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# CD neurotox: An integrated approach for the study of cadmium carcinogenesis and neurotoxicity

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## Foreword

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## Abstract

Cadmium is a well-recognised carcinogen, primarily released into the environment by anthropogenic activities (around 30,000 tons/year). In the effort to understand the early events responsible for cadmium carcinogenesis, we propose the use an *in vitro* biological system (the Cell Transformation Assay, CTA), that has been shown to closely model some key stages of the conversion of normal cells into malignant ones. Cadmium-triggered early responses in CTA will be analysed through microarray-based toxicogenomics. Protein expression of specific and non-specific targets of cadmium will be investigated consequently to transcripts analysis. The comparison of early events with those features in transformed cells (*foci*) will suggest the triggering mechanisms, and will provide hints on possible protective and/or preventive substances.

Along with carcinogenesis, a role of cadmium in neurotoxicity and neurodegenerative diseases is emerging. For example, amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting the voluntary motor nervous system. Genetic factors play a major role in the familial form, represented by 10% of cases. Whereas, in the remaining 90% sporadic cases a multifactorial origin is supposed, in which environmental factors may be involved. Particularly, the involvement of metal toxicity is proposed, based on epidemiologic data and on the increasing number of case reports of ALS-like syndromes associated to metal exposure.

Studies of neurodegenerative diseases mainly rely on the use of animal models and on stem cells. Already ten years ago in a Nature Report the validity of neurodegenerative disease animal models was questioned, and direction towards the reduction of transgenic models is mandatory.

In this direction, to investigate cadmium neurotoxic effects a human neuronal cell line have been used be used. We have applied a toxicogenomics approach to identify deregulated pathways in SH-SY5Y cells exposed to Cd, to unravel neuronal specific and non-specific responses to this toxic metal, and to recognize early genes and processes involved upon Cd exposure.All the results from different techniques and approaches (toxicogenomics, specific markers expression, in silico and in vitro methods) are integrated to better understand the carcinogenic and neurotoxic potential of cadmium. The results of this stud have been published in a peer–review paper (Forcella et al. 2020).

# 1 Introduction

Cadmium (Cd) is a widespread environmental contaminant able to enter the human body primarily by inhalation and ingestion, and to accumulate in various organs. Cadmium ions ($Cd^{2+}$) enter the cells through channels and transport pathways dedicated to essential ions, in what has been named a "Trojan horse mechanism" (Martelli et al., 2006). Once absorbed, Cd is trapped in the body and evades detoxification leading to an estimated biological half-life of more than 26 years. The accumulation of this metal contributes to the increase of oxidative stress and to the alteration of divalent ions homeostasis, primarily $Zn^{2+}$ and $Ca^{2+}$ (Choong et al., 2014; Thevenod, 2010; Urani et al., 2015; Callegaro et al., 2018). Cd is a well-known carcinogen as classified by the International Agency for Research on Cancer, but the mechanisms underpinning the molecular processes are not completely clarified.

Cadmium carcinogenicity has been well demonstrated both in *in vitro* biological systems (Urani et al., 2009), such as the Cell Transformation Assays (CTAs), and in humans and animal models (Hartwig, 2013). The CTA is the most advanced in vitro assay for human carcinogenicity prediction induced by chemicals (Vanparys et al., 2012). C3H10T1/2Cl8 mouse embryo fibroblasts are among the suitable cells. Cell transformation assays (CTAs) are available since decades as in vitro tools for carcinogenicity prediction and offer several advantages in comparison to the in vivo bioassays: i) are fast and cost efficient; ii) allow to identify not only genotoxic, but also some non-genotoxic compounds; iii) allow the clarification of in vitro genotoxic positive results; iv) provide a means to investigate tumour promotion activities and efficacy of chemopreventive agents; and v) support the 3Rs principles of replacement, reduction and refinement (EURL ECVAM, 2012). In vitro CTAs have been shown to closely mimic some stages of the in vivo carcinogenesis process. These assays rely on the ability of specific cell cultures to form foci of morphologically transformed cells upon exposure to carcinogens. The endpoint in CTAs are the number and type of foci formed, scored and classified by trained experts under light microscopy based on standard morphological features, leading to the estimate of the transformation frequency (i.e. the carcinogenic potential) of the test compound (Sasaki et al., 2012). The CTAs are currently used by academia, the chemical, agro-chemical, cosmetic, pharmaceutical, and tobacco industries for, e.g. mechanistic studies, the investigation of tumour promotion, the screening of chemo-preventing activities. These assays are also reported in the list of accepted methods within the new European chemical regulation REACH for screening purposes, and are listed in various recent guidelines and testing strategies (SCCP 2010; Jacobson-Kram and Jacobs, 2005; ECHA, 2008; Pfuhler et al., 2010).

Furthermore, exposure to and consequent effects of metals are of great concern as are among the top ten hazardous chemicals for human health (ATDSR, 2017). Heavy metals and metalloids (e.g., As, Pb, Hg, Cd) have also been associated to many neurodegenerative diseases, but their mechanism of action are far from being identified.

We have applied a toxicogenomics approach to identify deregulated pathways in SH-SY5Y cells exposed to Cd, to unravel neuronal specific and non-specific responses to this toxic metal, and to recognize early genes and processes involved upon Cd exposure.

SH-SY5Y is a neuroblastoma cell line, widely used as in vitro model for neurotoxicity studies and neurodegenerative diseases (Cheung et al., 2009). In addition, following the recommendations of the National Research Council of the National Academy of Sciences described in a recent report on toxicity testing in the 21st century, SHSY5Y cells were used in this work as they are from human origin.

The results of this study have been published in a peer–review paper (Forcella et al. 2020).

# 2 Experimental procedure

The present project aims at evaluating in an integrated approach the biological effects of heavy metals, and specifically of cadmium, on carcinogenesis processes and on neurotoxicity.

Suitable cell models are proposed to perform the experiments. The cell transformation assay have been performed using recommended cell models (e.g., C3H10T1/2 mouse embryo fibroblasts, OECD, 2007).

At the end of the assays, *foci* of transformed cells and all relevant controls collected were be analysed for:

- Gene expression through transcriptomic analysis.
- An in-depth analysis on specific sub-cellular functions (e.g., energetic metabolism),.
- A comparison of different foci induced by the same stimulus (metal) or by different metals, and an analysis at different time-points and recovery.

In parallel, experiments on specific cell markers and functions have been performed and compared with transcriptomic data and to validate them.

On the side of neurotoxicity, human neuronal cells (SH-SY5Y) have been used. The cells were exposed to different cadmium concentrations to identify the dose-responses, threshold concentrations, metal accumulation and transcriptomic responses and altered molecular pathways.

The transcriptomic analysis was performed with Agilent G2565BA Microarray Scanner (AgilentTechnologies Inc.), present in the nanobiotechnology laboratory of JRC.

As in carcinogenesis experiments, transcriptomic data have been validated by qRT-PCR and by protein expression analysis. Aims of the project are represented in addition by identification of a "tissue-specific or non-specific" mechanism by cadmium and cell and molecular targets. For this reason, cells from different target organs (e.g., pulmonary cells) could be analysed, as well as different metals.

# 3 Results

Cadmium induces a strong deregulation of specific transcripts. A total of 85 genes were significantly up-regulated and 11 genes were down-regulated (ANOVA limma, p value adjusted by the Benjamini and Hochberg's method equals or smaller than 0.05) (Smyth, 2004). The first 25 up-regulated genes are shown in Table 1 with log2 fold changes values and statistical significance (adjusted p value). Table 2 shows all down-regulated genes, as published in Forcella et al., 2020.

**Table 1:** Top up regulated genes in SH-SY5Y cells treated with 10 or 20 µM Cd for 48h

| Gene | Cd10 µM log2 fold change | Cd20 µM log2 fold change | adj.P.Value | Description |
|---|---|---|---|---|
| MT1M | 9,71 | 9,92 | 2,61E-05 | metallothionein 1M |
| MT1X | 7,11 | 7,53 | 7,53E-05 | metallothionein 1X |
| MT1F | 7,10 | 7,37 | 8,98E-05 | metallothionein 1F |
| MT1 L | 7,04 | 7,43 | 3,84E-05 | metallothionein 1 L (gene/pseudogene) |
| MT1HL1 | 6,95 | 7,42 | 3,84E-05 | Metallothionein 1H-like protein 1 |
| MT1B | 6,74 | 7,25 | 2,61E-05 | metallothionein 1B |
| ENST00000567054 | 6,63 | 7,02 | 3,84E-05 | metallothionein 1C (pseudogene) |
| HMOX1 | 6,08 | 7,26 | 0,001731748 | heme oxygenase (decycling) 1 |
| MT2A | 5,42 | 5,45 | 0,000160204 | metallothionein 2A |
| MT1A | 4,92 | 5,29 | 0,00014948 | metallothionein 1A |
| GADD45β | 4,82 | 7,05 | 0,000545108 | growth arrest and DNA-damage-inducible, beta |
| MT1E | 3,95 | 4,15 | 0,000250941 | metallothionein 1E |
| ZFAND2A | 3,64 | 5,20 | 0,006524353 | zinc finger, AN1-type domain 2A |
| TEX19 | 3,47 | 4,15 | 0,003753672 | testis expressed 19 |
| GDF15 | 3,24 | 4,22 | 0,002993824 | growth differentiation factor 15 |
| AKR1C3 | 2,94 | 3,14 | 0,002993824 | aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II) |
| TGFBI | 2,90 | 3,59 | 0,013115332 | transforming growth factor, beta-induced, 68kDa |
| MT1G | 2,70 | 3,36 | 0,034682665 | metallothionein 1G |
| HSPA1A | 2,67 | 4,38 | 0,005651168 | heat shock 70 kDa protein 1A |
| HSPA6 | 2,53 | 4,36 | 0,000545108 | heat shock 70 kDa protein 6 (Hsp70B') |
| RRAD | 2,53 | 4,24 | 0,00208082 | Ras-related associated with diabetes |
| HSPA1B | 2,29 | 4,10 | 0,014428639 | heat shock 70 kDa protein 1B |
| DNAJB1 | 2,23 | 3,57 | 0,001731748 | DnaJ (Hsp40) homolog, subfamily B, member 1 |
| DDIT3 | 2,21 | 3,45 | 0,000545108 | DNA-damage-inducible transcript 3 |
| S100A2 | 1,98 | 2,91 | 0,001731748 | S100 calcium binding protein A2 |

**Table 2:** Complete list of down regulated genes in SH-SY5Y cells treated with 10 or 20µM Cd for 48h

| Gene | Cd10 µM log2 fold change | Cd20 µM log2 fold change | adj.P.Value | Description |
|---|---|---|---|---|
| SLC35D3 | −1,41 | −2,15 | 0,03018534 | solute carrier family 35, member D3 |
| GREM2 | −1,02 | −1,32 | 0,02198818 | gremlin 2 |
| SLC39A10 | −0,88 | −1,07 | 0,04704006 | solute carrier family 39 (zinc transporter), member 10 |
| GLCCI1 | −0,84 | −1,42 | 0,02971039 | glucocorticoid induced transcript 1 |
| GALNT6 | −0,82 | −1,48 | 0,03741771 | UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 6 (GalNAc-T6) |
| NEK3 | −0,77 | −1,28 | 0,04518064 | NIMA (never in mitosis gene a)-related kinase 3 |
| UNG | −0,72 | −1,00 | 0,04826738 | uracil-DNA glycosylase |
| TXNIP | −0,65 | −1,77 | 0,01403976 | thioredoxin interacting protein |
| LOC642366 | −0,53 | −1,24 | 0,04997033 | uncharacterized LOC642366 |
| PDGFRL | −0,51 | −1,53 | 0,02586179 | platelet-derived growth factor receptor-like |
| KIF15 | −0,42 | −1,09 | 0,03768701 | kinesin family member 15 |

# 4 Conclusions

The results on human SHM- SY5Y neuronal cells confirm that cadmium induces the expression of genes belonging to a carcinogenic effect even on brain-derived cells.

The results demonstrate that Cd deregulates the expression of genes involved in specific neuronal functions and pathways, giving an overall picture strongly associated to a metal dyshomeostasis and to a damage of neuronal functions and dynamics.

The results of this study provide new insights and links on Cd-induced neurotoxicity, and support further in-depth studies on remedies to counteract the induced essential metal dyshomeostasis. As concluding remarks, we highlight that toxicogenomics approach is invaluable for mechanistic studies as it provides information on all possible dysregulated genes upon a specific environmental insult. The identification and systematic analysis of up- and down-regulated genes not only provide evidence on functions related to neurodegeneration at a single gene level, but also give a comprehensive vision of possible altered processes. In addition, this analysis will help to clarify whether metal-induced cell deregulations are the consequence rather than the cause of neurodegeneration. Deregulated pathways, even not cell-specific, could represent the early triggers for subsequent metabolic and structural unbalances and for neurodegeneration.

Finally, the analysis in a controlled environment and standardized neuronal cell model, along with further investigations in more complex models such as co-cultures or 3D models with repeated or longer exposure time, could help in identifying potential biomarkers to be studied in exposed individuals or in the general population.

All the results obtained with this study are discussed in the peer review article Forcella et al. 2020.

## References


G. Callegaro, M Forcella, P Melchioretto, A Frattini, L Gribaldo, P Fusi, M Fabbri, C Urani, Toxicogenomics applied to in vitro Cell Transformation Assay reveals mechanisms of early response to cadmium, Toxicology in Vitro, Volume 48,2018,Pages 232-243,

Z H. Cheung, N Y. Ip Autophagy deregulation in neurodegenerative diseases – recent advances and future perspectives Volume118, Issue3, August 2011, Pages 317-325

G. Choong, Y. Liu, D. M. Templeton, Interplay of calcium and cadmium in mediating cadmium toxicity, Chemico-Biological Interactions, Volume 211, 2014, Pages 54-65,

M. Forcella, P. Lau, M. Oldani, P. Melchioretto, A. Bogni, L. Gribaldo, P. Fusi, C. Urani, Neuronal specific and non-specific responses to cadmium possibly involved in neurodegeneration: A toxicogenomics study in a human neuronal cell model Neurotoxicology 76 (2020) 162–173

Hartwig, A. (2013). Cadmium and Cancer. In: Sigel, A., Sigel, H., Sigel, R. (eds) Cadmium: From Toxicity to Essentiality. Metal Ions in Life Sciences, vol 11. Springer, DordrechtVanparys et al., 2012

Jacobson-Kram D, Jacobs A. Use of Genotoxicity Data to Support Clinical Trials or Positive Genetox Findings on a Candidate Pharmaceutical or Impurity …. Now What? International Journal of Toxicology. 2005;24(3):129-134.

A. Martelli, E. Rousselet, C. Dycke, A. Bouron, J.-M. Moulis, Cadmium toxicity in animal cells by interference with essential metals, Biochimie, Volume 88, Issue 11, 2006, Pages 1807-1814,

K Sasaki, S Bohnenberger, K Hayashi, T Kunkelmann, D Muramatsu, P Phrakonkham, A Poth, A Sakai, S Salovaara, N Tanaka, B. C Thomas, M Umeda, Recommended protocol for the BALB/c 3T3 cell transformation assay, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, Volume 744, Issue 1, 2012, Pages 30-35,

Thévenod, F. Catch me if you can! Novel aspects of cadmium transport in mammalian cells. Biometals 23, 857–875 (2010).;

Urani, C., Melchioretto, P., Bruschi, M., Fabbri, M., Sacco, M.G., Gribaldo, L., 2015. Impact of cadmium on intracellular zinc levels in HepG2 cells: quantitative evaluations and molecular effects. Biomed Res. Int. article ID 949514, 1–11.

Urani, C.; Melchioretto, P.; Gribaldo, L. Regulation of Metallothioneins and ZnT-1 Transporter Expression in Human Hepatoma Cells HepG2 Exposed to Zinc and Cadmium. Toxicol. In Vitro 2010, 24, 370–374.;

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