine at the cell surface of apoptotic cells using flow cytometry.

Detection of apoptosis was carried out using annexin V–FTC staining according to the manufacturer's instructions (Immunostep). HT-29 and HepG2 cells (2×10^5 well) were seeded in 6-well plates in 2 ml serum-free medium and incubated for 24 h; the cells were then treated with

lanthanum and cerium at the indicated concentration for 24 and 48h. A negative control was prepared by untreated cells. Following incubation, floating cells were collected and adherent cells were digested by trypsin without EDTA to detach them. The cells were washed twice in temperate phosphate-buffered saline (PBS) and resuspended in 1X Annexin-binding buffer at a concentration of $1x10^6$ cells/ml. 5 µl of Annexin V-FITC and 5 µl of propidium iodide were added to each 100 µl of cell suspension. The cells were incubated at room temperature for 15 min in the dark, and then 400 µl of 1x Annexin binding buffer was added. The samples were analyzed by flow cytometer (BD Accurri C6); 20,000 events were collected per sample. Results are provided as mean ± SD of 2 experiments conducted in triplicatesand the rela.

Cell viability was also evaluated by using RealTime-Glo™ MT Cell Viability Assay (Promega Italia, MI). HepG2 cells and HT-29 cells were plated into 96 well opaque-walled assay plates ($10x10^4$ cells/well), treated with lanthanum/cerium single and mixed compounds and incubate for 24 and 72h. After treatment, the cells were incubated for 10 min in the cell culture incubator with a RealTime-Glo™ reagent according to the manufacturer's protocol. Luminescence was then measured on a Glomax Multi Detection System Promega with an integration time of 0.5 second per well. The luminescent signal correlates with the number of metabolically active cells**.** Results are provided as mean of luminescence values (RLU) ± SD of 2 experiments conducted in 6 replicates.

For cell cycle analysis, HepG2 and HT-29 cells (2×10^5 well) were seeded in 6-well plates in 2 ml serum-free medium and incubated for 24 h; the cells were then treated with lanthanum and cerium for 24, 48 and 72h. A negative control was prepared by untreated cells. Following incubation, floating cells were collected and adherent cells were digested by trypsin without EDTA to detach them. The cells ($1x10^6$/ml) were then centrifuged at 3000 rpm for 10 min at 4°C. The pellets obtained were resuspended in 70% ethanol, incubated for 1h at 4°C and then centrifuged at 1000 rpm for 10 min at room temperature. The cells were resuspended in 600 µl of PBS, incubated with

100 µl RNAase (4 mg/ml) for 30 min at room temperature and then with propidium iodide (1.8 mg/ml) for 10 min at room temperature. The samples were analyzed by flow cytometer (BD Accurri C6). Results are provided as mean ± SD of 2 experiments conducted in triplicates. Sub G0-G1 phase identify events not included in the normal cell cycle phase distribution but observed by flow cytometry.

2.6. Gene expression profiling using oxidative stress qPCR-based arrays

The modulation of 84 genes, targeting pathways of oxidative stress in humans was studied using arrays based on quantitative RT-PCR (qPCR)-analysis (RT^2 Profiler™; SA Biosciences, Milan, Italy), performed according to the manufacturer's protocol. The array used in this study (Ref. PAHS-065ZC) consisted of a 96-well plate containing primers optimized for amplification of the targeted genes, plus five housekeeping genes and seven internal controls, including evaluation of reverse transcription, qPCR and gDNA removal efficiencies, as described in previous work (Rouimi et al., 2012; Shah et al., 2016). RNA extraction was performed on each sample (containing $7x10^5$ – $2x10^6$ cells) by miRNeasy Plus mini kit (Qiagen, Milan, Italy). RNA concentration was estimated by Q-Bit fluorimetric assay (Thermo Scientific, Wilmington, DE, USA), while RNA integrity and quality were controlled using an Agilent 2100 Bioanalyzer. 0.5 µg of total RNA from each individual was converted into cDNA using the RT^2 First Strand Kit (SA Biosciences/QIAGEN). RT^2Real-Time SYBR Green/ROX PCR Master Mix was added to the cDNA and an equal volume of 25 µl was distributed to each of the 96 wells of the array. Amplification was performed on an ABI StepOne Plus thermocycler with an initial 10-min hot start step at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. A dissociation curve was run after 40 cycles to verify single product formation.

Cycle thresholds from the real-time PCR was exported to an excel file and analyzed using web-based PCR Array Data Analysis Software available at

www.SABiosciences.com/pcrarraydataanalysis.php, allowing determination of normalized average C_t values, paired *t* test *p*-value and fold change calculations, based on 2−ΔΔCt method (Livak and Schmittgen, 2001). Experiment were performed in triplicate for each treatment. Genes differentially expressed were retained when the *P*-value was < 0.05 and fold change > 2 following the different treatments.

Statistical analysis

Statistical analysis was performed by Student's t-test or ANOVA for analysis of variance when appropriate ($p < 0.05$ was considered significant).

3. Results

3.1 Effects on cell proliferation

Effects of increasing concentrations of cerium (Ce) and lanthanum (La), alone or in combination, on human colorectal (HT-29) and hepatocellular (HepG2) cancer cells were measured using MTT colorimetric assay. The results obtained for HepG2 cells are summarized in Table 1. The highest cell proliferation rate was observed in the group exposed for 48 and 72 hours to 25 µM of La+Ce when compared with control cells group, while 250 µM of La+Ce halved cell proliferation at all time point. Considering Ce and La separately, proliferation trends in HepG2 cells exposed to lanthanum were described in all groups stimulated with doses below 70 µM for 48 and 72 h, while at concentration of 180 µM, La inhibited cell proliferation. Ce stimulation of HepG2 cells revealed in general lower proliferation trends and concentration of 300 µM halved proliferation rate after 48 h exposure.

In HT-29 cell line, the supplementation of low concentration of lanthanides as a mixture or as a single compound (La 20 µM/24h group and La+Ce 25 µM/72h group respectively) increased the relative ratio of absorbance detected by MTT assay, indicating a considerable growth-promoting effect (Table 1). The inhibitory effects of Ce appeared only at concentration tested of 300 µM and

250 µM of La+Ce), while La as single compound induced a significant inhibition of cell growth at 370 µM at all time considered. In addition, after 48-72h exposure with 300 µM Ce, the initial inhibition of growth resulted overcome and cell proliferation restarted, reaching levels near to untreated controls. The tendency observed by using MTT assay was also confirmed by the analysis with RealTime-Glo™ MT Cell Viability Assay (Figure 1).

The influence of Lanthanides on cell proliferation was also evidenced in Figure 2 in which the expression of proliferation marker PCNA (Proliferating Cell Nuclear Antigen) was reported. According to previous results, in HepG2 cells PCNA expression was augmented at low concentration of La (70 µM) and Ce (120 µM) as well as at 25 µM of La+Ce, while it was reduced at high concentrations.. The same trend was detected in HT-29 cells: 20 µM La and 25 µM of La+Ce induced PCNA expression, while high dosed of La and Ce as mixture or as a single compound, exerted inhibitory activity.

3.2 Effects on cell cycle distribution and apoptosis induction

HepG2 and HT-29 cells were treated for 24, 48 and 72 h with tested compounds at a range of concentration able to interfere with cell proliferation. Since the inhibition of cell growth may result from either cell cycle arrest or cell death, we performed cell cycle analysis by flow cytometry after treatment with various dosages of La and Ce (Table 2). Annexin V flow cytometry analysis was applied to quantify the apoptotic profile (Table 3). The results obtained in HepG2 cells showed a significant presence of apoptotic cells after 48h exposure to both La (180 and 370 µM) and Ce (300 and 600 µM), with the highest level of apoptosis of about 29%. In this cell-line, lanthanides did not significantly alter cell cycle distribution, as shown in Table 2.

On the contrary, in HT-29 cells none of the tested compounds induced apoptotic cell death (data not shown). According to viability data, cell cycle analysis showed that increasing dosage of both La and Ce caused a gradually and significative reduction of cells in S phase, accompanied with a simultaneous increase of the population in G0-G1 phase (Table 2).

3.3 Gene expression profiling

Gene expression analysis on HepG2 and HT-29 cell lines, stimulated with single and combined Lanthanum and Cerium doses, was limited to 48h time point, where a significant reduction in proliferation rates, established by MTT analysis, was recorded (as shown in Table 1).

From analysis pipeline applied to HT-29 expression profiles in treated and untreated control cell lines 26 out of 84 genes resulted significantly up/down regulated. Gene list and fold changes were reported in Figure 1A and 1B. Supplemental material with fold regulation of each single gene is also provided. The major alterations in selected genes from basal expression levels recorded in respective control groups were found in the 600 μM cerium exposed (48h).

In HepG2 cell-lines, the expression analysis was limited to six groups at 48h, chosen because of previous proliferation tests. Gene list and fold changes were reported in Figure 2A and 2B; 47 out of 84 genes resulted significantly up/down regulated. Like for HT-29, also in HepG2 cell lines the main alterations in considered gene panel were found in higher cerium exposed replicates.

4. Discussion

The analysis of the effects of lanthanides on cell proliferation revealed that the concentration used represents the pivotal factor for switching the biological effects of lanthanides from toxicity to activity.

According to Chen and coauthors (Chen et al., 2000) the effects of lanthanides on cell growth is bi-directional, depending on the concentration used and cell lines. In our study, the results obtained from MTT and RealTime-Glo™ MT Cell Viability Assay evidence how La produces growth-promoting effects in HT-29 cells in a range of relative low concentrations (20 μM). Ce does not seem to promote HT-29 growth, since the main effect of Ce is indeed inhibition of cell proliferation, higher than that observed for La, being present within 24 h at high exposure concentrations.

Interestingly, after prolonged exposure of cells to high concentration (300 µM) of Ce, the initial inhibition of growth resulted overcome and cell proliferation restarts, reaching levels near to untreated controls. This evidence seems to suggest a reversible block of cell proliferation, consistent with cell cycle analysis that revealed the presence of a percentage of cells in G2-M phase similar to that detected in control cells. In this regard, both Ce and La block HT-29 cells in the G0-G1 phase, consistently with the inhibition of cell proliferation, while at low doses increase the percentage of cells in S and G2-M phases, according to promoting effects.

Recently, La was found to inhibit the proliferation and promote the apoptosis in some cancer cells (Wang et al., 2016; Yu et al., 2015b). The results obtained in our study seem to suggest that in HT-29 cells the inhibition of proliferation by lanthanides is related to a cytostatic rather than a cytotoxic effect, without apoptotic induction.

The combination of Ce and La causes a boosted promoting effect on cell proliferation at low dose (25 µ), while at high dose (250 µM) La+Ce exert a more significative inhibitory effect.

In HepG2 cells, La promoted cell growth in a larger range of concentrations especially on 48 h and 72 h groups, while the inhibition of cell proliferation needed doses of 180 µM to be recorded. Ce exposure of HepG2 lines does not provide any growth-promoting effects, but at the highest concentration tested it induced the inhibition of cell growth at all time point. At these doses, the inhibitory effect of both lanthanides seems to be related to apoptotic cell death induction, as revealed by annexin V test.

Finally, cell exposure to La and Ce in combination produces an increase of the proliferation rate after 48-72 h incubation at the lowest tested concentrations, while only prolonged incubation with 250 µM lanthanides is related to a significant increase of cell inhibition.

In both cell lines, the tendency of growth promoting and inhibiting effects exerted by Lanthanides was confirmed by the evaluation of the expression of Proliferating Cell Nuclear Antigen, an auxiliary protein of DNA polymerase delta involved in the control of DNA replication (Wang,

2014). According to proliferation data, our results evidenced that the expression levels of PCNA was increased at low doses of lanthanides and down-regulated at high doses.

Studies on toxicities of REE elements by different intake pathways showed that the liver was the target organ of REE element toxicity in mice (Arvela et al., 1991; Chen et al., 2000), and then we choose the hepatocellular (HepG2) cancer cell line to provide a unique and powerful experimental system to study differential gene expression in humans. In addition, since no data exists on the effects of lanthanides in colon cancer cells, the analysis was also performed on human colorectal (HT-29) cancer cell line.

Among cerium treated HT-29 cell lines, the Heme oxygenase 1 (HMOX1), Thioredoxin reductase 1 (TXRND1), Glutamate-cysteine ligase (GCLC), Myoglobin (MB) and Ferritin (FHT1) were the most interesting upregulated genes after 48 h exposure to Ce 600 µM. As previously reported (Valdes et al., 2013), the corresponding enzymes/proteins usually exert early cytoprotective effects against different toxic elements and molecules, promoting the phase II excretion and/or redox inactivation of such toxic compounds to reduce injure of biomolecules such as nucleic acids, proteins and lipids (Martin et al., 2004). Another gene significantly upregulated in HT-29 after Ce exposure was Glutathione peroxidase-3 (GPX3), coding for a selenoprotein that is the extracellular member of a family of plasma glutathione peroxidases (GPXs). In a recent study performed on different REEs in hepatocyte nuclei, superoxide dismutase (SOD) and catalase (CAT) activities decreased after cerium exposure while GPXs activities, GSH and malondialdehyde (MDA) levels increased compared to the control group (Huang et al., 2011). The authors found opposite effects in hepatocyte mitochondria, where GPXs activities and GSH levels were significantly decreased, underlining how REEs could selectively accumulate in different cellular compartments inducing oxidative damage.

SQSTM1 encoding the p62 protein (sequestosoma 1), which plays an important role in the process of autophagy (Salminen et al., 2012), a process that provides to protect the cell by removing protein deposits altered and was found mutated in neurodegenerative diseases (Alzheimer's disease). In our

study, this protein was found to be up regulated in all treated groups and being involved in the elimination of toxic substances could indicate the necessity that the cells have to detoxify by the presence of toxic levels of cerium.

Considering the downregulated genes in HT29 cell lines, the major alteration was recorded in 48 h Cerium 600 µM group: the expression of Forkhead box M1 (FOXM1), Methionine sulfoxide reductase A (MSRA), Prostaglandin-endoperoxide synthase 2 (PTGS2), Cytoglobin (CYGB) and Myeloperoxidase (MPO) resulted strongly inhibited. Among these genes only CYGB was constitutively inhibited also in all groups treated with single cerium and lanthanium after 48 hour exposure, suggesting a common effect of different single REE elements on CYGB expression in HT-29 cell lines. CYGB is a known oxidative stress-responsive gene, due to its capacity to bind ROS and nitric-oxid (Liu et al., 2013). Hypoxic upregulation of CYGB as well as its altered expression in various human cancers suggest a possible role of this globin in tumor cell response under low oxygen tension (Chakraborty et al., 2014). Conversely, CYGB inhibition was not recorded with combined stimulation with La and Ce. Moreover, the same target, analyzed in HEPG2 cell lines, shown a completely different behaviour, with a strong upregulation within 48 hours exposure to both La and Ce (Figure 2).

Indeed a different range of effects was recorded for HEPG2 cells when compared to HT-29.

In HEPG2 cell lines lower amounts of single REEs (70 µM of La, 120 µM of Ce respectively) induced few alterations in analyzed genes when compared with higher doses (180 µM of La, 600 µM of Ce). Interestingly in HEPG2 cell exposed to Ce 120 µM only inhibition effects on 19 target was recorded (Figure 2), with no upregulation of any of 84 considered genes. However, when Ce concentration was 600 µM, an upregulation of 7 maker was observed, with the highest expression levels of lactoperoxidase - LPO (up to 25 fold when compared with untreated control group), as well as chemokine (C-C motif) ligand 5 (CCL5), myeloperoxidase (MPO) and glutamate-cysteine ligase (GCLC). This reproduce a known pathway of oxidative stress response in HEPG2 cells (Pyo et al., 2016) based both on the production of antioxidant enzymes and phase II enzymes and on the

excretion of inflammatory chemokines like CCL5 that are critical for macrophage recruitment to the liver tissues (Barashi et al., 2013).

Another upregulated marker of REE exposure, not only in HT29 but also in HEPG2 (Figure 1 and 2), is Myoglobin (MB). MB is the oxygen carrier of skeletal and heart muscle but it is known to contribute in nitric oxide homeostasis and in reducing oxidative stress by scavenging hydrogen peroxides increased by hypoxia and following re-oxygenation after hypoxic events (Helbo et al., 2013). Recent studies on hypoxia-regulated expression of the MB gene in different cancer cell lines and breast cancer tissues revealed how this globin could exert a cancer-suppressive role by impairing mitochondrial activity (Bicker et al., 2014). The ectopic tumor-suppressor function of MB found in hypoxia-responsive cancer cell lines studied by Bicker and coauthors, may partly explain the effects seen on proliferation decrease of both HEPG2 and HT29 cell lines when exposed to higher concentration of REE (Table 1).

Among considered glutathione peroxidases (GPXs), previous studies shown that both over-expression (Guariniello et al., 2015) and catalytic activities (Guerriero et al., 2015) of GPX4 and GPX7 resulted strictly related to human hepatocellular carcinoma progression. The down regulation of both GPX4 and GPX7 on HEPG2 cell lines after 48h exposure to higher cerium concentration (Figure 2) confirmed the anti-tumoral effects of this rare element through inhibition of selenium-containing peroxidases. The radically different behaviour of GPXs expression in HT-29 (GPX4 strongly up-regulated by all REE exposure protocols) suggest important differences between the two cell lines considered in current study.

Among downregulated markers in HEPG2 cell lines following single La or combined La and Ce stimulation the most inhibited gene was PXDN, respectively from 16 to 22 fold lower in HEPG2 exposed to La 70 and 180 μM, and from 21 to 20 fold lower when exposed to combined La+Ce 100 μM and La+Ce 250 μM (Figure 3).

On the contrary, in all Ce only exposed groups no alteration of PXDN was recorded. PXDN (peroxidasin) is a secreted heme-containing peroxidase primary involved in the extracellular matrix

393 formation, but it shares similar function of Phagocyte-derived myeloperoxidase (MPO) and other
394 secreted peroxidases (Soudi et al., 2015), representing a versatile class of enzymes necessary to
395 manage both physiological and pathogenic functions, linked to immune and oxidative stress
396 response. In our experimentation the radically different behaviour of heme-peroxidases like
397 MPO/LPO (highly induced) and PXDN (strongly inhibited) suggest a controversial implication of
398 peroxidases in response to lanthanides induced oxidative stress, confirming how peroxidase
399 families, also if characterized by high conserved catalytic domain, have been described to evolve
400 diverging during evolution (Li et al., 2012).
401 Regarding mitochondrial genes included in the study major alteration were recorded in expression
402 of UCP2, the Uncoupling protein 2 (mitochondrial, proton carrier), MPV17 (mitochondrial inner
403 membrane protein) and mitochondrial isoform of super oxide dismutase (SOD2).
404 UCP2 is known to suppress mitochondrial reactive oxygen species (ROS) generation, mitigating
405 oxidative stress-induced apoptosis. Recent studies in different hepatocellular carcinoma cells
406 (HCC), including HEPG2, show a basal high expression of UCP2, related to chemotherapeutic
407 agents' resistance (Valle et al., 2010). The specific knockdown of UCP2 expression by targeted
408 siRNA enhanced the Gemcitabine induced inhibition of HCC growth (Yu et al., 2015a). The similar
409 inhibition of UCP2 promoted by REE exposure in HEPG2 found in current study confirm the key-
410 role of this mitochondrial proton carrier in HCC proliferation. Conversely, in REE treated HT29
411 lines UCP2 expression did not resulted inhibited and the up-regulation of UCP2 (only in 250 μM
412 La+Ce group) was recorded. This demonstrate the different behaviour of Colon adenocarcinoma
413 cell lines compared to hepatocellular carcinoma lines, also if they similarly shown a basal UCP2
414 overexpression (Derdak et al., 2008; Santandreu et al., 2009).
415 Super Oxide Dismutases (SOD) are a family of evolutionarily conserved enzymes that dismutate
416 superoxide free radicals: SOD2 isoform is localized in the mitochondrial matrix, in association with
417 mtDNA (Xu et al., 2008). Reduced SOD2 mRNA is strongly associated with poor survival in HCC
418 patients, correlated with larger tumor size, multiple tumor nodules and tumor emboli, and cancer

recurrence (Wang et al., 2016). Increased expression levels of SOD2 was recorded in HEPG2 stimulated for 48 h with La 180 μM, La+Ce 100 μM and La+Ce 250 μM but its overexpression doesn't seem to prevent the strong inhibition of HEPG2 proliferation found performing MTT tests (Table 1). The controversial effects on oxidative stress response genes during the performed REE stimulation protocols need to be further investigated in order to understand if activated ROS pathways found in present study are exclusively related to specific cancer phenotype of used cell lines, or could be extended to normal cells.

In conclusion, the expression profiles of certain genes in La and Ce treated cells differed markedly from those of control cell lines. This is consistent with the wide biological effect spectrum of REE elements. Multiple genes may act together to play a same role, and further analysis of the differentially expressed genes would be helpful for understanding the wide biological effect spectrum of REE elements.

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Figure captions

Figure 1: Effect of lanthanides on cell viability

HepG2 (panel **A**) and HT-29 cells (panel **B)** were plated ($10x10^4$ cells/well) in a 96-well plate, treated with the test compounds for the indicated times and then incubated for 10 min at 37°C with RealTime-Glo™ reagent. Luminescence was measured with an integration time of 0.5 second per well on a Glomax Multi Detection System Promega. The fold change in cell viability of lanthanides-treated cells was compared to control cells. Data are expressed as mean of luminescence values (RLU) ± SD (Standard Deviation) of 2 experiments conducted in 6 replicates. *= $p<0.05$; **= $p<0.01$; ***=$p<0.001$.

Figure 2: Effect of lanthanides on Proliferating Cell Nuclear Antigen (PCNA) expression.

Western Blotting analysis of PCNA performed on total extracts of HepG2 (panel **A**) and HT-29 (panel **B**) cells treated or not with different concentration of Lanthanides for indicating time. Equal loading was evaluated by re-probing membranes with β-actin. Densitometry values are indicated.

Figure 2A

HT-29 - Genes differentially expressed at 48 hours of treatment (all groups with at least one of the treatments with -2<ΔCt>2)

Figure 2B

HT-29 - Genes differentially expressed at 48 hours of treatment (only groups with -2<ΔCt>2)

Figure 3A

HepG2 - Genes differentially expressed at 48 hours of treatment (all groups with at least one of the treatments with -2<ΔCt>2)

Figure 3B

HepG2 - Genes differentially expressed at 48 hours of treatment (only groups with -2<ΔCt>2)

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