# PROTEOMIC ANALYSIS OF WILD TYPE AND LUCIFERASE REPORTER HEPG2 CELLS EXPOSED TO TCDD

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## Abstract

Luciferase reporter gene assays are more and more used to screen for dioxins in feed and food samples. Other bioassays could be developed to detect a contamination with dioxins, based on specific biomarkers. In order to identify changes in human proteins after exposure to dioxin, we have studied the effect of TCDD on the protein profile of the human hepatoma HepG2 cell line. Furthermore, we have compared the wild type HepG2 (WT-HepG2) cell line with a HepG2 cell line stably transformed with a luciferase reporter gene (Luc-HepG2) in order to study the possible difference in the cell response due to the transformation.
Differential expression analysis of stably transformed (LUC-HepG2) and untransformed (WT-HepG2) cells showed significant changes for 42 proteins. We identified seven of these proteins, which are all involved in DNA repair and/or protein degradation or stabilization.
When comparing TCDD treated and untreated WT-HepG2 cells, we observed significant changes for 22 proteins.
When comparing TCDD treated and untreated Luc-HepG2 cells, 26 protein changes appeared.
Furthermore, our data indicate that dioxin treatment induces different protein changes in WT-HepG2 and in transformed Luc-HepG2 cells.

## Introduction

Dioxins act through direct binding to the AhR receptor that subsequently migrates into the nucleus of the cell to regulate transcription of specific target genes. Human hepatoma HepG2 cells have been widely used to characterize the effects of dioxins on human liver cells. Many studies have been performed by measuring induction of the detoxification enzyme cytochrome C oxydase CYP1A1 using the enzymatic EROD test. More recently, transfection of a plasmid containing the CYP1A1 promoter driving the expression of a reporter enzyme was used either in transient expression experiments or to obtain stably transformed reporter cell lines. Here, we used proteomic analysis of HepG2 cells to identify proteins whose abundance is affected by dioxin treatment, with the aim to detect further biomarkers for dioxin intoxication. Furthermore, we compared original, unmodified HepG2 (WT-HepG2) cells to transformed, luciferase –expressing LUC-HepG2 cells.

## Material and methods

*Cell culture* and *sample preparation*: HepG2 human hepatoma were stably transfected with a construct containing 4 copies of a DRE synthetic oligonocleotide inserted upstream of the thymidine kinase promoter and the luciferase reporter gene (Luc-HepG2). Non transformed cells are named here WT-HepG2.
Cells were treated or not during 24h with 30 nM TCDD in culture medium (containing 0.4% DMSO) and then carefully scraped from the flask. Briefly the protein extracts were prepared by resuspending and homogenizing the cells in hypotonic buffer (Hepes 10 mM, NaCl 10 mM, KH2PO4 1mM, NaHCO3 5 mM, EDTA 5 mM, CaCl2 1mM, MgCl2 0.5 mM containing a protease inhibitor cocktail). The total cell lysate was centrifuged at 21000g for 75 minutes. Total and luciferase protein concentrations were determined in the supernatant.

*2D-DIGE and image analysis :*Protein extracts (25 µg) were labelled using fluorescent Cy2, Cy3 and Cy5 and analyzed by two Dimensional-Differential In Gel Electrophoresis (2D-DIGE). Images were analyzed with the DeCyder software 6.5 (G.E. Healthcare) according to the manufacturer. Each gel was performed in triplicate.
*Protein Identification***.** Spots that showed a significant variation in their abundance were excised from the gel using the Ettan Spot Picker and submitted to tryptic digestion following protein reduction (135 mM DTT) and alkylation (55 mM iodoacetamide). The resulting digested peptides were analyzed with an UltraFlex II MALDI-TOF-TOF (Bruker Daltonics) by MS fingerprint. Protein identifications were carried out using the biotools software (Bruker) using the Mascot search engine ($p < 0.05$).

**Results and discussion**

*1. Comparison of untreated WT- and Luc-HepG2 cells*
Image analysis revealed significant changes in 42 spots (fold change ≥ 1.5, $p< 0.05$): the intensity of 24 of these spots was increased in transformed untreated LUC-HepG2 cells, while it decreased for 18 of these spots (figure 1). Nine of these spots were identified by MALDI-MS (table 1). Five spots, which are increased in the transformed LUC-HepG2 cells, correspond to the same group of proteins, the Heterogeneous nuclear ribonucleoproteins C1/C2 (proteins involved in DNA repair[1]).
Two other over expressed proteins after transformation with the reporter gene were identified as the splicing factor, arginin/serin-rich protein 1 and the mitochondrial precursor of the aldehyde dehydrogenase X (an enzyme involved in protection of the cell against oxidative stress[2]) Four proteins seem to be down regulated after cell transformation: the elongation factor 2 (EF-2), the oxygen-regulated protein precursor (chaperone protein of the endoplasmic reticulum[3]), the heat shock protein HSP 90-beta (cytoplasmic chaperone for many different signalling proteins[4]) and the proteasome activator complex subunit 2 (protein involved in intracellular protein degradation[5]).

*2. Comparison of TCDD treated and untreated WT-HepG2 cells*
Twenty two spots changed their intensity after TCDD treatment of WT-HepG2 cells (figure 1). Among these 22 spots, only 5 were increased while 17 were decreased. The major effect of dioxin treatment seems thus to be an inhibition of protein synthesis.
Two of the 17 proteins which are up-regulated after dioxin treatment have been identified: both belong to the aldehyde dehydrogenase family (the mitochondrial precursor of the aldehyde dehydrogenase X and the retinal dehydrogenase 1) (table 1).

*3. Comparision of TCDD treated and untreated LUC-HepG2 cells*
Twenty six spots showed a change in intensity after TCDD treatment of LUC-HepG2 cells. Among these 26 spots, only 9 were increased while 17 were decreased. Here again, the major effect of dioxin treatment seems to be an inhibition of protein synthesis.
Two of the 9 proteins which are down-regulated after dioxin treatment have been identified: the splicing factor, arginine/serine-rich-1 and the serum albumin precursor (table 1).

*4. Comparison of TCDD treated LUC-HepG2 and WT-HepG2 cells.*
We observed 66 spots presenting a difference of intensity in TCDD treated LUC-HepG2 when compared to TCDD treated WT-HepG2 cells. 29 spots were up-regulated while 37 spots were down-regulated.
From these 37 down-regulated spots, three protein have been identified: the endoplasmin precursor, the liver carboxylesterase 1 precursor and the serum albumin precursor (table 1).
Only 8 of the 66 spots are common to the list of spots showing a change in transformed LUC-HepG2 when compared to WT-HepG2 (both untreated) (table 2). The variations of these 8

spots are consistent in both comparisons: 5 spots are increased in the transformed LUC-HepG2 cells, treated or not with dioxin, and 3 spots are decreased. Unfortunately, none of them could be identified.
The remaining 34 spots showing a change in LUC-HepG2 cells, when compared to WT-HepG2, are unchanged when comparing both dioxin treated LUC-HepG2 and WT-HepG2 (Table 2). It seems that the dioxin treatment masks or reverses these changes.

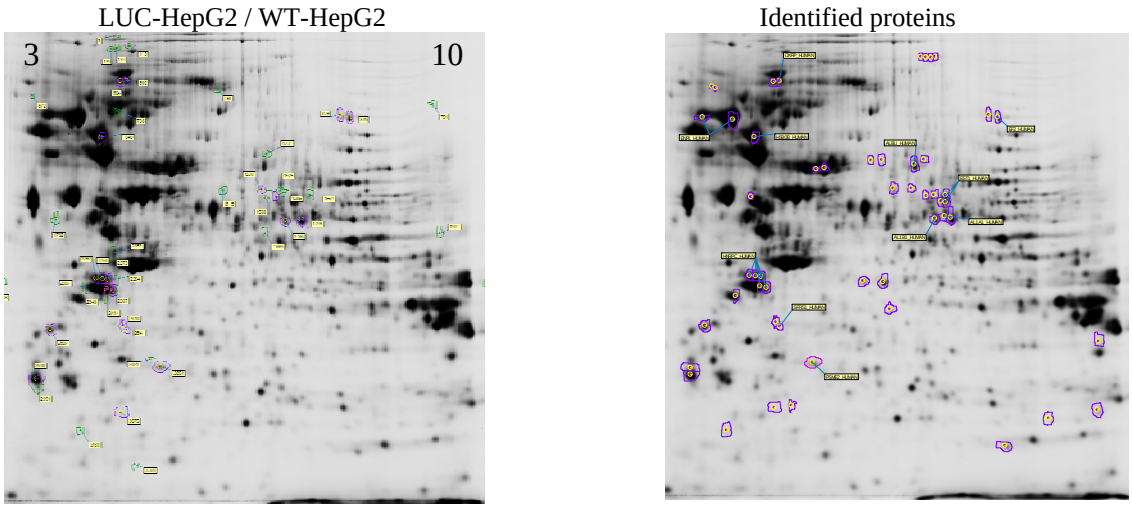


Figure 1 : 2D-DIGE analysis of HepG2 cells proteome. LUC-HepG2 / WT-HepG2 : comparison between WT-HepG2 and LUC-HepG2 cells. Both samples are on the same gel, proteins being differently labelled. Spots arounded in green are up-regulated and spots arounded in magenta are down regulated in the WT-HepG2 cells. WT-HepG2 cells : untransformed and untreated HepG2 cells. LUC-HepG2 cells : HepG2 cells transformed with a luciferase reporter vector, untreated with TCDD.

Surprisingly, the cytochrome P450 CYP1A1 was not detected as an induced protein in any of these cell lines. Similarly, no up-regulated spot corresponding to luciferase was detected in dioxin treated LUC-HepG2 cells, although a 10-fold induction was observed using the enzymatic test. By comparing to a luciferase calibration test, we measured a concentration of luciferase of 0.013% in the soluble protein extract from TCDD treated LUC-HepG2 cells. Theoretically, about 3 ng of luciferase were thus loaded on the 2D-DIGE gel. Even if the detection limit of the identification system is 125 pg, it seems that the amount of these enzymatic proteins is too low relative to the identified high abundance proteins. Fractionation of the extracts followed by 2D-DIGE analyses of specific fractions will possibly resolve this issue.

Finally, when comparing the effects of dioxin treatment on WT-HepG2 and LUC-HepG2 cells, it appears that only one spot is common to both lists (spot n°2014, table 2). In addition, it is regulated in opposite directions in the two cell lines. Our data clearly indicate that dioxin treatment induces different protein changes in WT-HepG2 and in transformed Luc-HepG2 cells (table 2).

References

1. Carpenter B., MacKay C., Alnabulsi A., MacKay M., Telfer C., Melvin W.T., Murray G.I. The roles of heterogeneous nuclear ribonucleoproteins in tumour development and progression. Biochim. Biophys. Acta, 2006, 1765, 85-100.
2. Yoshida A., Rzhetsky A., Hsu L.C., Chang C., Human aldehyde dehydrogenase gene family, European Journal of Biochemistry, 1998, **251**, 549-557.
3. Hoshino T., Nakaya T., Araki W., Suzuki K., Suzuki T., Mizushima T. Endoplasmic reticulum chaperones inhibit the production of amyloid-β peptides. Biochem. J. (2007) 402 (581–589).
4. Rutherford S, Knapp JR, Csermely P. Hsp90 and developmental networks. Adv Exp Med Biol. 2007;594:190-7.
5. Huang L, Burlingame AL. Methods Enzymol. 2005;405:187-236. Comprehensive mass spectrometric analysis of the 20S proteasome complex. .

| Spot ID | Protein name | Code | Mw | PI | Variation | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | LUC-HepG2/ WT-HepG2 | WT-HepG2+TCDD/ WT HepG2 | LUC-HepG2+TCDD/ LUC-HepG2 | LUC-HepG2+TCDD WT-HepG2+TCDD |
| 2268/ 2273 2262 2348 2367 | Heterogeneous nuclear ribonucleoproteins C1/C2 | HNRPC | 33707 | 4,95 | ↗ | | | |
| 911 / 854 | Endoplasmin precursor | ENPL | 92696 | 4,76 | | | | ↘ |
| 1049 | Heat shock protein HSP 90-beta | HS90B | 83423 | 4,82 | ↘ | | | |
| 1633 1638 | Liver carboxylesterase 1 precursor | EST1 | 62766 | 6,15 | | | | ↘ |
| 1314 | Serum albumin precursor | ALBU | 71317 | 5,88 | | | ↘ | ↘ |
| 2641 | Splicing factor, arginine/serine-rich 1 | SFRS1 | 27711 | 10,83 | ↗ | | ↘ | |
| 879 | Elongation factor 2 (EF-2) | EF2 | 96115 | 6,42 | ↘ | | | |
| 1765 | Retinal dehydrogenase 1 | AL1A1 | 55323 | 6,32 | | ↗ | | |
| 1789 | Aldehyde dehydrogenase X | AL1B1 | 57637 | 6,44 | ↗ | ↗ | | |
| 2854 | Proteasome activator complex subunit 2 | PSME2 | 27384 | 5,33 | ↘ | | | |
| 500 | 150 kDa oxygen-regulated protein precursor (Orp150) | OXRP | 111494 | 5,02 | ↘ | | | |

Table 1 : Identified proteins (MALDI-TOF-MS) showing a variation in HepG2 cells transformed with a luciferase reporter vector and/or treated with TCDD. WT-HepG2 cells : untransformed and untreated HepG2 cells. WT-HepG2 + TCDD : TCDD (30nM, 24h) treated cells. LUC-HepG2 cells : HepG2 cells transformed with a luciferase reporter vector, untreated with TCDD. LUC-HepG2 + TCDD : transformed and TCDD (30nM, 24h) treated cell.

| Spot No. | LUC-HepG2/ WT-HepG2 | WT-HepG2+TCDD/ WT-HepG2 | Luc-HepG2+TCDD/ LUC-HepG2 | Luc-HepG2+TCDD/ WT-HepG2 + TCDD | Identification |
|---|---|---|---|---|---|
| 2288 | -2.97 | | | | |
| 1888 | -2.21 | | | | |
| 879 | -1.86 | | | | EF2 HUMAN |
| 876 | -1.85 | | | | |
| 500 | -1.80 | | | | OXRP HUMAN |
| 1554 | -1.80 | | | | |
| 798 | -1.63 | | | | |
| 1049 | -1.57 | | | | HS90B HUMAN |
| 1201 | -1.57 | | | | |
| 1901 | -1.55 | | | | |
| 2854 | -1.54 | | | | PSME2 HUMAN |
| 1509 | -1.51 | | | | |
| 1515 | -1.50 | | | | |
| 2337 | 1.52 | | | | |
| 3183 | 1.53 | | | | |
| 2262 | 1.54 | | | | HNRPC HUMAN |
| 2367 | 1.55 | | | | HNRPC HUMAN |
| 2414 | 1.55 | | | | |
| 2268 | 1.61 | | | | HNRPC HUMAN |
| 2264 | 1.63 | | | | |
| 2821 | 1.64 | | | | |
| 2298 | 1.65 | | | | |
| 672 | 1.67 | | | | |
| 2348 | 1.70 | | | | HNRPC HUMAN |
| 1792 | 1.75 | | | | |
| 2273 | 1.75 | | | | HNRPC HUMAN |
| 3075 | 1.69 | | | | |
| 3381 | 2.26 | | | | |
| 751 | -2.76 | | 2.43 | | |
| 1525 | -1.73 | | 1.54 | | |
| 591 | 1.52 | | -1.61 | | |
| 2610 | 1.52 | | -1.62 | | |
| 2641 | 1.68 | | -2.07 | | SFRS1 HUMAN |
| 504 | -1.77 | | | -1.63 | |
| 1589 | -1.75 | | | -2.31 | |
| 1588 | -1.66 | | | -1.77 | |
| 2900 | 1.57 | | | 2.18 | |
| 2951 | 1.63 | | | 2.46 | |
| 2063 | 1.88 | | | 2.87 | |
| 1798 | 1.70 | 1.58 | | | |
| 1789 | 1.90 | 1.8 | | | AL1B1 HUMAN |
| 2637 | 1.52 | -1.74 | | 3.23 | |
| 2014 | | -1.55 | 1.53 | 1.78 | |
| 1039 | | -3.72 | | 6.85 | |
| 3449 | | -2.13 | | 2.16 | |
| 1585 | | -1.69 | | 1.64 | |
| 1141 | | -1.65 | | 1.55 | |
| 2188 | | -1.57 | | 1.53 | |
| 2215 | | -1.54 | | 1.58 | |
| 1256 | | -1.98 | | -1.73 | |
| 1720 | | -1.96 | | | |
| 1250 | | -1.83 | | | |
| 1515 | | -1.76 | | | |
| 1114 | | -1.67 | | | |
| 1519 | | -1.63 | | | |
| 2046 | | -1.63 | | | |
| 1567 | | -1.62 | | | |
| 1176 | | 1.51 | | | |
| 1765 | | 1.68 | | | AL1A1 HUMAN |
| 2842 | | 1.76 | | | |
| 2299 | | 2.28 | | | |
| 1246 | | | -2.93 | -3.12 | |
| 1314 | | | -2.82 | -2.72 | ALBU HUMAN |
| 2413 | | | -2.02 | -2.59 | |
| 2442 | | | -1.73 | -1.84 | |
| 2313 | | | -1.68 | -2.08 | |
| 2519 | | | -1.63 | -1.96 | |
| 2322 | | | -1.61 | -1.69 | |
| 752 | | | -1.53 | -1.76 | |
| 2774 | | | 1.52 | | |
| 3062 | | | 1.53 | | |
| 1897 | | | 1.56 | | |
| 1354 | | | 1.66 | | |
| 2662 | | | 2.37 | | |
| 3091 | | | 3.14 | | |
| 3391 | | | -2.18 | | |
| 255 | | | -1.84 | | |
| 253 | | | -1.75 | | |
| 245 | | | -1.69 | | |
| 250 | | | -1.69 | | |
| 1184 | | | -1.67 | | |
| 340 | | | | 1.56 | |
| 446 | | | | -2.04 | |
| 563 | | | | -2.56 | |
| 568 | | | | -2.79 | |
| 602 | | | | -1.81 | |
| 820 | | | | -1.54 | |
| 911 | | | | -1.64 | ENPL HUMAN |
| 1017 | | | | 1.97 | |
| 1119 | | | | -3.22 | |
| 1128 | | | | -1.51 | |
| 1130 | | | | -1.54 | |
| 1160 | | | | -1.61 | |
| 1211 | | | | -1.72 | |
| 1255 | | | | -1.53 | |
| 1290 | | | | -1.76 | |
| 1439 | | | | 1.55 | |
| 1526 | | | | -1.63 | |
| 1633 | | | | -2.54 | EST1 HUMAN |
| 1638 | | | | -2.42 | EST1 HUMAN |
| 1693 | | | | -1.57 | |
| 1720 | | | | 1.69 | |
| 1813 | | | | -1.61 | |
| 1860 | | | | -1.9 | |
| 1866 | | | | -1.51 | |
| 2192 | | | | 1.57 | |
| 2290 | | | | -2.05 | |
| 2309 | | | | -1.54 | |
| 2314 | | | | -1.63 | |
| 2499 | | | | -1.59 | |
| 2530 | | | | 1.92 | |
| 2553 | | | | 1.6 | |
| 2704 | | | | 2.54 | |
| 2870 | | | | 1.67 | |
| 2871 | | | | 1.9 | |
| 2895 | | | | 1.92 | |
| 2902 | | | | 2.26 | |
| 2910 | | | | 1.6 | |
| 2970 | | | | 1.81 | |
| 2999 | | | | 1.51 | |
| 3081 | | | | 1.77 | |
| 3144 | | | | 2.26 | |
| 3200 | | | | 1.72 | |
| 3252 | | | | -1.6 | |

Table 2 : List of observed changes in protein spots intensity. WT-HepG2 cells : untransformed and untreated HepG2 cells. WT-HepG2 + TCDD : TCDD (30nM, 24h) treated cells. LUC-HepG2 cells : HepG2 cells transformed with a luciferase reporter vector, untreated with TCDD. LUC-HepG2 + TCDD : transformed and TCDD (30nM, 24h) treated cells. For example, in the column LUC-HepG2/WT-HepG2, “-2,97” for the spot n°2288 means a decrease of intensity in the WT-HepG2 cells.