Phenobarbital Induces Alterations in the Proteome of Hepatocytes and Mesenchymal Cells of Rat Livers

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Abstract

Preceding studies on the mode of action of non-genotoxic hepatocarcinogens (NGCs) have concentrated on alterations induced in hepatocytes (HCs). A potential role of non-parenchymal liver cells (NPCs) in NGC-driven hepatocarcinogenesis has been largely neglected so far. The aim of this study is to characterize NGC-induced alterations in the proteome profiles of HCs as well as NPCs. We chose the prototypic NGC phenobarbital (PB) which was applied to male rats for a period of 14 days. The livers of PB-treated rats were perfused by collagenase and the cell suspensions obtained were subjected to density gradient centrifugation to separate HCs from NPCs. In addition, HCs and NPC isolated from untreated animals were treated with PB in vitro. Proteome profiling was done by CHIP-HPLC and ion trap mass spectrometry. Proteome analyses of the in vivo experiments showed many of the PB effects previously described in HCs by other methods, e.g. induction of phase I and phase II drug metabolising enzymes. In NPCs proteins related to inflammation and immune regulation such as PAI-1 and S100-A10, ADP-ribosyl cyclase 1 and to cell migration such as kinesin-1 heavy chain, myosin regulatory light chain RLC-A and dihydropyrimidinase-related protein 1 were found to be induced, indicating major PB effects on these cells. Remarkably, in vitro treatment of HCs and NPCs with PB hardly reproduced the proteome alterations observed in vivo, indicating differences of NGC induced responses of cells at culture conditions compared to the intact organism. To conclude, the present study clearly demonstrated that PB induces proteome alterations not only in HCs but also in NPCs. Thus, any profound molecular understanding on the mode of action of NGCs has to consider effects on cells of the hepatic mesenchyme.

Introduction

Screening assays, which enable the early detection of potential carcinogenic activities, are of crucial importance for safe drug development strategies. Chemical compounds may cause cancer by directly affecting DNA and are thus called genotoxic carcinogens. This type of compounds is easily detectable as such by the application of well-established in vitro assays, such as Ames bacterial reverse mutation assay, mammalian forward mutation assays and detection of chromosomal aberrations. Furthermore, in vivo assays are routinely used which include rodent erythrocyte micronucleus assay, mammalian bone marrow chromosomal aberration assay and assays for somatic cell gene mutation in endogenous genes. Chemical carcinogens which do not affect DNA directly are called non-genotoxic carcinogens (NGCs) [1]. In contrast to genotoxic carcinogens, there are no sufficiently accurate and validated short-term assays that may allow detection of NGCs [2–6]. Currently employed assays necessitate long-term rodent carcinogenicity assays causing high efforts, costs and time requirement as major drawbacks. In order to overcome these problems, deeper insights into NGC-relevant mechanisms are urgently required, which may be obtained by the application of a screening technology such as proteome profiling.

According to current knowledge, a characteristic effect of many NGCs is a deviation of tissue homeostasis resulting in organ growth based on a dysbalance between cell replication and cell death by apoptosis [7,8]. This dysbalance acts also on mutated/initiated cells. By this mechanism NGCs enhance the
selective proliferation of preneoplastic cells and exert tumour promoting effects. Possible molecular mechanisms of the tumour promoting effects of NGCs comprise epigenetic changes such as hypo- and hypermethylation of CpG sites, chromatin modifications, and miRNA regulated mechanisms [9], but also endocrine effects, inhibition of gap junctional intercellular communications, immune modulation, and/or profound disturbances in the epithelial-mesenchymal interactions [6]. It is known that during carcinogenesis the microenvironment may gain a pivotal role supporting preneoplastic and neoplastic cell growth via an altered vasculature, deviated immunological activities and altered interstitial extracellular matrix (ECM) [10,11]. Further possible modes of NGC actions in rodents may be cytotoxicity followed by regenerative growth and a pro-inflammatory status. Involvement of inflammatory mechanisms may be accompanied by enhanced production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [7,8]. These two species may have relevance in carcinogenesis via signalling function and possibly cause endogenous DNA damage, which may be responsible for a weak genotoxic potential of NGCs.

The most common target organ of NGCs in rodent models is the liver, i.e., about 40% of all NGC tested so far are hepatocarcinogens. Hitherto, research on the action of NGCs has been focusing mainly on hepatocytes (HCs), the major parenchymal cells of the liver which eventually give rise to liver cancer. A role of non-parenchymal liver cells (NPCs) in NGC-driven hepatocarcinogenesis has been mostly neglected, because these cells do not transform [12,13]. However, NPCs, which consists mainly of Kupffer, endothelial, and stellate cells, may also be targeted by NGCs and may contribute considerably to the selective proliferation of preneoplastic and neoplastic HCs via release of paracrine growth factors or other growth-enhancing stimuli. Here, as a model NGC we chose phenobarbital (PB), a barbiturate known to be a potent tumour promoter in rodent liver [14–18]. PB has been described to interact with the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) triggering a signal transduction cascade leading to an induction of cytochrome P450 genes such as members of the CYP2B and CYP3A subfamily [9,19–23]. Furthermore, it was shown that PB acts through other mechanisms such as oxidative stress [24], which may correlate with the P450 induction [25], driving tumour promotion by inducing proliferation [26,27] in HCs, non-receptor mediated endocrine modifications and inhibition of gap junction intercellular communications, regulating growth and differentiation [18].

Proteome profiling is a powerful technique to observe molecular consequences of drug action. Cells may respond to drug actions via the synthesis of new proteins. Cells synthesize proteins in order to overcome biological challenges. Therefore, the identification of drug-induced proteins may give important hints to better understand the way of action of drugs. Furthermore, if the drug-induced proteins display restricted expression patterns, they may be used as indicative marker proteins. For comprehensive investigation, we analysed subcellular fractions including the secretome of isolated HCs and NPCs separately applying LC-MS/MS analysis. We hypothesized that NGC-driven hepatocarcinogenesis may involve NPCs and specifically considered a potential contribution of inflammatory activities of this tissue compartment, as known for the pathogenesis of hepatocellular carcinoma in humans [28]. Therefore we treated HCs and NPCs with pro-inflammatory cytokines in vitro in order to identify proteome signatures characteristic for such events. Induction of such a signature by PB treatment could thus indicate the involvement of inflammatory processes in drug action. Furthermore, we investigated PB effects induced by in vivo treatment of animals in comparison to PB effects observed upon in vitro treatment of isolated primary cells, including both HCs and NPCs. This strategy provided the unique opportunity to differentiate direct drug effects on the isolated cell types in vitro from indirect drug effects modulated by complex epithelial-mesenchymal interactions in the intact organism. This approach may thus give novel insights into the mode of action of this prototypic NGC. To conclude, the aim of the present study was to investigate proteome alterations in HCs as well as NPCs, which are caused by the non-genotoxic carcinogen phenobarbital (PB) in vitro as well as in vivo.

**Material and Methods**

**Animals and treatment**

Male Wistar rats were obtained from and kept at the “Division for Decentralized Biomedical Facilities of the Medical University of Vienna” under standardized SPF-conditions. Phenobarbital (PB, 5-Ethyl-5-phenyl-barbituric acid-sodium salt; Fluka, 04712) [29,30], admixed to drinking water, was administered to three rats which were 8 to 10 weeks old at a daily dose of 50mg/kg bodyweight. Two times a week the PB concentrations in the drinking water were adjusted to the body weights and the amount of water consumed. Five controls and four animals for the in vitro experiments were treated with tap water only. Animals were sacrificed by exsanguination under CO₂-asphyxiation during the liver perfusion. The experiments were approved by the “Committee of Animal Protection of the Austrian Ministry of Sciences” (permission number 66009/157 II10b/2009) and performed according to Austrian regulations in accordance with the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” by the National Academy of Sciences.

**Separation of liver cells and primary cultures**

The following procedure was used for the in vitro experiments. To isolate and cultivate hepatocytes (HCs) and non-parenchymal cells (NPCs) at a functional state as naïve as possible, livers of rats were perfused with collagenase (Worthington CLS-2) as described before [31,32]. In the resulting cell suspension HCs were isolated from NPCs by low speed centrifugation followed by discontinuous density gradient centrifugation using Percoll [33]. The purity was found to be an average of 95.4% for hepatocyte fractions and 99.8% for NPC fractions. Cell preparations were used for further experimentation when the viability exceeded 90%, as determined by the trypan blue exclusion assay. HCs and NPCs...
were subsequently seeded on collagen-coated 6-well plates. HCs were seeded at a density of 4x10^5 cells/well in Williams’ medium E (Invitrogen) supplemented with glutamax, HEPES, gentamycin, H2 mix, ascorbat and 10% FCS. NPCs were seeded at a density of 3-4x10^5 cells/well in RPMI 1640 medium (Gibco Ltd.) supplemented with gentamycin and 10% FCS at 37°C for 2 hours. To determine the purity of the isolated cell fractions, cells were counted using microscope pictures and the ImageJ software (National Institutes of Health). After an attachment period of 2 hours cells were switched to serum-free medium (Williams medium E and RPMI 1640 medium, both supplemented as above without the 10% FCS) and kept at 37°C for further 24 hours in order to collect cell supernatants.

In vitro treatment of primary HCs and NPCs

In addition to the procedure described above, the in vitro treatment of cultures commenced 2h after plating of cells deriving from four untreated rats. Two rats were used for the cytokine treatment, while the other two were used for the PB treatment. HCs were treated with 10ng/ml interleukin-1β (R&D Systems) and 5ng/ml interleukin-6 (R&D Systems) [34] for 24 hours, which induce the acute phase plasma protein synthesis in HCs [35–38]. 10ng/milliliter lipopolysaccharide (LPS, Sigma-Aldrich) was applied to the medium of NPCs for 24 hours [39–43]. HCs and NPCs were treated with 1mM PB (Fluka) for 24 hours.

Cellular sub-fractionation and protein sample processing (see also 44)

The serum-free supernatants were sterile filtered using a 0.2µm cellulose acetate filter (Whatman). One part of this filtrate was precipitated by adding 5x volume of -20°C tempered p.a. ethanol (Merck) and subsequent storing at -20°C for at least overnight. The other part was directly stored at -80°C for subsequent analyses by ELISA. During all steps samples were kept on ice. For harvesting of cytoplasmic and nucleic protein fraction, cells were gathered in isotonics buffer (10mM HEPES/NaOH pH=7.4, 10mM NaCl, 3.5mM MgCl2, 1mM EGTA, 0.25M Sucrose and 0.5% Triton X-100) and protease inhibitor mix (1mM PMSF; aprotinin, leupeptin and pepstatin, [1µg/ml] each). The cells were disrupted by sheer stress caused by syringing the cell lysates through 23G needles. The cytoplasmic fraction, in the supernatant, was separated from nuclei and membrane proteins as well as debris by centrifugation at 2300xg and 4°C for 5min and was subsequently precipitated by ethanol tempered to -20°C. The nucleic protein fraction was extracted from the remaining residue via a 10min incubation with an extraction buffer (10mM Tris/HCl pH=7.4, 1mM EDTA, 500mM NaCl) which act through osmotic pressure followed by a 1:10 dilution with a NP40 buffer (10mM Tris/HCl pH=7.4, 1mM EDTA, 0.5% NP40), to reduce the final NaCl concentration, for 15min. Nucleic proteins were separated from debris by centrifugation at 2328xg and 4°C for 5min and precipitated by ethanol tempered to -20°C.

After precipitation all fractions were centrifuged at 4700xg and 4°C for 25min. The resulting protein pellets were dissolved in sample buffer (7.5M urea, 1.5M thiourea, 4% CHAPS, 0.05% SDS, 100mM DTT) in a volume according to the protein amount / pellet size.

Shotgun analysis - 1D-SDS PAGE, in-gel tryptic digestion & MS analysis (‘bottom up’)

For 1D-SDS PAGE we used a 4% stacking and a 12% resolving polyacrylamide gel. Protein samples were loaded onto the gel and the electrophoresis ran until complete separation of a pre-stained molecular marker (Dual Color, Biorad, Hercules, CA) was visible. Gels were fixated with 50% methanol/10% acetic acid and MS compatible silver stained as described by Mortz, E. et al [45]. For tryptic digestion samples were cut into lanes to group proteins with a similar molecular weight, which improved the following LC-MS/MS analyses. These lanes were cubed and the proteins were destained, reduced and alkylated before digestion with trypsin (sequencing grad, Roche) at 37°C over night as described before [46]. After elution the peptide solutions were analysed by LC - MS/MS.

For reversed-phase chromatography we used a nano-flow LC (1100 Series LC system, Agilent) combined with the HPLC chip technology (Agilent). The chips consist of a 40nl Zorbax 300SB-C18 trapping column and a Zorbax 300SB-C18 (75µm x 150mm) separation column. The flow rate was 400nl/min, using a gradient from 0.2% formic acid and 3% ACN to 0.2% formic acid and 50% ACN over 40min or 60min for supernatants or the other two fractions, respectively.

Peptide identification was performed by MS/MS fragmentation analysis using an ion-trap mass spectrometer (XCT-Ultra, Agilent) combined with already described HPLC chips, which are eventually an orthogonal nanospray ion source (Agilent). For peak list-generation and spectrum identification, of the MS/MS data we used Spectrum Mill MS Proteomics Workbench software (Version A.03.03, Agilent). We searched our MS/MS data against the UniProtKB/Swiss-Prot protein database (Version 10th August 2010; 519,348 entries). The settings were as follows: max. of 2 missed cleavages, minimum scored peak intensity (%SPI) of 70%, precursor mass tolerance of +/-1.5Da, product mass tolerance +/- 0.7. Two types of modification were considered, both arising during sample preparation. Carbamidomethylation of cysteine (deliberately) was set as fixed modification and oxidized methionine (artefact) was set as post-translational modification.

Generating protein maps and their interpretation

The resulting peptide-protein assignment list was reviewed by hand considering the following parameters. Spectrum Mill peptide sequence score values (sequence matching probability) of >13 counted as valid sequence assignments. Peptide sequences were valid, when at least one peptide sequence scored as much and did not occur in other proteins of this cell type. An estimated error rate was calculated by searching the sequences against a non-sense reversed database. Furthermore we included peptides scoring between 9 and 13 only if precursor m/z value, retention time and MS2 pattern were found similarly in at least one of our previous experiments and the peptide was thereby scoring above 13. With respect to protein inference, we chose the smallest
number of proteins required to explain all observed peptides as described for ProteinProphet [47]. As our protein identification algorithm includes manual selection, we cannot calculate an exact false discovery rate.

Proteome analysis using emPAI (exponentially modified protein abundance index) values[48], comparisons and PB specific alterations were recorded and interpreted using the GPDE (Griss Proteomics Database Engine), a database specifically engineered for the identification and characterization of marker proteins [49].

ELISA
Arginase-1 protein levels were determined by a kit (Uscn Life Science Inc.; Houston, TX 77036) according to the user manual.

Results

Isolation and proteome profiling of primary rat hepatocytes and non-parenchymal cells
Primary rat cells were obtained from untreated rats and rats treated with PB in vivo. Cell isolation was performed by liver perfusion followed by separation into hepatocytes (HCs) and non-parenchymal cells (NPCs) (see Figure 1). Cells were cultured for 24 hours to allow accumulation of secreted proteins. Subsequently, cells were fractionated into cytoplasm and nuclear extract. The protein fractions were further separated by SDS-PAGE and forwarded to proteome profiling via LC-MS/MS as described.

We restricted our analyses to proteins which were identified by two peptides or more in two or more independent experiments. By this approach, we identified 1148 proteins in HCs and 1213 proteins in NPCs with an overlap of 966 proteins in both cell compartments. The 182 proteins being apparently specific for HCs indeed comprise a large number of known liver-specific proteins including, apolipoproteins and other serum proteins, cytochrome P450 isoenzymes, sulfotransferases, UDP-glucuronosyltransferases and many others (table 1-5, table S1 & S2). Furthermore, the 247 proteins identified in NPCs included many proteins known to be expressed in stromal cells. Representative for leukocytes are the surface antigens CD37, CD47, CD96 and CD166 as well as the chemokines CXCL1, CXCL2, and CCL6. Amongst known marker proteins for endothelial cells we identified endothelial cell-specific molecule 1, endothelial nitric oxide synthase and septin-2 (vascular endothelial cell specific protein 11), while MMP-3 and various collagens are characteristic for the stellate cells.

In vitro treatment of isolated primary cells
HCs were treated with IL-6 and NPCs with LPS, respectively. Upon treatment for 24 hours, the induction of several pro-inflammatory proteins was observed which confirms the responsiveness of these cells to stress stimuli under our experimental conditions (Figure 2).
To investigate direct drug effects, primary untreated cells were treated with 1mM PB for 24 hours and analysed by proteome profiling. Remarkably, this treatment had hardly any measurable effect on the proteome composition of HC and

![Figure 1. Microscope images of HCs and NPCs in culture.]( doi: 10.1371/journal.pone.0076137.g001 )

These pictures depict representative areas of untreated, PB in vitro as well as in vivo treated HCs and NPCs in culture extracted from microscopic images of equal magnification.
NPCs. A single protein was apparently induced in HC and two proteins only in NPCs, respectively. Proteins indicating stress such as chaperones including the heat shock protein family, stress-induced phosphoprotein, heme oxygenase 1 and DNAJ homolog subfamily members were not or hardly induced.

In vivo treatment of animals with phenobarbital

13 proteins were found to be newly induced in the cytoplasmic fraction of HCs by PB, including phase I drug metabolizing enzymes such as amine oxidase, phase II enzymes such as UDP-glucuronosyltransferases and glutathione S-transferases, the chaperone rotamase D, glutathione synthetases and the proto-oncogen c-Raf. The induction of estradiol 17-beta-dehydrogenase 8 may indicate alterations in steroid hormone homeostasis. The induction of CYP2B, probably via CAR, is a known positive control for PB action.

Table 1. Selected proteins found up-regulated in the secretome of HCs and NPCs isolated from rat livers, when treated with PB in vivo.

| Accession number | Protein name | GO - biological process |
|------------------|--------------|------------------------|
| P24090 | Alpha-2-HS-glycoprotein | acute-phase response |
| P06238 | Alpha-2-macroglobulin | acute-phase response, response to glucocorticoid stimulus |
| P02650 | Apolipoprotein E | cellular response to growth factor stimulus |
| P02454 | Collagen alpha-1(I) chain | cellular response to transforming growth factor beta stimulus, response to corticosteroid stimulus |
| P06759 | Apolipoprotein C-III | inflammatory & drug response |
| P02680 | Fibrinogen gamma chain | inflammatory response |

| Accession number | Protein name | GO - biological process |
|------------------|--------------|------------------------|
| P29534 | Vascular cell adhesion protein 1 (V-CAM 1) | acute & chronic inflammatory response |
| P12346 | Serotransferrin (Transferrin) (Beta-1 metal-binding globulin) (Liver regeneration-related protein LRRG03) | acute-phase response |
| P07154 | Cathepsin L1 (Major excreted protein) (MEP) (Cystic protein 2) (CP-2) | autophagic cell death, proteolysis, response to organic cyclic compound |
| P22985 | Xanthine dehydrogenase/oxidase | bone resorption, contributes to the generation of reactive oxygen species. |
| P11232 | Thioredoxin (Trx) | cellular response to drug |
| P02761 | Major urinary protein (MUP) (Alpha-2u-globulin) | cellular response to lipid, positive regulation of gene expression |
| P10760 | Adenosylhomocysteinase (AdoHcyase) (S-adenosyl-L-homocysteine hydrolase) | chronic inflammatory response to antigenic stimulus |
| P07152 | Stromelysin-2 (SL-2) (Matrix metalloproteinase-10) (MMP-10) | Collagen degradation |
| P18484 | AP-2 complex subunit alpha-2 (Alpha2-adaptin) | endocytosis, intracellular protein transport |
| P15978 | Class I histocompatibility antigen, Non-RT1.A alpha-1 chain | immune response |
| Q63228 | Glia maturation factor beta (GMF-beta) | inhibition of proliferation of tumor cells |
| P31720 | Complement C1q subcomponent subunit A | innate immune response |
| Q11102 | Isoamyl acetate-hydrolyzing esterase 1 homolog | lipid catabolic process |
| P14841 | Cystatin-C (Cystatin-3) | positive regulation of cell proliferation, response to drug and toxin |
| P20961 | Plasminogen activator inhibitor 1 (PAI-1) (Endothelial plasminogen activator inhibitor) (Serpin E1) | response to reactive oxygen species, tissue regeneration |

These up-regulated proteins of interest were selected out of 666 and 1044 distinct proteins, whereby the selected proteins had a relative difference of emPAI values of at least 40% and 50% for HCs and NPCs, respectively. Furthermore, the proteins had to fulfill the criteria of being represented in at least 50% of the experiments with at least 2 peptides. For convenience, the proteins are sorted according their Go terms.

1. Evidence at transcript level only

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NPCs. A single protein was apparently induced in HC and two proteins only in NPCs, respectively. Proteins indicating stress such as chaperones including the heat shock protein family, stress-induced phosphoprotein, heme oxygenase 1 and DNAJ homolog subfamily members were not or hardly induced.
Table 2. Selected proteins found up-regulated in the cytoplasmic protein fraction of HCs and NPCs isolated from rat livers, when treated with PB *in vivo*.

| Hepatocytes / cytoplasmic protein fraction | Accession number | Protein name | GO - biological process                                                                 |
|------------------------------------------|------------------|--------------|----------------------------------------------------------------------------------------|
|                                          | P09034          | Argininosuccinate synthase | acute-phase response, cellular response to interferon-gamma, response to drug, liver development |
|                                          | P12346          | Serotransferrin | acute-phase response, response to organic cyclic compound                               |
|                                          | Q6IP791         | Regulator complex protein LAMTOR1 | cell growth, cholesterol homeostasis, positive regulation of MAPK & TOR signaling cascade |
|                                          | Q8K561          | Thioredoxin domain-containing protein 9 | cell redox homeostasis                                                                  |
|                                          | P15709          | Bile salt sulfotransferase | drug metabolic process                                                                 |
|                                          | P00176          | Cytochrome P450 2B1 | drug metabolic process                                                                 |
|                                          | P05178          | Cytochrome P450 2C6 | drug metabolic process                                                                 |
|                                          | P04903          | Glutathione S-transferase alpha-2 | drug metabolic process                                                                 |
|                                          | P17988          | Sulfotransferase 1A1 | drug metabolic process                                                                 |
|                                          | P08011          | Microsomal glutathione S-transferase 1 | drug metabolic process                                                                 |
|                                          | P09875          | UDP-glucuronosyltransferase 2B1 | drug metabolic process                                                                 |
|                                          | P19488          | UDP-glucuronosyltransferase 2B37 | drug metabolic process                                                                 |
|                                          | P97675          | Ectonucleotide pyrophosphatase / phosphodiesterase family member 3 (E-NPP 3) (B10) | immune response                                                                        |
|                                          | P07151          | Beta-2-microglobulin | immune response, antigen processing and presentation of peptide antigen via MHC class I |
|                                          | P80254          | D-opachrome decarboxylase | inflammatory response                                                                   |
|                                          | P51647          | Retinal dehydrogenase 1 | response to oxidative stress, response to organic cyclic compound                      |
|                                          | P55053          | Fatty acid-binding protein, epicardial | response to wounding                                                                   |
|                                          | Q66HA8          | Heat shock protein 105 kDa | stress response                                                                         |
| Non-parenchymal cells / cytoplasmic protein fraction | Accession number | Protein name | GO - biological process                                                                 |
|                                          | P11497          | Acetyl-CoA carboxylase 1 | acetyl-CoA metabolic process, fatty acid biosynthetic process, response to organic cyclic compound |
|                                          | Q4KM31          | Pleckstrin | actin cytoskeleton reorganization, hemopoietic progenitor cell differentiation, positive regulation of platelet activation |
|                                          | P12346          | Serotransferrin | acute-phase response, response to organic cyclic compound                               |
|                                          | P70831          | G-protein coupled receptor family C group 6 member A | calcium-mediated signaling, response to amino acid stimulus                           |
|                                          | P85972          | Vinculin | cell adhesion                                                                           |
|                                          | Q91Y81          | Septin-2 (Vascular endothelial cell specific protein 11) | cell division                                                                         |
|                                          | Q6EH61          | Glutaredoxin-1 | cell redox homeostasis                                                                  |
|                                          | P85845          | Fascin | cellular response to cell-matrix adhesion, liver development, cell motility             |
|                                          | Q2OQA9          | Kinesin-1 heavy chain | cytoplasm organization, vesicle transport along microtubule                            |
|                                          | P00176          | Cytochrome P450 2B1 | drug metabolic process, response to organic cyclic compound                             |
|                                          | P07322          | Gamma-ENOS | glycolysis, gluconeogenesis, response to organic cyclic compound                       |
|                                          | P97675          | Ectonucleotide pyrophosphatase/phosphodiesterase family member 3 | immune response                                                                        |
|                                          | P67779          | Prohibitin | organ regeneration, response to cytokine stimulus, response to drug, response to stress |
|                                          | Q64244          | ADP-ribosyl cyclase 1 | positive regulation of B cell proliferation, cell growth & vasoconstriction and response to drug |
|                                          | Q9JU82          | Integrin-linked protein kinase (cell-cell junction) | positive regulation of MAPK/PPCK cascade, positive regulation of cell migration, positive regulation of cell proliferation |
|                                          | Q5U204          | Regulator complex protein LAMTOR3 | positive regulation of TOR signaling cascade, cellular protein localization |
|                                          | P13832          | Myosin regulatory light chain RLC-A | protein targeting to plasma membrane, regulation of cell shape |
|                                          | Q59536          | Dihydroxyimidazolase-related protein 1 | pyrimidine base catabolic process                                                      |
|                                          | Q5FVC7          | Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 2 | regulation of ARF GTPase activity                                                   |
|                                          | P05943          | Protein S100-A10 | regulation of cell differentiation, regulation of cell growth                          |
Table 2 (continued).

| Accession number | Protein name |
|------------------|--------------|
| P25235           | Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2 | response to drug |
| P20961           | Plasminogen activator inhibitor 1 (PAI-1) | response to reactive oxygen species, tissue regeneration, positive regulation of receptor-mediated endocytosis |
| Q88600           | Heat shock 70 kDa protein 4 | stress response |
| Q7TP47           | Heterogeneous nuclear ribonucleoprotein Q (hnRNP Q) (Liver regeneration-related protein LRRG077) | tissue regeneration, mRNA processing |
| Q68FP1           | Gelsolin (Actin-depolymerizing factor) | tissue regeneration, regulation of cell adhesion |
| P50399           | Rab GDP dissociation inhibitor beta | vesicle-mediated protein transport |
| Q5U2R7           | LDLR chaperone MESD | Wnt receptor signaling pathway |

These up-regulated proteins of interest were selected out of 1283 and 1336 distinct proteins, whereby the selected proteins had a relative difference of emPAI values of at least 50% for HCs and NPCs, with the same exclusion criteria as table 1. For convenience, the proteins are sorted according their Go terms.

1. Evidence at transcript level only, PB – found only in PB treated rats

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Pathway analysis of the in vivo data via Reactome

The alterations in the proteome were further analysed by Reactome, a public peer-reviewed pathway database from CSHL, OICR and EBI, which is cross-referenced to bioinformatics databases and allows the assignment of a given protein to one or more molecular pathways. When searching for the positively PB-induced proteome alterations, in both HCs and NPCs, proteins assigned to the categories of “gene expression”, “metabolism of proteins” and “3’UTR-mediated translational regulation” were most prominent among the top listed events (see table 6 & 7). With respect to molecular pathways, in HCs “Peroxisomal lipid metabolism”, “Class I MHC mediated antigen processing & presentation” and “Asparagine N-linked glycosylation” were listed on top (see table 8). In contrast, in NPCs “Protein folding”, “Dissolution of Fibrin Clot” and “Platelet Adhesion to exposed collagen” were listed on top (see table 9).

ELISA verification of arginase-1 variations

To verify selected LC-MS/MS data in a quantitative fashion, we conducted an ELISA for rat arginase-1. Arginase-1 was chosen, because of its ability to diminish anti-tumour immunity by interfering with the activation of T-cells [50]. LC-MS/MS results indicated a PB induced decrease in protein secretion of arginase-1 by HCs in case of in vivo treatment. The ELISA results confirmed our LC-MS/MS results as demonstrated in Figure S1. In the secretome arginase-1 concentration was found decreased by a factor of 2.3. In the cytoplasmic protein fraction, arginase-1 abundance was found induced by PB more than three-fold, which corresponds very well to the LC-MS/MS results.

Discussion

The aim of this study was to investigate molecular mechanisms induced by treatment of rats with the non-genotoxic carcinogen phenobarbital (PB) by means of proteome profiling. This approach was used to pinpoint crucial...
events not previously recognised by other technical approaches. Current mechanistic considerations on non-genotoxic carcinogenesis include altered cell-cell interactions, epigenetic changes, endocrine effects, inhibition of gap junctional intercellular communications and immune modulation [6], which may be of crucial importance for initiation as well as promotion and progression [10]. However, the significance of

| Accession number | Protein name | GO - biological process |
|------------------|--------------|------------------------|
| P28064           | Proteasome subunit beta type-8 (Proteasome subunit beta-5i) | anti-gen processing and presenting, fat cell differentiation |
| P13383           | Nucleolin (Protein C23) | associated with transcription |
| Q6TRW4           | Sister chromatid cohesion protein PDS5 homolog B | cell division |
| Q5IQH9           | Protein disulfide-isomerase A5 | cell redox homeostasis, response to stress |
| Q9WUL0           | DNA topoisomerase 1 | cellular response to stress |
| P27008           | Poly [ADP-ribose] polymerase 1 | DNA damage response, detection of DNA damage |
| P05183           | Cytochrome P450 3A2 | drug metabolic process, oxidative demethylation |
| O09171           | Betaine–homocysteine S-methyltransferase 1 | methionine biosynthetic process, protein methylation |
| Q4KM6S           | Cleavage and polyadenylation specificity factor subunit 5 | mRNA polyadenylation |
| Q62780           | Probable ATP-dependent RNA helicase DDX46 | mRNA processing |
| P17136           | Small nuclear ribonucleoprotein-associated protein B (snRNP-B) | mRNA processing |
| Q35B21           | Myb-binding protein 1A (PAR-interacting protein) (PIP) | nucleocytoplasmic transport, transcription (DNA-dependent) |
| Q6LED0           | Histone H3.1 | nucleosome assembly |
| Q00715           | Histone H2B type 1 | nucleosome assembly |
| Q8P747           | Heterochromatin protein 1-binding protein 3 | nucleosome assembly |
| P62914           | 60S ribosomal protein L11 | protein localization to nucleus, translation |
| P07695           | Superoxide dismutase [Mn], mitochondrial | removal of superoxide radicals |
| Q6AY65           | Signal recognition particle 54 kDa protein | SRP-dependent cotranslational protein targeting to membrane |
| Q6PF78           | Estradiol 17-beta-dehydrogenase 12 | steroid biosynthetic process |
| Q63536           | Activated RNA polymerase II transcriptional coactivator p15 | transcription, DNA-dependent |
| Q6PDV7           | 60S ribosomal protein L10 | translation |
| P05765           | 40S ribosomal protein S21 | translation |
| P24050           | 40S ribosomal protein S5 | translation |
| Q71TY3           | 40S ribosomal protein S27 | translation |
| P43244           | Matrin-3 (Nuclear scaffold protein p130/MAT3) | chromatin organisation |

Phenobarbital Induces Alterations in the Proteome

Table 3. Selected proteins found up-regulated in the nuclear extract protein fraction of HCs and NPCs isolated from rat livers, when treated with PB in vivo.

| Accession number | Protein name | GO - biological process |
|------------------|--------------|------------------------|
| P41516           | DNA topoisomerase 2-alpha | DNA topological change, response to drug |
| O06829           | Transcription intermediary factor 1-beta (TIF1-beta) (epithelial to mesenchymal transition) | epithelial to mesenchymal transition; positive regulation of transcription (DNA-dependent) |
| Q68FY1           | Nucleoporin NUP53 | mRNA & protein transport |
| Q6AY87           | THO complex subunit 6 homolog (WD repeat-containing protein 58) | mRNA processing |
| Q4V898           | RNA-binding motif protein, X chromosome | mRNA splice site selection, positive regulation of transcription (DNA-dependent) |
| Q00566           | Methyl-CpG-binding protein 2 (MeCP2-2 protein) | negative regulation of transcription from RNA polymerase II promoter, transcription (DNA-dependent) |
| Q9Z2Y1           | Protein timeless homolog (rTIM) | positive regulation of circadian rhythm, response to DNA damage stimulus |
| Q5JL3            | Interleukin enhancer-binding factor 3 | protein methylation, transcription (DNA-dependent) |

These up-regulated proteins of interest were selected out of 1081 and 957 distinct proteins, whereby the selected proteins had a relative difference of emPAI values of at least 50% for HCs and NPCs, with the same exclusion criteria as table 1. For convenience, the proteins are sorted according their Go terms.

* Exact isoform could not be distinguished with our resources

† Evidence at transcript level only, PB – found only in PB treated rats

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Table 4. Selected proteins found down-regulated in HCs isolated from rat livers, when treated with PB in vivo.

| Accession number | Protein name | GO - biological process |
|------------------|--------------|-------------------------|
| Secreted protein fraction | | |
| Q2TL32 | E3 ubiquitin-protein ligase UBRA4 (N-recognin-4) (Zinc finger UBR1-type protein 1) | Ubl conjugation pathway; together with clathrins, forms meshwork structures involved in membrane morphogenesis and cytoskeletal organization |
| Cytoplasmic protein fraction | | |
| P08516 | Cytochrome P450 4A10 (CYP4A10) (Cytochrome P450-LA-omega 1) | arachidonic acid metabolic process |
| P09606 | Glutamine synthetase (GS) (Glutamate−ammonia ligase) (Glutamate decarboxylase) | ammonia assimilation cycle, glutamine biosynthetic process |
| P04182 | Ommithine aminotransferase, mitochondrial (Ommithine−oxo-acid aminotransferase) | L-proline biosynthetic process |
| Q5PPL3 | Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating | Cholesterol biosynthesis, Steroid biosynthesis |
| Nuclear extract protein fraction | | |
| Q9ES53 | Ubiquitin fusion degradation protein 1 homolog (UB fusion protein 1) | proteasomal ubiquitin-dependent protein catabolic process |

These down-regulated proteins of interest were selected out of 389 proteins. These proteins were found exclusively in the livers of untreated rats and had to fulfill the criteria of being represented in at least 50% of the experiments with at least 2 peptides.

1. Evidence at transcript level only,
doi: 10.1371/journal.pone.0076137.t004

mutations [51]. Laskin et al and Roberts et al have found that NGCs, such as PB and peroxisome proliferators, may also activate Kupffer cells to release the pro-inflammatory cytokine TNFa [12,25]. Furthermore, stromal liver cells may be induced to secrete survival factors, which may act as tumour promoters [52,53]. Therefore, an improved understanding of non-genotoxic compounds has to consider drug effects on stroma cells, even if these cells are not transformed to cancer cells. In order to assign molecular events caused by NGCs to the different cell types of the liver, we isolated primary cells by liver perfusion and separated them into parenchymal HCs and NPCs as described previously [54,55]. Here, we isolated cells from untreated and PB-treated animals in order to investigate in vivo effects.

Cells obtained from untreated livers were also treated with PB in vitro. This experimental approach is limited mainly due to the de-differentiation of primary cells during prolonged in vitro cultivation. To avoid this issue, we chose a 24 hours treatment period to ensure a meaningful data interpretation. In the in vitro part, functional activation by interleukin-6 or LPS resulted in well detectable proteome alterations in HCs or NPCs (data not shown). Any cytotoxic substance will cause a cell stress response, which results in an increased expression of chaperones, especially the heat shock protein family. Under the present experimental conditions PB application in vitro hardly induced such a stress response and generally exerted marginal effects. A reason for that may be that the dedifferentiation of HCs in culture during the first 24 hours hampers the reaction of HCs to PB, namely the induction of drug metabolising enzymes. Nevertheless, proteins induced in vitro were also found to be induced in the in vivo experiments, some of which are presented in Figure 2A. Figure 3 highlights the diverse response of HCs and NPCs upon in vivo (A) and in vivo (B) PB treatment. This observation suggests that in vivo PB treatment has effects being more profound and largely different from those obtained by in vitro treatment. This may be due to the fact that the milieu in the intact organism is required to enable the full response of liver cells to NGCs and that the disrupted cell-cell interactions in vitro and the tendency towards de-differentiation under artificial culture conditions compromise such a response.

The presently observed PB-induced proteome alterations in the stromal cells of the liver indeed suggest profound functional alterations. There was strong induction of pleckstrin, which is a positive regulator of platelets causing an aggregation of these sensitive cells, indicating that PB may trigger a wound healing cascade. ADP-ribosyl cyclase 1 (CD38) is an immunity-related protein induced in NPCs upon PB action. This protein usually regulates B cell function by influencing the intra-cellular Ca2+ concentration [56]. It was also demonstrated that it affects the migration capabilities of dendritic cells and as a consequence it also affects T cells [57].

The increased secretion of PAI-1, cathepsin L1, MMP-10 and V-CAM 1 indicates some inflammatory activation of these cells. Remarkably, however, a profound inflammatory activation as observed upon LPS-treatment was not evident (Figure 2). Cox-1 (Q63921), an important mediator of inflammatory signalling, was observed induced by PB in stroma cells in one animal only, which may be due to low protein concentration at the limit of detection. The expression of the inflammation-related protein MX-1 [58] in the control stroma cells, however, indicates that the presently employed cell manipulation steps may also have altered the inflammatory activity state in an artificial way, rendering clear conclusions, with respect to inflammatory pathways, difficult. The present observations still indicate some complex regulatory actions of PB which are related to but still distinct from classical inflammatory activation warranting further investigation.

Many effects of PB on HCs, as observed in our in vivo experiments, have already been identified applying techniques other than proteomics, e.g. the induction of phase-I and phase-II drug-metabolising enzymes [59], redox-regulating enzymes...
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Table 5. Selected proteins found down-regulated in NPCs isolated from rat livers, when treated with PB in vivo.

| Accession number | Protein name                                                                 | GO - biological process                                      |
|------------------|-------------------------------------------------------------------------------|--------------------------------------------------------------|
| Secreted protein fraction |                                                                          |                                                              |
| Q5X1Z12         | Acetyl-CoA acetyltransferase, cytosolic (Cytosolic acetoacetyl-CoA thiolase) | liver development, cellular response to nutrient             |
| P52844          | Estrogen sulfotransferase, isoform 1 (EST-1)                                 | estrogen metabolic process                                   |
| P09606          | Glutamine synthetase (GS) (Glutamate–ammonia ligase) (Glutamate decarboxylase) | ammonia assimilation cycle, glutamine biosynthetic process   |
| P14095          | Growth-regulated alpha protein (C-X-C motif chemokine 1) (Cytokine-induced neunphil chemoattractant 1) (CINC-1) | acute inflammatory response, immune response                 |
| P108688         | Guanidinoacetate N-methyltransferase                                          | S-adenosylhomocysteine metabolic process                     |
| P04176          | Phenylalanine-4-hydroxylase (PAH) (Phe-4-monooxygenase)                       | L-phenylalanine metabolic process                            |
| Cytoplasmic protein fraction |                                                                          |                                                              |
| O5DWV2          | Cadherin-7                                                                    | homophilic cell adhesion                                     |
| O35796          | Complement component 1 Q subcomponent-binding protein, mitochondrial (Glycoprotein gC1qBP) | negative regulation of interferon-gamma & interferlin-12 production, positive regulation of apoptotic process |
| Q64811          | Cysteine sulfenic acid decarboxylase (Sulfinoalanine decarboxylase)           | carboxylic acid metabolic process                            |
| P108180         | Cytochrome c oxidase subunit 6A1, mitochondrial (Cytochrome c oxidase polypeptide V1a-liver) | mitochondrial respiratory chain complex IV                   |
| P06683          | Cytochrome P450 2C11 (CYP11C1) (Cytochrome P-450(M-1))                        | xenobiotic metabolic process                                 |
| P63095          | Guanine nucleotide-binding protein G(a) subunit alpha isomers short           | adenylate cyclase-activating dopamine receptor signaling pathway |
| P14659          | Heat shock-related 70 kDa protein 2 (Heat shock protein 70.2)                | multicellular organismal development, response to stress     |
| P27881          | Hexokinase-2 (Hexokinase type II) (HK II)                                    | cellular glucose homeostasis, apoptotic mitochondrial changes |
| Q920F3          | KH domain-containing, RNA-binding, signal transduction-associated protein 2 (SLM-1) | regulation of transcription, DNA-dependent                   |
| O088160         | Neural Wiskott-Aldrich syndrome protein (N-WASP)                              | actin polymerization or depolymerization                     |
| P04182          | Ornithine aminotransferase, mitochondrial (Ornithine--o xo-acid aminotransferase) | L-proline biosynthetic process                               |
| P22062          | Protein-L-isospartate(D-aspartate) O-methyltransferase (PIMT)                | S-adenosylhomocysteine metabolic process                     |
| P12928          | Pyruvate kinase isozymes R/L (L-PK)                                         | ATP biosynthetic process                                     |
| Q53890          | Ras-related protein Rab-43                                                   | protein transport                                           |
| Q6BB18          | Ubiquitin-fold modifier-conjugating enzyme 1 (Ufm1-conjugating enzyme 1)    | protein ufmylation                                          |
| Nuclear extract protein fraction |                                                                          |                                                              |
| P21531          | 60S ribosomal protein L3 (L4)                                                | translation                                                  |
| P08753          | Guanine nucleotide-binding protein G(k) subunit alpha (G(i) alpha-3)         | cell cycle, adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway |
| P63095          | Guanine nucleotide-binding protein G(s) subunit alpha isomers short (G-alpha-8) | adenylate cyclase-activating dopamine receptor signaling pathway, heterotrimeric G-protein complex |
| P14659          | Heat shock-related 70 kDa protein 2 (Heat shock protein 70.2)               | multicellular organismal development, response to stress     |
| P179550         | Nuclear pore glycoprotein p62 (62 kDa nucleoporin) (Nucleoporin Nup62)        | cell death, negative regulation of cell proliferation        |
| P62961          | Nuclear-sensitive element-binding protein 1 (CCAAV-binding transcription factor I subunit A) (DNA-binding protein B) (EFI-A) (YB-1) | CRD-mediated mRNA stabilization                             |
| Q49845          | SAP domain-containing ribonucleoprotein (Nuclear protein Hcc-1)              | regulation of transcription, DNA-dependent                   |

These down-regulated proteins of interest were selected out of 484 proteins. These proteins were found exclusively in the livers of untreated rats and had to fulfill the criteria of being represented in at least 50% of the experiments with at least 2 peptides.

1. Evidence at transcript level only,

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as well as the proto-oncogen c-RAF were observed by proteome profiling (table 1-3) [60–62]. As part of the drug metabolism, we found Cytochrome P450 2B1 and other isoforms, sulfotransferase 1A1 and glutathione S-transferase alpha-2 (GST-A2) up-regulated in HCs. Increased glutathione S-transferase levels may indicate increased oxidative stress [63]. It has been proposed that an accumulation of ROS has many effects on cells such as an increased proliferation, DNA mutation rates [64] and genetic instability [65]. Reproducing these and other well-known PB effects by our currently employed proteome profiling analyses supports the notion that this strategy was valid. Furthermore, we were able to observe a few molecular events which have not yet been described. This includes the induction of estradiol 17-beta-dehydrogenase 8 in HCs (table 2) and the down-regulation of estrogen sulfotransferase 1 in NPCs (table 5) by PB, which may indicate alterations of the estrogen metabolism. Estrogens are important growth factors and potential tumour promoters [66].
and may exert anti-inflammatory activities [67]. Whether PB may act via estrogen activity modulation deserves more detailed investigations, e.g. by including female rats in the PB study. Furthermore, ectonucleotide pyrophosphatase 3 (E-NPP 3) was found to be induced, which has been observed in association with neoplastic bile duct diseases [68].

The nuclear proteins presently observed to be induced by PB actually indicate that cells were finally exposed to DNA stress. This interpretation is supported by the PB-induced expression of DNA topoisomerase I, poly [ADP-ribose] polymerase 1 and sister chromatid cohesion protein PDS5 homolog B. These proteins are known to be involved in DNA damage sensing as well as DNA-repair. These findings were somewhat unexpected, as PB is known to be non-genotoxic. However, it may increase the hepatocellular production of ROS by Cyp450 induction or ROS production by activated NPCs and may thus evoke some marginal DNA repair activity. This demonstrates the potential difficulties to group chemical compounds unequivocally to the categories of genotoxic or non-genotoxic carcinogen.

Interestingly it was demonstrated that inflammation-related ROS formation and signalling may lead to carcinogenesis of...
epithelial cells and a phenotypic change. This includes dissolution of cell-cell contacts, cytoplasmic redistribution of E-cadherin and up-regulation of integrins, as evidenced in the present experiments by the up-regulation of integrin-linked protein kinase and matrix metalloproteinases such as MMP-10. Furthermore, under these conditions MMP activity may correlate with invasiveness as determined by Matrigel invasion assays [69]. An PB-induced formation of ROS is evidenced by the observed increase of ROS responding enzymes such as retinal dehydrogenase 1 and mitochondrial superoxide dismutase [Mn] in HCs and plasminogen activator inhibitor 1 (PAI-1) in NPCs, as well as by the induction of DNA repair proteins described above.

The present investigation of primary cells by proteome profiling may be considered as essential for the achievement of biological relevance of the employed model, but on the other hand, also accounts for the experimental limitation of the present study with respect to reliable quantification of potential marker proteins. Shotgun proteomics as presently employed using nano liquid chromatography and ion trap mass spectrometry may give a quite comprehensive overview to cell activities, but results in rather semi-quantitative data hardly accessible to stringent statistical analysis. However, the present observation of PB-induced proteome alterations especially in NPCs is a first but important step to improve our understanding of the molecular events in response to PB treatment.

### Table 6. Events represented by up-regulated proteins found in HCs upon PB treatment of rats.

| name of the event                           | un-adjusted probability of seeing N or more genes in this event by chance | number of genes in the query which map to this event | total number of genes involved in this event |
|--------------------------------------------|---------------------------------------------------------------------------|------------------------------------------------------|---------------------------------------------|
| Metabolism                                 | 7.40E-17                                                                  | 62                                                   | 1033                                        |
| Gene Expression                            | 3.40E-03                                                                  | 25                                                   | 654                                         |
| Metabolism of proteins                     | 1.90E-10                                                                  | 26                                                   | 283                                         |
| 3' -UTR-mediated translational regulation  | 4.60E-11                                                                  | 19                                                   | 134                                         |
| Signal Recognition (Preprolactin)          | 1.40E-08                                                                  | 15                                                   | 112                                         |
| Signal Recognition (Preproinsulin)         | 1.60E-08                                                                  | 15                                                   | 113                                         |
| DNA Replication                            | 2.80E-01                                                                  | 7                                                    | 241                                         |
| Cell Cycle                                 | 3.20E-01                                                                  | 11                                                   | 422                                         |
| Apoptosis                                  | 2.00E-02                                                                  | 9                                                    | 185                                         |
| Signal Transduction                        | 1.00E+00                                                                  | 14                                                   | 1710                                        |
| Cdc20:Phospho-APC/C mediated degradation of Cyclin A | 2.50E-03                                                              | 7                                                    | 85                                          |
| Developmental Biology                      | 9.60E-01                                                                  | 5                                                    | 418                                         |
| Immune System                              | 9.30E-01                                                                  | 4                                                    | 319                                         |
| Membrane Trafficking                       | 1.70E-02                                                                  | 5                                                    | 69                                          |

This indicates effects on molecular events via positively PB-induced proteome alterations.

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### Table 7. Events represented by up-regulated proteins found in NPCs upon PB treatment of rats.

| name of the event                           | un-adjusted probability of seeing N or more genes in this event by chance | number of genes in the query which map to this event | total number of genes involved in this event |
|--------------------------------------------|---------------------------------------------------------------------------|------------------------------------------------------|---------------------------------------------|
| Metabolism of proteins                     | 4.90E-08                                                                  | 20                                                   | 283                                         |
| Gene Expression                            | 7.90E-03                                                                  | 20                                                   | 654                                         |
| 3' -UTR-mediated translational regulation  | 2.80E-06                                                                  | 12                                                   | 134                                         |
| Metabolism                                 | 1.60E-04                                                                  | 33                                                   | 1033                                        |
| Signal Recognition (Preprolactin)          | 1.30E-04                                                                  | 9                                                    | 112                                         |
| Signal Recognition (Preproinsulin)         | 1.40E-04                                                                  | 9                                                    | 113                                         |
| Apoptosis                                  | 3.30E-04                                                                  | 11                                                   | 185                                         |
| DNA Replication                            | 2.50E-01                                                                  | 6                                                    | 241                                         |
| Cell Cycle                                 | 3.20E-01                                                                  | 9                                                    | 422                                         |
| Signal Transduction                        | 1.00E+00                                                                  | 18                                                   | 1710                                        |
| Cdc20:Phospho-APC/C mediated degradation of Cyclin A | 3.50E-03                                                              | 6                                                    | 85                                          |
| Developmental Biology                      | 1.90E-01                                                                  | 10                                                   | 418                                         |
| Muscle contraction                         | 1.10E-01                                                                  | 3                                                    | 66                                          |
| Membrane Trafficking                       | 1.20E-01                                                                  | 3                                                    | 69                                          |
| Cell-Cell communication                    | 6.80E-01                                                                  | 2                                                    | 133                                         |

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understanding of the complex mode of action of non-genotoxic carcinogens. It is evident from the current results that the analysis of drug effects on isolated cell model systems will hardly represent the mode of action in vivo. Furthermore, the contribution of tumour-promoting molecules associated with ROS to the effect of PB is clearly evidenced by the induction of several marker proteins signifying oxidative stress and associated DNA damage. Only the complete comprehension of direct and indirect consequences will enable a strategy to identify a panel of marker molecules with sufficient specificity for the unequivocal indication of non-genotoxic carcinogen activity.

Table 8. Molecular pathways represented by up-regulated proteins found in HCs upon PB treatment of rats.

| Pathway name                                | Total number of proteins | Matching proteins in data | % in data |
|---------------------------------------------|--------------------------|---------------------------|-----------|
| Peroxisomal lipid metabolism                | 21                       | 4                         | 19%       |
| Class I MHC mediated antigen processing & presentation | 16                       | 3                         | 18%       |
| Asparagine N-linked glycosylation          | 24                       | 3                         | 12%       |
| Bile acid and bile salt metabolism         | 27                       | 3                         | 11%       |
| Eukaryotic Translation Elongation           | 109                      | 12                        | 11%       |
| Eukaryotic Translation Initiation           | 145                      | 16                        | 11%       |
| Eukaryotic Translation Termination         | 104                      | 12                        | 11%       |
| RAF/MAP kinase cascade                     | 10                       | 1                         | 10%       |
| SRP-dependent cotranslational protein targeting to membrane | 129                      | 14                        | 10%       |
| Translation                                | 178                      | 18                        | 10%       |
| Platelet Adhesion to exposed collagen       | 11                       | 1                         | 9%        |
| Phase I conjugation                        | 67                       | 6                         | 8%        |
| Biological oxidations                      | 149                      | 11                        | 7%        |
| Lipid digestion, mobilization, and transport | 26                       | 2                         | 7%        |
| Membrane Trafficking                       | 69                       | 5                         | 7%        |
| Metabolism of proteins                     | 283                      | 21                        | 7%        |
| Processing of Capped Intronless Pre-mRNA   | 14                       | 1                         | 7%        |
| Metabolism of amino acids and derivatives   | 194                      | 12                        | 6%        |
| Fatty acid, triacylglycerol, and ketone body metabolism | 90                       | 5                         | 5%        |
| Formation of Fibrin Clot (Clotting Cascade) | 36                       | 2                         | 5%        |
| Metabolism of non-coding RNA               | 19                       | 1                         | 5%        |
| Phase 1 - Functionalization of compounds   | 84                       | 5                         | 5%        |
| Regulation of Apoptosis                    | 79                       | 4                         | 5%        |
| Signaling by Wnt                            | 69                       | 4                         | 5%        |

PB treatment of rats may exert a perturbation or up-regulation of the pathways, listed in this table, in HCs. Total number of proteins states the number of different proteins present in the pathway in this database. The matching column gives the proteins number of the number of different proteins also found in our data. The last column shows the calculated percentage value of the previous two columns.

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Table 9. Molecular pathways represented by up-regulated proteins found in HCs upon PB treatment of rats.

| Pathway name                                         | Total number of proteins | Matching proteins in data | % in data |
|------------------------------------------------------|--------------------------|----------------------------|-----------|
| Protein folding                                      | 35                       | 5                          | 14%       |
| Dissolution of Fibrin Clot                           | 10                       | 1                          | 10%       |
| Platelet Adhesion to exposed collagen                | 11                       | 1                          | 9%        |
| Signaling by Wnt                                      | 69                       | 6                          | 8%        |
| Asparagine N-linked glycosylation                    | 24                       | 2                          | 8%        |
| Lipid digestion, mobilization, and transport         | 26                       | 2                          | 7%        |
| Regulation of Apoptosis                              | 79                       | 6                          | 7%        |
| Regulation of DNA replication                        | 87                       | 6                          | 6%        |
| Eukaryotic Translation Termination                   | 104                      | 7                          | 6%        |
| Fatty acid, triacylglycerol, and ketone body metabolism | 90                       | 6                          | 6%        |
| Eukaryotic Translation Elongation                    | 109                      | 7                          | 6%        |
| Class I MHC mediated antigen processing & presentation | 16                       | 1                          | 6%        |
| Rap1 signalling                                      | 16                       | 1                          | 6%        |
| Eukaryotic Translation Initiation                    | 145                      | 9                          | 6%        |
| SRP-dependent cotranslational protein targeting to membrane | 129                      | 8                          | 6%        |
| APC/C-mediated degradation of cell cycle proteins    | 100                      | 6                          | 6%        |
| Regulation of mitotic cell cycle                     | 100                      | 6                          | 6%        |
| Metabolism of nucleotides                            | 69                       | 4                          | 5%        |
| Metabolism of amino acids and derivatives            | 194                      | 11                         | 5%        |
| Metabolism of proteins                               | 283                      | 16                         | 5%        |
| Translation                                          | 178                      | 10                         | 5%        |
| Signal amplification                                 | 18                       | 1                          | 5%        |
| Semaphorin interactions                              | 79                       | 4                          | 5%        |
| Synthesis of DNA                                     | 120                      | 6                          | 5%        |

PB treatment of rats may exert a perturbation or up-regulation of the pathways, listed in this table, in NPCs. (description see table 6).
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Figure 3. Distribution of distinct proteins, when comparing controls with PB-treatment from the \textit{in vitro} and \textit{in vivo} sample pools, respectively. This figure demonstrates the distribution of distinct proteins found in HCs and NPCs during the pooled A) \textit{in vitro} and B) \textit{in vivo} experiments, while including only proteins found with at least 2 peptides. The up- and down-regulation of proteins were neglected in this qualitative comparison.

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Supporting Information

Figure S1. ELISA verification of arginase-1 variations. This figure depicts the arginase-1 variations in 1) secretome and 2) cytoplasm of HCs using the quantitative data from the ELISA and 3) the semi-quantitative data from the LC-MS/MS analyses (description of how to read this sort of presentation see figure 2), which are altered accordingly. Arginase-1 concentration decreases in the secretome and increases upon PB treatment of rats. Part 4) presents the values used to generate these figure, whereby the emPAI values were used for part 3). (TIF)

Table S1. Summary of proteome profiling results obtained with HCs derived from PB treated rats (in vivo). Column names, which are labelled ‘analysis’ and ‘reference’, refer to the experiments of the isolated primary cells, HCs and NPCs, deriving from rats treated in vivo with PB (analysis) and untreated animals (reference). “found”, specificity of protein identification; accession, Uniprot accession numbers; name, protein names; “analysis_fractions”, subcellular fractions in which the protein was identified in untreated samples; “analysis_peptides”, number of distinct peptides identified per proteins in untreated samples; “analysis_nuclei_expcount”, number of positive identifications in nuclear fractions compared to the total number of experiments; “analysis_nuclei_empai”, calculated emPAI values in the nuclear fraction; “analysis_nuclei_empai_stdev”, standard deviation thereof. These terms are used in the same way referring to the cytoplasm as well as the cytoplasm. After the values obtained for the treated samples (analysis) all values are listed again referring to untreated samples (reference). The last three columns list difference values for the emPAI values obtained for nuclei, secretomes and cytoplasms, respectively. (XLSX)

Table S2. Summary of proteome profiling results obtained with NPCs derived from PB treated rats (in vivo). Column names, which are labelled ‘analysis’ and ‘reference’, refer to the experiments of the isolated primary cells, HCs and NPCs, deriving from rats treated in vivo with PB (analysis) and untreated animals (reference). “found”, specificity of protein identification; accession, Uniprot accession numbers; name, protein names; “analysis_fractions”, subcellular fractions in which the protein was identified in untreated samples; “analysis_peptides”, number of distinct peptides identified per proteins in untreated samples; “analysis_nuclei_expcount”, number of positive identifications in nuclear fractions compared to the total number of experiments; “analysis_nuclei_empai”, calculated emPAI values in the nuclear fraction; “analysis_nuclei_empai_stdev”, standard deviation thereof. These terms are used in the same way referring to the secretome as well as the cytoplasm. After the values obtained for the treated samples (analysis) all values are listed again referring to untreated samples (reference). The last three columns list difference values for the emPAI values obtained for nuclei, secretomes and cytoplasms, respectively. (XLSX)

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Author Contributions

Conceived and designed the experiments: CG BGK VHP SS. Performed the experiments: PK CG SS MP. Analyzed the data: CG BGK. Wrote the manuscript: CG BGK PK.

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