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Generation of hydrogen peroxide-resistant murine neuroblastoma cells: a target discovery platform for novel neuroprotective genes

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Generation of hydrogen peroxide-resistant murine neuroblastoma cells: a target discovery platform for novel neuroprotective genes

Abstract
Oxidative stress has been suggested to play an important role in the pathogenesis of various neurodegenerative diseases including Alzheimer's disease (AD). Hydrogen peroxide (H_2O_2), one of the main reactive oxygen species, is converted into the highly toxic ·OH radical in the presence of redox-active transition metals, which then oxidises nucleic acids, lipids and proteins, leading to neurodegeneration and cell death. There is an urgent need to gain more knowledge about relevant therapeutic targets to combat oxidative stress and it neurotoxic effects, and how this knowledge can be utilized to develop novel neuroprotective therapies for AD. One way to identify new mechanisms combating oxidative stress was via the creation of H_2O_2-resistant cell lines and identification of the mechanisms responsible for their resistance. However, in most cases catalase overexpression or increased glutathione content was identified as the primary mode of H_2O_2 resistance in these cell lines. In this study, we have generated six different resistant neuronal cell lines or populations (from the same original murine Neuro2a neuroblastoma line) by exposing cells to increasing concentrations of H_2O_2 and performing continuous selection for survivors over a period of several months, which appear to have acquired H_2O_2 resistance based on other, novel mechanisms. These six populations showed a significant, but differential resistance against H_2O_2 when compared with the parental cell line. Using combinations of catalase-, glutathione synthesis- and glutathione peroxidase-inhibitors it was shown that the increased resistance of Neuro2a-HR cells is not solely based on an increased activity of catalase or the glutathione system, suggesting that their resistance might be based on yet unknown, novel defence mechanisms.

Keywords
generation, neuroprotective, hydrogen, genes, peroxide, resistant, murine, neuroblastoma, cells, target, discovery, platform, novel, CMMB

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GENERATION OF HYDROGEN PEROXIDE RESISTANT MURINE NEUROBLASTOMA CELLS – A TARGET DISCOVERY PLATFORM FOR NOVEL NEUROPROTECTIVE GENES

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Keywords: Hydrogen peroxide, oxidative stress, resistance, glutathione peroxidase, catalase

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Introduction

Oxidative stress has been suggested to play an important role in the pathogenesis of various neurodegenerative condition including Parkinson's and Alzheimer's disease (AD) (Behl, 1997; Butterfield and Kanski, 2001; Jenner, 1991; Jenner and Olanow, 1996, 2006). Oxidative damage in AD brain is manifested by increased protein oxidation (elevated levels of protein carbonyls and nitrated tyrosine residues), lipid peroxidation (elevated levels of thiobarbituric acid reactive substances malondialdehyde, 4-hydroxy-2-trans-nonenal, isoprostanes), DNA and RNA oxidation (elevated levels of 8-hydroxy-2-deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHG)) and increased levels of glycoxidation products (Vitek et al., 1994).

Hydrogen peroxide (H_2O_2) is one of the most prominent and frequent representatives of reactive oxygen species (ROS). H_2O_2 itself is not very reactive and on its own not a very potent oxidizing threat to cells. However, it is easily converted into the highly reactive $^{\cdot}OH$ radical via the Fenton reaction, which then oxidises nucleic acids, lipids and proteins. Chronic treatment of mammalian cells with H_2O_2 has shown that cells are able to adapt to oxidative stress. The most common resistance mechanisms are based on an increase in glutathione GSH content or activity of antioxidative enzymes such as superoxide dismutase (SOD), catalase or glutathione peroxidase (GPX) (Bose Girigoswami et al., 2005; Cantoni et al., 1993; Spitz et al., 1992; Spitz et al., 1988b).

Cellular detoxification systems such as GSH, thioredoxin, (SOD) or catalase which counteract oxidative stress are pushed to their limits (Lovell et al., 1995; Marcus et al., 1998). In brains of AD patients the GSH content was found to be significantly

decreased, while the expression of catalase, Cu/Zn-SOD, (GPX) and glutathione reductase (GR) was elevated (Aksenov et al., 2001; Aksenov et al., 1998; Liu et al., 2004). Despite increased levels of catalase mRNA, the activity of catalase in AD affected brains was also found to be decreased (Gsell et al., 1995).

It has been proposed that H_2O_2 resistant cell lines can be used to unravel novel H_2O_2 protection-conferring genes (or more correctly, the proteins they code for), and that such novel genes can contribute to the development of novel neuroprotective drugs. To date, a number of studies have reported the generation of H_2O_2 resistant cells (Andley and Spector, 2005; Bose Girigoswami et al., 2005; Spector et al., 2000; Spitz and Sullivan, 2010). One of them, a murine hippocampal HT22 cell line, was generated by Schafer*et al* (Schafer et al., 2004) and showed a cross-resistance to glutamate, while the simultaneously created glutamate resistant cell line showed a similar resistance against H_2O_2. Further investigation revealed that both cell lines contained high levels of phosphorylated GSK-3β, resulting in an inactivation of this apoptosis-inducing protein.

The aim of this study was to generate H_2O_2 resistant cells using the murine neuronal cell line Neuro2a for further studies on their differential gene expression. H_2O_2 resistant cells are likely to exhibit changes in gene expression, allowing for the identification of novel genes and mechanisms that confer neuroprotection against oxidative stress.

Materials and Methods

Cell Culture

Neuro2a cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) containing 5% foetal bovine serum (FBS), 200 U/mL Penicillin, 200 µg/mL Streptomycin, 2.6 µg/mL Fungizone®, and 2 mM glutamine or GlutaMAX™-I (Invitrogen, Carlsbad, USA). Cells were maintained in a 5% CO_2 incubator at 37°C. To passage confluent cells medium was aspirated and the cells were washed with sterile Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS), and incubated with Tryple Express (Invitrogen, Carlsbad, USA) at 37ºC until cells detached from the flask. Cells were resuspended in DMEM and transferred into new flask in a suitable ratio.

Generation of H_2O_2 resistant cells by chronic H_2O_2 treatment

High level weekly treatment to generate Neuro2a-HR_HL cells: Neuro2a cells were cultured in T175 flasks and treated weekly with 500 µM H_2O_2 in a volume of 40 mL growth medium. This medium was removed the day after treatment and fresh growth medium was added. Cells were split as required, so cells had a confluency of 80-90% on the day of treatment. H_2O_2 concentrations were gradually increased to a final concentration of 1 mM. Those cells were named Neuro2a-HR_HL, clones B8, E3 and F5 were isolated by dilution to approximately 0.8 cells/well from this population after six months of treatment.

Low level daily treatment to produce Neuro2a-HR_LL cells: Neuro2a-HR_LL cells were generated by growth in 6-well plates and treated with 30 µM H_2O_2 in a volume of 2 mL growth medium daily. H_2O_2 concentrations were slowly increased up to 200 µM. Cells were split as required. For Neuro2a-HR_LL, subpopulations E525,

E725 and E735 were selected by dilution to approximately 0.8 cells/well in 96-well plates after four months.

Assessment of H_2O_2 resistance of Neuro2a cells

In order to only test constitutively expressed genes, cells were withdrawn from H_2O_2 treatment for at least 2 weeks prior to testing them for H_2O_2 resistance. In brief, 5×10^4 cells/well were seeded in 96-well plates and incubated overnight. Medium was aspirated, replaced with 100 µL growth medium containing differing amounts of H_2O_2 and cells were incubated at 37°C and 5% CO_2. The cell viability was determined 24 hr later using the resazurin (Alamar Blue) assay (O'Brien et al., 2000).

Assessing contribution of catalase and glutathione system to H_2O_2 resistance of Neuro2a cells

The two major H_2O_2 detoxification systems in mammalian cells are the catalase system and the GSH system. To investigate the contribution of each of these systems to the H_2O_2 resistance of the resistant neuron-like cells, cells were seeded and incubated overnight. Cells were incubated with 10 mM catalase inhibitor 3-Amino-1,2,4-triazole (3-AT) and/or 10 mM GPX inhibitor mercaptosuccinic acid (MS) for 2 hr and/or 1 mM γ-glutamylcysteine synthetase inhibitor L-Buthionine-sulfoximine (BSO) for 22 hr. To obtain comparable results for all tested conditions all plates were seeded on the same day, the next day the medium containing 5% FBS was replaced with DMEM with 1% FBS with or without 1 mM BSO for 22 hr. Thereafter all medium was replaced with DMEM containing 1% FBS with or without the other inhibitors or combinations of inhibitors for 2 hr. After removal of the inhibitor(s) containing medium, fresh medium with 5% FBS containing different

amounts of H_2O_2 was added and cells were incubated at 37°C and 5% CO_2. Cell viability was determined 24 hr later using the resazurin reduction assay. All preparations of the inhibitor in DMEM were sterile filtered using 0.22 µm filters; preparations containing MS were pH adjusted to 7.5.

Cell viability assay

Cell viability was measured as mitochondrial metabolic activity using resazurin to resorufin reduction (John O'Brien, 2000). Briefly, 100 µL DMEM containing 0.125 mg/L resazurin were added to each well of a 96-well plate and incubated for 2 hr at 37°C. Fluorescence was then measured at 560EX nm/590EM nm in a BMG Labtech POLARstar Omega fluorescent plate reader (BMG LABTECH, Ortenberg, Germany). Wells containing resazurin and no cells were used to determine background fluorescence. All measurements were done in triplicate and expressed as a percentage of control cells. Experiments were repeated once.

Determination of H_2O_2 in cell culture medium via PCA-Fox assay

To measure H_2O_2 clearance from the medium, 1 mM H_2O_2 in 100 µL DMEM were added to each well of a 96-well plate containing 5×10^4 cells. After incubation for 0-90 min at 37° the conditioned media was removed from the cells and diluted 1:10 in PBS. The diluted samples (180 µL) were transferred into a new 96-well plate and 20 µL Fox-reagent (2.5 mM ferrous ammonium sulphate, 2.5 mM xylenol orange, 1.10 M $HClO_4$) were added and mixed. The plate was incubated on a rocking platform for 30 min and the absorbance was measured at 545 nm in a BIORAD microplate reader (Bio-Rad Laboratories, Hercules, US).

Statistical Analysis

All measurements were done in triplicate and expressed as a percentage of untreated control cells. One-way ANOVA was performed using SPSS® Statistics 18 software (IBM, Armonk, USA) on data expressed as percentage of control cells for each experiment and the Games-Howell posthoc test was used when variances were significant different within groups; for homogeneity of variance the Tukey posthoc test was used. Results were presented as the mean ± SD using GraphPad Prism® Version 5.04 (GraphPad Software, La Jolla, USA).

Results

Generation and selection of H_2O_2 resistant murine neuronal cells

The aim of these experiments was to create a neuron-like cell line derived from murine Neuro2a cells with increased resistance against H_2O_2, in which novel neuroprotective genes that confer protection against H_2O_2, could be identified.

The murine neuroblastoma cell line Neuro2a was used to generate two different types of H_2O_2 resistant cell populations. The basic principles of this process were to treat Neuro2a cells with increasing amounts of H_2O_2 over long time periods and to select for the "best survivors" under these conditions (Spitz et al., 1988a).

For the generation of Neuro2a-HR_HL (high level) subclones: Parental Neuro2a cells were treated with 250 µM H_2O_2 once a week. This concentration of H_2O_2 led to 95-99% cell death within 24 hr of treatment, after which the surviving cells regrew to confluence within a week. However, cells adapted to the treatment with 250 µM H_2O_2, and the rate of cell death decreased upon subsequent challenges. Therefore, H_2O_2concentrations were slowly increased so that still a significant rate of cell death was seen (highest concentration of 1 mM H_2O_2). After six months of H_2O_2 treatment three single cell clones, termed Neuro2a-HR_HL B8, E3 and F5 were isolated using the dilution method.

For the generation of the Neuro2a-HR_LL subpopulations, parental Neuro2a cells were treated with 30 µM H_2O_2 daily. At this concentration cells seemed to be mostly unaffected by the treatment and no cell death was observed by microscopy. The H_2O_2 concentration was increased slowly by ~10 µM per week until reaching 200 µM daily within four months. Three subpopulations of cells, termed Neuro2a-HR_LL E525, E725 and E735, were isolated after fourmonths as daily treatment with H_2O_2

Determination of H_2O_2 resistance of the Neuro2a-HR cells

The H_2O_2 resistance of Neuro2a-HL and Neuro2a-LL cells in comparison with the parental cells was tested after challenge with hydrogen peroxide. Cells were incubated with H_2O_2 at concentrations up to 10 mM, and cell viability determined after 24 hours. Parental Neuro2a cells exhibited an LC_{50} (lethal concentration of H_2O_2 to cause 50% cell death) value of 214 ± 10µM (n=3, in triplicate), while the LC_{50} for all the H_2O_2 resistant Neuro2a cells was > 1mM, reaching 10.5 ± 0.71 mM in the most resistant subpopulation, LL E725 (Figure1 A and B Table 2).

Contribution of catalase activity to the resistance of Neuro2a-HR cells

For the successful identification of novel H_2O_2 resistance-conferring genes in the resistant cells it is necessary that the Neuro2a-HR populations would still show an increased H_2O_2 resistance when compared to parental cells, even when the H_2O_2 detoxification systems such as catalase and/or GPX are inhibited. To exclude that the resistance of the generated Neuro2a-HR cells was simply based on an increase in catalase activity, the H_2O_2 resistance of the selected six H_2O_2 resistant clones or subpopulations was tested after inhibition of catalase. Cells were pre-incubated with the irreversible catalase inhibitor 3-AT (10 mM) for 2 hr before being challenged with H_2O_2. This treatment has been previously found to completely inhibit catalase activity in astrocytes (Dringen and Hamprecht, 1997). Cell viability was determined after 24 hr.

After catalase inhibition the LC_{50} of parental cells dropped by approximately 70%, from 214 ± 10 µM to 75.2 ± 29.0 µM (n=3, in triplicate). Among the resistant cells, the Neuro2a-HR_LL cells reached the LC_{50} at approximately 400 - 600 µM, indicating a

possible five to almost eight-fold higher resistance than the parental cells. Neuro2a-HR_HL clones appeared not as homogenous in this assay, with LC_{50} ranging from 310 µM for clone B8 to approximately 600 µM for clones E3 and F5 (Fig. 2) . This "resistance gap" of 4-8fold compared to the parental cells even at complete inhibition of catalase activity indicates that the increased resistance of all six Neuro2a-HR populations is not simply caused by overactivity of the catalase system.

Contribution of glutathione peroxidase (GPX) activity to the resistance of Neuro2a-HR cells

GPX is a further enzymatic system involved in the direct detoxification of H_2O_2. GPX was inhibited to assess the contribution of this enzyme to the increased resistance of Neuro2a-HR cells towards H_2O_2. Cells were pre-incubated for 2 hr with 10 mM MS, a reversible inhibitor of GPX. Cells were then challenged as previously described and cell viability was determined. In astroglial cells these treatment conditions were found to fully inhibit GPX activity (Dringen et al., 1998; Kussmaul et al., 1999).GPX inhibition resulted in a decrease in the LC_{50} of parental and all Neuro2a-HR cells.The LC_{50} of parental cells decreased from 214± 10 µM to 132 ± 64 µM, a decrease of almost 50%. Neuro2a-HR_LL subpopulations had LC_{50} values that ranged from 341 µM to ≥ 450 µM. Similar findings were made for Neuro2a-HR_HL cells, which displayed LC_{50} values between 194 µM to ≥ 311 µM. As seen in prior experiments, the B8 clone did not perform as well as the E3 and F5 clones, reaching a LC_{50} of 194 ± 43 µM while all other clones reached LC_{50} values of ≥ 311 µM. This "resistance gap" indicates that an increased GPX activity is also not responsible for the increased resistance of Neuro2a-HR cells.

Contribution of the total GSH pool to the resistance of Neuro2a-HR cells

Increased resistance of Neuro2a-HR cells could be based on a faster production or a higher level of free available GSH (the cofactor for GPX, but also may other GSH dependent enzymes). To examine this possibility, cells were pre-incubated with 1 mM BSO for 22 hr and then challenged with 0-400 µM H_2O_2 as previously described. BSO is an irreversible inhibitor of γ glutamylcysteine synthetase, the rate limiting enzyme in GSH synthesis(Drew and Miners, 1984). BSO treatment reduced the LC_{50} of parental cells by 48% to 119 ± 24 µM, while Neuro2a-HR_LL cells showed LC_{50} values of 233 to 564 µM. Neuro2-HR_HL cells showed comparable resistance, with LC_{50} values ranging from 233 ± 51µM for clone B8 to ≥ 400 µM for both other clones (Table 5).

Combined contribution of catalase and the GSH pool to the resistance of Neuro2a-HR cells

In the previous sections, it was shown that the higher resistance of Neuro2a-HR cells is not solely due to an increased catalase activity, GPX activity or GSH pool of the cells, as inhibition of both pathways still showed an increased resistance against H_2O_2 when compared to parental cells. This indicates that Neuro2a-HR cells might have acquired additional defence systems. To confirm this hypothesis, two treatments from previous experiments were combined and cells were treated with 3-AT and BSO to inhibit catalase and GSH synthesis (which includes GSH as the rate-limiting cofactor for GPX activity) before assessing their H_2O_2 resistance.

It was found that the parental tolerance against H_2O_2 decreased by more than 90%, showing an LC_{50} of 20.5 ± 5.4 µM. Neuro2a-HR_LL cells showed LC_{50} values between 125 µM and ≥ 200µM, suggesting an at least 6-fold higher resistance than the parental cells (Table 6). Comparable results were observed when catalase and GSH production were inhibited in Neuro2a-HR_HL cells, with LC_{50} values ranging between 34 µM for clone B8 and ≥ 80 µM for F5. This "resistance gap" suggests again that an increased catalase activity and higher GSH content is not responsible for the increased resistance of Neuro2a-HR cells, but that other mechanisms must play a role in the acquired resistance mechanism.

Comparison of the rate of detoxification of H_2O_2 between Neuro2a and Neuro2a-HR cells

The previous sections suggest that the resistance mechanism of Neuro2a-HR cells is not simply based on an increased detoxification of H_2O_2 by catalase and GPX. To further test this hypothesis, it was investigated if Neuro2a-HR cells detoxify H_2O_2 more effectively than the original parental cell line. For this purpose, the rate of clearance of 1 mM H_2O_2 exogenously added to the cell culture medium, was measured using the PCA-Fox assay (Gay et al., 1999). No significant differences in the rate of detoxification of exogenous added H_2O_2 (calculating half-life assuming first order kinetics) were detected between the parental and Neuro2a-HR cells. All cells clones were capable of detoxifying 1m M H_2O_2, with a $T_{1/2}$ of 30.2 ± 2.0 min for the parental cells, and between 26.0 and 31.6 min for the resistant clones or subpopulations (Fig. 3). This supports our hypothesis that the increased resistance of Neuro2a-HR cells is not simply based on a higher activity of the detoxification systems for H_2O_2.

Discussion

Identification of novel defence mechanisms against H_2O_2 could lead to the development of new drug targets for the treatment of neurodegenerative diseases, and AD particularly, as oxidative stress plays an important role in these diseases (Boll et al., 2008; Butterfield et al., 2002; Shamoto-Nagai et al., 2007). Slowly adapting cells against a toxin has proven to be beneficial in order to find such protection-conferring mechanisms against such a toxin. This method, called conditioning, has shown to often lead to an increased tolerance or total resistance of those cell populations against the toxin (Ramakrishnan et al., 2010). These resistant cell lines can then be further examined to find the protection-conferring genes (Alborzinia et al., 2011; Bose Girigoswami et al., 2005).

The future overall aim of this study is to find novel drug targets for diseases involving oxidative stress by comparison of the gene expression pattern between parental and H_2O_2 resistant cells. It was therefore necessary to generate a H_2O_2 resistant neuron-like cell line. The murine neuroblastoma cell line Neuro2a was chosen as the neural cell model for this purpose.

AD is a human disease that mostly affects glutamatergic and cholinergic neurons and therefore it would be more than justifiable to use human primary cells (Greenamyre et al., 1987; Lewis et al., 2010; Pearson et al., 1983). Those cells would need to be made immortal by stable transfection of telomerase-based or oncogene-containing vectors (Davies et al., 2003). Constant passaging and immortalisation of such primary cells often affects their natural characteristics (Georgopoulos et al., 2011). Further characterisation of this newly created cell line would therefore be unavoidable. Another disadvantage of this approach would be the limited access to human brain samples. Instead, neuroblastoma cell lines are a

commonly used tool for neurotoxicity studies (LePage et al., 2005). Although these cells might not have an identical phenotype as the original cell line, they have the advantage of being well established and characterised. Depending on the aim of the study, they may be a valuable and valid alternative.

Several different cell types, such as murine lens, monkey kidney or hamster ovarian cells, have been found to develop resistance after conditioning with H_2O_2 (da Silva et al., 1996; Spector et al., 2000). This suggests that the resistance is based on a universal mechanism, which potentially could be found in any cell line. Based on this hypothesis a murine neuroblastoma cell line was used for this project, as this allows the subsequent development of a mouse model as the next step in drug discovery.

Two different approaches of H_2O_2 treatment were used, as acute high concentrations of H_2O_2 can lead to a different expression pattern of protective genes when compared to low chronic exposures (Cantoni et al., 1993).

All Neuro2a-HR cells displayed a high H_2O_2 resistance compared to parental cells when challenged with H_2O_2 concentrations of up to 1 mM. This gap in resistance was still present when catalase and/or the glutathione system were inhibited. It did not make a difference whether cells had been created by treatment with low or high levels of H_2O_2, as all Neuro2a-HR cells were resistant to equal amounts of H_2O_2. This is an opposite finding to prior studies where treatment of cells with higher concentrations of H_2O_2 also induced higher catalase activity (Cantoni et al., 1993).

Inhibition of catalase decreased the LC_{50} by approximately 70% to 75 ± 29 µM, indicating that catalase is indeed an important defence system in mammalian cells, accounting for approximately 70% of H_2O_2 defence. Inhibition of GPX let to a decrease in LC_{50} by 42% (132 ± 64 µM) and depletion of GSH by γ-glutamylcysteine synthetase, the rate limiting enzyme in GSH synthesis, reduced the LC_{50} by

approximately 48%, indicating that catalase and the glutathione system contribute strongly to the detoxification of H_2O_2.

Prior findings by Giblin *et al* indicate that the glutathione system detoxifies low levels of H_2O_2, while high levels are dismutated by catalase (Giblin et al., 1990). Giblin *et al* suggested that the function of the glutathione system mainly lies in the detoxification of secondary peroxidation products and is therefore vital for cell survival. As catalase and glutathione are already known and well characterized as antioxidant defence systems, it would be especially interesting to find other, so far unknown detoxification systems which could be developed as drug targets for AD and other related conditions. To investigate the contribution of unknown mechanisms, catalase as well as γ-glutamylcysteine synthetase were inhibited, as this approached would allow observation of even minor contributing mechanisms to the detoxification of H_2O_2.

To address the question of whether the resistance of Neuro2a-HR cells is due to a more effective detoxification or to a higher tolerance to H_2O_2 before becoming apoptotic, the clearance of exogenously added H_2O_2 from the supernatant was measured. No dramatic difference in H_2O_2 detoxification between parental cells and Neuro2a-HR cells could be found, suggesting that the resistance mechanism is not based on a better H_2O_2 degradation.

In summary, it can be stated that the created Neuro2a-HR cells show an at least 4-fold higher resistance against H_2O_2 than the parental cell line and that both methods of cell conditioning, 1 mM weekly or 200 µM daily, lead to resistant clones or subpopulations. Furthermore, it was shown that the increased resistance of Neuro2a-HR is not based on catalase or glutathione related mechanisms. This indicates the contribution of additional, so far unknown, mechanisms which allow Neuro2a-HR cells to survive at higher levels of oxidative stress. The mechanism(s)

are most likely not based on faster detoxification of extracellular H_2O_2 as the clearance rate for exogenously added 1 mM H_2O_2 was similar between parental and resistant cells. These resistant clones might serve as interesting target discovery tools for the identification of neuroprotective genes.

Figure Legends:

Figure 1. Relative resistance of Neuro2a-HR cells. Relative resistance of Neuro2a-HR_LL subpopulations E525, E725 and E735 (A) and Neuro2a-HR_HL cell clones B8, E3 and F5 (B). Results presented as mean ± SD, (* $p < 0.05$).

Figure 2: Rate of detoxification of 500 µM H2O2 by the different clones / subpopulations

All cells were capable of detoxifying 500 µM H_2O_2 with a $t_{1/2}$ of 26-32 min and an almost complete clearance of H_2O_2 was achieved after 90 min Half-life of H_2O_2 added to Neuro2a and Neuro2a-HR cells assuming a first order kinetic decay is presented (n=2, in triplicate)

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Figures

Figure 1 A

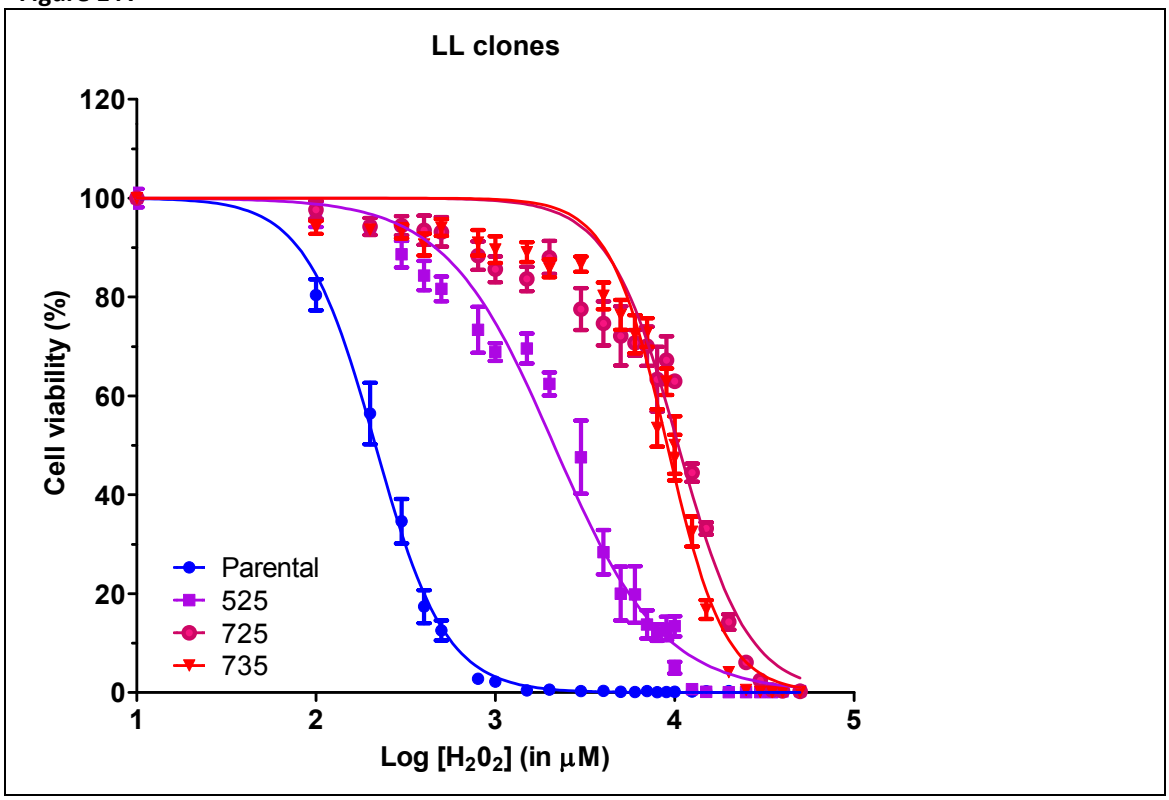

Figure 1B

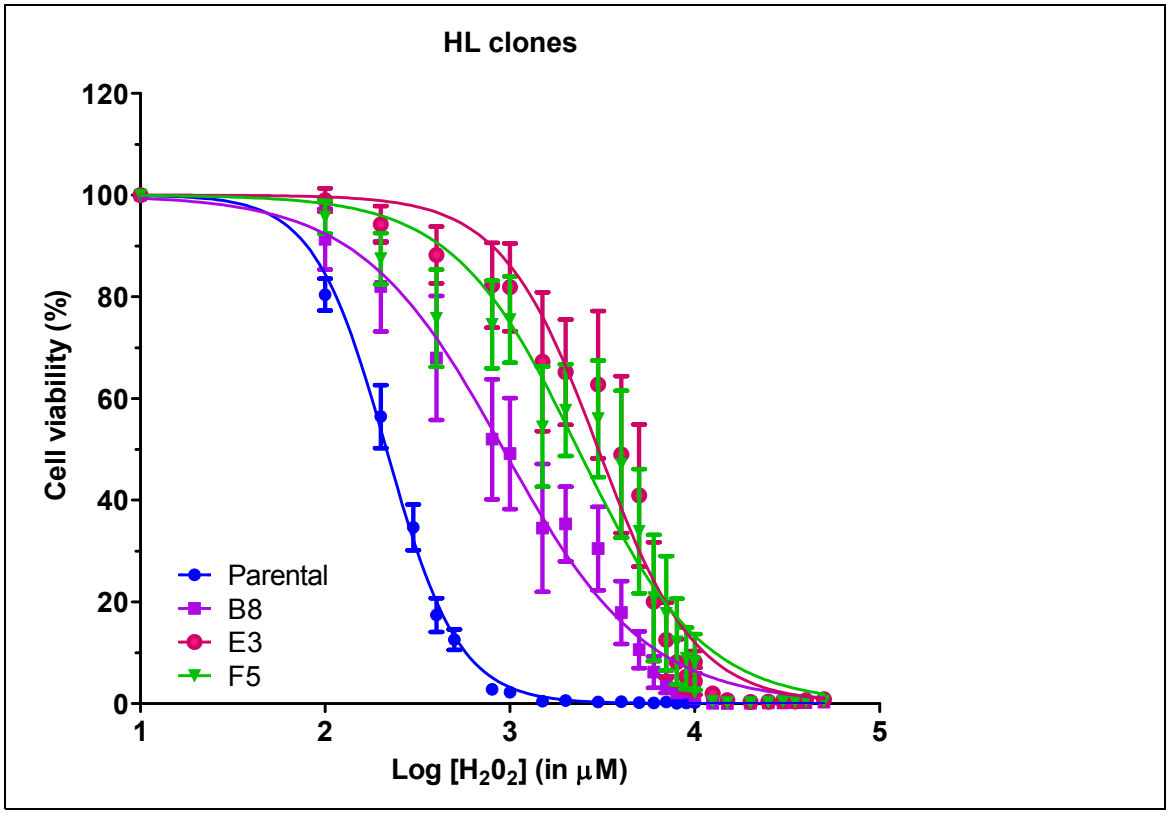

Figure 3

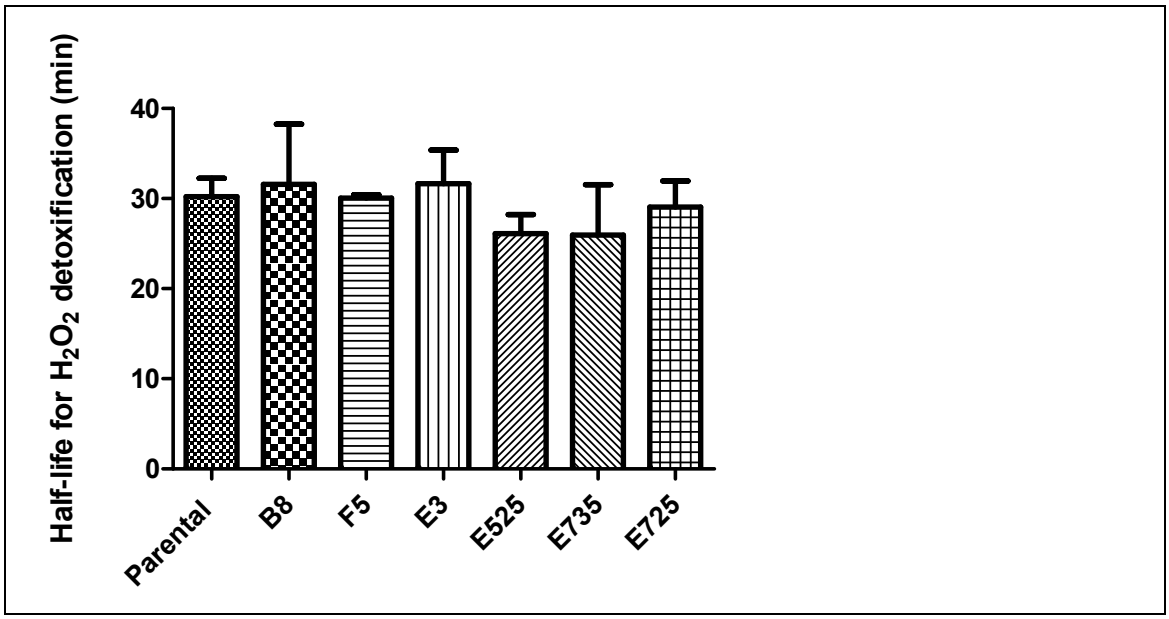

Table 1. Generated Neuro2a hydrogen peroxide resistant cells

Neuro2a-HR_HL clones (≤1 mM H_2O_2 weekly)	**Neuro2a-HR-LL subpopulations (≤200 µM H_2O_2 daily)**
Neuro2a-HR_HL B8	Neuro2a-HR_LL E525
Neuro2a-HR_HL E3	Neuro2a-HR_LL E725
Neuro2a-HR_HL F5	Neuro2a-HR_LL E735