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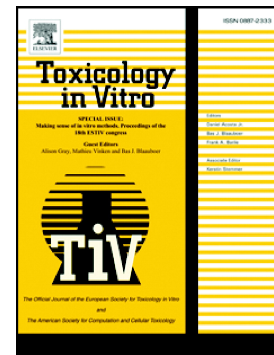

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In vitro hydroquinone–induced instauration of histone bivalent mark on human retroelements (LINE-1) in HL60 cells.

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Running title: Exposure to HQ low-dose and histone modifications

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Highlights

- *In vitro* exposure to low-doses HQ can alter the epigenetic signature of HL60 cells
- *In vitro* treatment with low-doses HQ determine instauration of histone bivalent mark
- Epigenetic modifications after *in vitro* treatment with HQ are reversible
- *In vitro* low-dose HQ determine no significant variation in DNA methylation on LINE-1

Abstract

Benzene is extensively used in industry despite its leukemogenic activity, representing a significant occupational hazard. We investigated if long-term treatment with low-doses hydroquinone (HQ), a benzene metabolite, might be sufficient to alter *in vitro* the epigenetic signature underlining LINE-1 sequences, a poorly explored step in health risks associated with benzene exposure. In HL-60 cell line, exploring the epigenetic events occurring in chromatin, we found the transient instauration of the distinctive signature combining the repressive H3Lys27 tri-methylation mark and the activating H3Lys4 tri-methylation mark (H3K27me3/H3K4me3), indicating a tendency toward a poised chromatin conformation. These alterations are lost in time after short-term treatments, while the long-term setting, performed using a concentration within the levels of total HQ in peripheral blood of benzene-exposed workers, showed a gradual increase in H3K4me3. We observed the absence of statistically significant variations in DNA methylation and expression levels of LINE-1, despite a decrease in protein levels of UHRF1, DNA methyl-transferases and histone methyl-transferases. In conclusion, *in vitro* treatment with low-dose HQ determined the instauration of a reversible poised state of chromatin in LINE-1 sequences, suggesting that prolonged exposure could cause persistent epigenetic alterations.

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Highlights

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- *In vitro* exposure to low-doses HQ can alter the epigenetic signature of AML cell line
- *In vitro* treatments with low-doses HQ determine instauration of histone bivalent mark
- *In vitro* exposures to low-doses HQ show no significant variation in DNA methylation on LINE-1
- Epigenetic modifications after *in vitro* treatment with HQ are transitory and reversible

Keywords

Benzene; histone modifications; bivalent mark; DNA methylation; AML; LINE-1.

Abbreviations

LINE-1 or L1, long interspersed nuclear elements 1; HQ, hydroquinone; AML, acute myeloid leukaemia; DNMTs, DNA methyl-transferases; HMTs, histone methyl-transferases.

1. Introduction

Benzene, a volatile aromatic hydrocarbon, is extensively used in industry even though it is recognized as a myelotoxin with leukaemogenic activity, representing a significant occupational risk (Khalade *et al.*, 2010): it is also a well-known environmental pollutant, due to its release in the air through cigarette smoke and fumes of motor vehicles (Wallace, 1989). Exposure to high concentrations of benzene (>100ppm) leads to toxicity in the hemopoietic system, while acute myeloid leukaemia (AML) is the major oncogenic disorder associated with chronic exposure, although other forms of leukaemia have been reported (Aksoy, 1989; Atkinson, 2009; Khalade *et al.*, 2010; Stenehjem *et al.*, 2015). Metabolism of benzene plays a fundamental role in its toxicity (Meek and Klaunig, 2010; Zolghadr *et al.*, 2012), and among the different metabolites, hydroquinone (HQ) is one of the most important (Snyder and Hedli, 1996), as it can induce several genetic and epigenetic changes (Liu *et al.*, 2012). Aberrant patterns of DNA methylation, including loss of imprinting (LOI), gene-specific hyper- or hypo-

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methylation and global hypo-methylation are all common in AML and other tumour types (Lübbert *et al.*, 1992; Bollati *et al.*, 2007; Brait and Sidransky, 2011; Hamilton, 2011; Liu *et al.*, 2011), and are important for transcriptional repression or activation of cancer-associated genes (Chen *et al.*, 2004; Wilson *et al.*, 2007). Nevertheless, the actual mechanism behind the influence of these aberrations on tumourigenesis remains unclear.

DNA methylation of repetitive elements is widely used in research and clinic as an indicator of global genomic methylation level, thanks to their high number of copies interspersed in the genome (Yang *et al.*, 2004; Sahnane *et al.*, 2015): the best-studied families are the long (LINE-1 or L1) and the short (SINEs, in particular Alu) interspersed nuclear elements (Byun *et al.*, 2013). In human studies, differences in DNA methylation of L1 and Alu have been consistently demonstrated in response to a wide range of environmental exposures, including airborne pollutants (Bollati *et al.*, 2007; Baccarelli *et al.*, 2009; Peluso *et al.*, 2012; Seow *et al.*, 2012). LINE-1 DNA hypo-methylation was demonstrated in benzene-exposed healthy workers and partially *in vitro* in HQ treated cells (Bollati *et al.*, 2007; Ji *et al.*, 2010; Liu *et al.*, 2012). Most of the studies conducted so far have been centred on DNA methylation, whereas only a few investigations have analysed the effects of environmental chemicals on histone modifications: only recently the relation with benzene exposure was explored (Baccarelli and Bollati, 2009; Philbrook and Winn, 2015).

DNA methylation is catalysed by three DNA methyl-transferases (DNMTs) (Portela and Esteller, 2010): aberrant expression levels of DNMTs induced by environmental carcinogens, accompanied by changes in global methylation, have been already observed during leukaemogenesis (Benbrahim-Tallaa *et al.*, 2007; Ji *et al.*, 2008; Liu *et al.*, 2011). DNA methylation has a role in directing histone methylation, and vice-versa, with these two marks supporting each other to establish the chromatin environment (Greer *et al.*, 2014; Rose and Klose, 2014): the bridge role between the two epigenetic levels is prevalently played by UHRF1 (ubiquitin-like with PHD and ring finger domains 1) (Bronner *et al.*, 2013).

Histone methylation occurs on all basic residues (Fischle *et al.*, 2008) and can happen at multiple different residues on the same histone catalysed by several histone methyl-transferases (Greer *et al.*, 2014). Some histone marks are mutually exclusive: one of the most studied combined methylation is the distinctive signature that associates the repressive H3K27 tri-methylation (me3) mark and the

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activating H3K4me3 mark. This bivalent condition, harboured by many promoters in embryonic stem cells and essential during development, was first proposed by Bernstein et (Bernstein *et al.*, 2006) as a mechanism to silence developmental genes while keeping them poised for activation, and is lost after differentiation. Nowadays it is known to be present also in cancer, where can lead both to repression of previously active genes (following loss of H3K4me3), or activation of previously repressed sequences/genes associated with cancer progression (following loss of H3K27me3) (Chapman-Rothe *et al.*, 2013; Voigt *et al.*, 2013; Hahn *et al.*, 2014).

The present work aims at addressing the epigenetic changes in response to sub-cytotoxic concentrations of HQ, by focusing prevalently on the possible alterations on chromatin and DNA methylation. Actually, *in vitro* demonstration of HQ activity on the epigenetic machinery is difficult to achieve due to the nature of the exposure: while acute effects (i.e. apoptosis activation and ROS production) are easily evaluable through single high-dose administrations of the agents (Zolghadr *et al.*, 2012), and are thus well-studied, long-term exposures at low doses are more difficult to reproduce in laboratory. In this study, by setting up a long-term treatment with low-doses of hydroquinone within the levels of total HQ found in peripheral blood of benzene-exposed workers, we explore *in vitro* the differences between short and long-term exposure, to evaluate if long-term treatment with low-doses of hydroquinone might be sufficient to alter the epigenetic signature, thereby describing a poorly explored step in the mechanism of toxicity associated with benzene exposure.

2. Materials and methods

2.1. Cell line and compounds

The MPO-positive AML cell line, HL60, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and routinely cultured in suspension at 37°C and 5% CO_2 in RPMI medium supplemented with 10% foetal bovine serum (Euroclone, MI, IT) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Hydroquinone (HQ, ≥99%) was purchased from Sigma-Aldrich, stocked at RT and dissolved in complete medium immediately before each treatment. Decitabine (DAC, Sigma-Aldrich) served as a positive control for global DNA methylation: stock solutions (50 mM) were prepared in DMSO and stored at -20°C. Working dilutions were prepared before use.

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2.2. Treatment settings

To analyse cell growth, viability and the epigenetic effects cells were seeded at low density and treated 24 hours later following the different settings (detailed treatment schemes are reported in Supplementary Figure 1):

Single treatment: cells were treated once with different concentrations of HQ (1, 5, 15 and 25 µM) and harvested 1, 2, 3, 5 and 7 days later; detailed analysis was performed only after treatments with HQ 5 and 15 µM.

Repeated treatment: cells were treated four times every 48 hours with different concentrations of HQ (1, 5 and 15 µM) and harvested 2 and 7 days after the fourth treatment; detailed analysis was performed only after treatments with HQ 1 and 5 µM.

Long-term treatment: cells were treated five times a week for five weeks with a single concentration of HQ (1 µM). Every seven days cells were counted, reseeded at low density and harvested. The dose of 1 µM of HQ used in our long-term exposure corresponds to 110 ng/mL HQ, a concentration within the range of total HQ found in the blood of workers exposed to airborne benzene (Kerzic et al., 2010); that was between 20 and 120 ng/mL, which correspond to free HQ between 2 and 16 ng/mL, for airborne benzene exposures ranging from 1 mg/m^3 (around 0,3 ppm) up to a maximum of 80 mg/m^3 (around 25 ppm).

2.3. Flow cytometry

Cell were harvested at the different end points, rinsed with PBS and fixed in 70% EtOH at -20 °C for at least 20 minutes. After a further rinse in PBS, DNA was stained with Propidium Iodide in PBS (PI, Sigma-Aldrich, final concentration 50 µg/ml) in the presence of RNAse (Sigma-Aldrich, 30 U/ml). Samples were analysed at the FACScan flow cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA) equipped with a 15 mW, 488 nm and air-cooled argon ion laser. At least 10,000 events were analysed for each sample and all data were processed using CellQuest software (Beckton Dickinson). Fluorescent emission of PI was collected through a 575 nm band-pass filter and acquired in log mode: the percentage of apoptotic cells was determined based on sub-G1 peaks detected in monoparametric histograms.

2.4. SDS page and Western Blot

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Cell lysates were prepared in RIPA buffer (Sigma-Aldrich) containing protease inhibitor cocktail (Roche, Basel, CH). Protein concentration was determined by Qubit method (quant-iT Protein Assay kit, Invitrogen, Life Technologies, Carlsbad, CA, USA) and equivalent amounts of total cell lysate (30 µg) were added with sample buffer 2X (Laemmli, Sigma-Aldrich), separated by 10% acrylamide SDS-page under denaturing conditions and transferred onto nitrocellulose membrane (Hybond-ECL, GE Healthcare, Fairfield, CT, USA). Membranes were incubated overnight with primary antibodies: anti-DNMT1, (Abcam, Cambridge, UK); anti-DNMT3a (Cell Signaling, Danvers, MA, USA); anti-DNMT3b (Active Motif, Carlsbad, CA, USA); anti-UHRF1 (IGBMC, FR); anti-EZH2 (Cell Signaling), anti-SUV39H1 (GeneTex, Irvine, CA, USA), anti-G9a (Cell Signaling) and anti-GAPDH (Millipore, Merck, Darmstatd, DE). After incubation with secondary anti-rabbit/mouse antibody conjugated to horseradish peroxidase (Sigma Aldrich), chemioluminescence on membranes was detected by ECL reagents (ECL detection reagent, GE Healthcare) and acquired on films (Hyperfilm-ECL, GE Healthcare).

2.5. DNA bisulfite conversion and pyrosequencing

Bisulfite modification of genomic DNA (300 ng) was performed with an EpiTect Bisulfite Kit (Qiagen, Hilden, DE) according to the manufacturer's protocol. PCR products were analysed by pyrosequencing using PyroGold reagents on a PyroMark Q96 ID system (Qiagen). Pyrogram outputs were analysed by the Pyromark ID 1.0 software (Qiagen) to determine the percentage of methylation of each of the four CpG sites analysed within the 5'UTR of LINE-1 elements (GenBank ID M80343.1, primers in Supplementary Table 1).

2.6. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed based on a modification of previously published methods from our laboratory (Babbio *et al.*, 2012). Briefly, cells were cross-linked by adding 1% formaldehyde to the culture medium: the cross-linking reaction was quenched by adding 0.125 M glycine and collected in PBS. Cell pellets were re-suspended in lysis buffer and sonicated 14 times for 10 seconds on ice (BRANSON S250 digital sonicator, Branson, Danbury, CT, USA). Sonicated chromatin was incubated overnight at 4 °C in dilution buffer with anti-IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-UHRF1 (IGBMC), anti-H3K9me3, anti-H3K9me2, anti-H3K27me3 and anti-H3K4me3 (Active Motif). Twenty percent of the total lysate was used for input control. DNA was extracted using phenol/chloroform/isoamyl alcohol, precipitated in ethanol and resuspended in H_2O.

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Chromatin immunoprecipitation products were amplified using GoTaq Hot-Start Polymerase (Promega, Madison, Wi, USA) and specific primers (Supplementary Table 1) following the manufacturer's protocol.

2.7. Statistical analysis

Statistical differences between mean values were determined by using unpaired t-test (since we considered differences between NT and each concentration) in GraphPad Prism. Values are expressed as mean ± SD of three replicate experiments.

3. Results

Since the aim of our study was to investigate processes that take place in bone marrow, both catechol and hydroquinone were good candidates: however, we chose HL60 cell line as our model, which is MPO positive and can thus potentially metabolize HQ to benzoquinone, to possibly analyze the combined effects of both compounds. Exploring *in vitro* the differences between short and long-term exposure to HQ, in the effort to investigate if low-doses might be sufficient to alter the epigenetic signature, we developed three treatment settings: detailed explanation of the settings is reported in material and methods and Supplementary Figure 1. We evaluated cell viability to chose sub-cytotoxic concentrations in each setting: HQ concentrations of 1, 5, and 15 μM were selected as representative of low dose HQ. In particular, the concentration of 1μM (110 ng/mL) used in the long-term treatment is within the total HQ levels found in workers exposed up to 80 mg/m^3 of airborne benzene (Kerzic *et al.*, 2010). The selected doses were used to assess the alterations in the epigenetic components, in DNA methylation levels and in chromatin modifications.

3.1. Effects on HL60 viability induced by different treatments with HQ

As a first step, we analysed the cytotoxic effect of HQ on HL60 cell line in the different settings, starting from literature data available exclusively for a single exposure (Terasaka *et al.*, 2005; Ji *et al.*, 2010; Liu *et al.*, 2012). In response to a single administration, with the exception of the highest concentration (25 μM), we could observe no significant difference in cytotoxicity shortly after the treatment (1d-3d) between concentrations, while at longer times (5d-7d) 15 μM slightly reduced cell proliferation (Figure 1A). Increasing the number of treatments up to four (excluding 25 μM), we

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detected high toxicity at 15 µM and a slight reduction in cell growth at 5 µM: 1 µM didn't affect cell proliferation (Figure 1B). In order to study HQ effects following prolonged exposure, we provided daily administrations of the lowest concentration (1 µM) for multiple weeks. We did not observe significant effects on cell growth for the first three weeks: a reduction in the cell number was observed at the fourth week and became massive the fifth week. The reduced growth affected only the treated cells, while non-treated cells showed no visible effect compared with the previous weeks (Figure 1C), underlying that cell toxicity was due to the compound, and not to prolonged cultivation (see Supplementary Figure 1). As in our experimental setting HL60 cells undergo a reduction in cell growth after 8 week of cultivation, possibly due to the accumulation of stress factors, it might be that low-dose HQ contributes to the stress load that leads the cells to arrest/undergo senescence/die in a shorter time with respect to controls.

Following these observations, we concluded that even a very low concentration of HQ such as 1 µM (within total HQ levels found in peripheral blood of benzene-exposed workers), could not be administered *in vitro* for long periods in the selected cell line due to its toxic effect. Analyses of the epigenetic alterations, therefore, were performed only on the doses and times where the effect on viability was not significant (single, 5 and 15 µM; repeated, 1 and 5 µM; long-term, 1 µM up to four weeks; the fifth week was not analysed).

We evaluated the effect of the selected sub-cytotoxic doses on cell cycle progression and apoptosis: flow cytometry analysis evidenced that single and repeated exposures are not able to alter the percentage of cycling cells and of apoptotic cells (Figures 1D and 1E). Long-term treatment showed a slight reduction in G0/G1 starting from the second week of treatment and a slight increase of the apoptotic cells in the fourth week of treatment only (Figure 1F). This feeble increase, however, does not seem to be sufficient to explain the decline in growth observed a week later: further analyses, not object of this study, are necessary.

3.2. Variations in DNMTs, UHRF1 and HMTs levels induced by different treatments with HQ

We explored the variations occurring at protein levels in the components necessary for the maintenance of DNA and histone methylation (UHRF1, DNMTs and some HMTs) following exposure to HQ. After a single treatment (5 and 15 µM), we could detect an effect prevalently 5 days after the

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exposure: a visible reduction of variable entity, more pronounced in UHRF1 and DNMT1, is visible. These differences were still partially present two days later (Figure 2A). When evaluating the effect of lower doses (1 and 5 μM) after a repeated exposure, we observed a dose dependent decrease in protein levels, especially of DNMT1 and G9a, 2 days after the 4th treatment (Figure 2B): five days later, independently from the used concentration, protein levels were comparable to non-treated samples, displaying the ability of HL60 to recover to basal level in the absence of HQ (Figure 2B). If we look at the effect of a prolonged exposure to a low dose of HQ (1 μM), it becomes clear that this benzene metabolite is indeed able to down-regulate the epigenetic components analysed (Figure 2C): the reduction was visible starting from the second week of treatment, and was maintained in the following weeks.

Concurrently we analysed the effect of HQ on transcriptional levels (Supplementary Figure 2), confirming the previously observed tendency towards a decrease in DNMTs transcription reported in literature (Liu *et al.*, 2012) and showing variable alterations of UHRF1 and the HMTs investigated.

We concluded that repeated administrations of very-low doses of HQ, such as 1 μM, are able to maintain down-regulation of important epigenetic modifiers.

3.3. Effect of the exposure to HQ on global methylation

Given the variations observed in UHRF1 and DNMTs proteins, as well as the evidences in literature (Bollati *et al.*, 2007; Ji *et al.*, 2010; Liu *et al.*, 2012), we investigated DNA methylation status on the 5'UTR region of LINE-1, vastly utilized in research and clinic for the evaluation of methylation levels (Sahnane *et al.*, 2015), after exposure to HQ. Analysing 5 and 7 days following the single treatment we could not detect statistically significant variations: we could observe only a slight tendency towards a dose dependent reduction 5 days after the exposure, reduction that was lost in the samples harvested two days later (Figure 3A). As Figure 3B shows, the effects of the repeated treatment on LINE-1 methylation 2 days after the 4th treatment are less linear, with a slight decrease only at the highest dose, while 5 days later the levels of methylation were again back to basal. Completing the methylation analysis on the long-term treatment (Figure 3C), we could not observe significant variations in any of the time-points analysed. In correspondence to the absence of alterations on DNA methylation we could observe no significant variations in LINE-1 5-UTR transcriptional levels (Figure 3D, E and F) in any of the treatment settings.

In conclusion, the treatment settings used in this work were not able to significantly alter DNA methylation and transcription of LINE-1, even when the exposure was prolonged up to four weeks.

3.4. Effect of the exposure to HQ on chromatin accessibility at LINE-1 loci

Focusing our attention on chromatin, given the variations observed in HMTs proteins, we performed ChIP experiments on LINE-1 5'UTR (the same region evaluated by pyrosequencing and qPCR, containing the promoter) aimed at evaluating covalent histone modifications and UHRF1 binding. In basal condition, we could observe the presence of repressive marks, such as UHRF1 binding and H3K27 tri-methylation. Data on H3K9 showed the prevalence of bi-methylation vs. tri-methylation: this could be due to the hypothesis that the sequences analysed by PCR are in fact the more conserved intronic LINE-1, where bi-methylation is prevalent, but also from the fact that HL60 possess very low levels of SUV39H1 (the HMT deputed to H3K9 tri-methylation). H3K4me3 was generally absent, as expected in transcriptionally-repressed and hyper-methylated sequences (Figure 4A).

Following exposure to HQ, Figure 4B shows the appearance of H3K4 tri-methylation, coupled with retention of H3K27 tri-methylation, 5 days after single treatment, meaning the instauration of a poised chromatin conformation. Variations in UHRF1 binding and of H3K9me2 were less reproducible and were not analysed. Two days later, however, the effect on chromatin was lost, with the restoration of the basal condition (appreciable both by PCR products visualization and densitometric analysis, Figure 4B). Similarly to what observed for the single treatment, an increase in H3K4me3, again coupled with preservation of H3K27me3, was visible 2 days after the 4th treatment, but resulted lost 5 days later (Figure 4C). Finally, analysing the variations in chromatin markers after long-term treatment, we could observe a progressive effect from week 1 through week 4 with a gradual increase in the levels of H3K4me3 (Figure 4D). Levels of H3K27me3 were stable at all time-points. These alterations, however, as shown above were not sufficient to determine significant variations in LINE-1 5-UTR transcriptional levels, probably due to the maintenance of repressive markers (H3K27me3 and DNA methylation, Figure 3).

In conclusion, this first analysis on chromatin modifications of LINE-1 sequences showed that even a single exposure to HQ is enough to perturb the structure of non-active DNA portion of the genome. However these changes, in the case of short-term treatments, are lost in time, suggesting the ability of HL60 to revert to the original conformation in the absence of HQ and thus the necessity of a prolonged

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exposure for the persistence of these alterations: we could not evaluate at long-term the persistence of this alterations in the absence of HQ due to the instauration of cytotoxicity.

4. Discussion

In the last few years, several investigations have examined the relationship between exposure to environmental chemicals and epigenetics, and have identified numerous agents able to modify epigenetic marks. Most of the studies conducted so far have been centred on DNA methylation, whereas only recently a few have focused on the effects on histone modifications (Bollati *et al.*, 2007; Wilson *et al.*, 2007; Baccarelli and Bollati, 2009). Benzene and its metabolites are among the most studied, due to their association with diverse hematopoietic malfunction as well as leukemogenesis: global DNA hypo-methylation, accompanied by gene-specific hyper-methylation, was demonstrated in benzene-exposed healthy workers (Bollati *et al.*, 2007). However, *in vitro* demonstration of benzene activity on the epigenetic machinery is complicated by the nature of the type of exposure behind these changes: while studies on benzene and its metabolites on acute effects (i.e. apoptosis activation and ROS production) are easily achievable using single high-dose administrations of the agents (Zolghadr *et al.*, 2012), chronic exposures at very low doses, comparable to the ones reached in occupational exposure, are more difficult to reproduce in laboratory.

Epidemiological studies have shown that airborne benzene concentrations ≤1ppm are able to give hematotoxicity in workers exposed for long periods (Lan *et al.*, 2004). Mild reduction of DNA methylation (-2,33%) at LINE-1 sequences occurs in workers exposed to ≤1ppm of airborne benzene for many hours per day and for at least one year (Fustinoni *et al.*, 2005). In the present work, we extended these studies in HL60 exposed cells, focusing firstly on finding sub-cytotoxic concentrations and setting different types of treatments in the effort to move towards a long-term treatment scheme that could somehow recall exposures that can be experienced in working environments. The HQ concentration we used (1 µM) corresponds to 110 ng/mL, a level very similar to the highest amount of total HQ (between 20 and 120 ng/mL, corresponding to 2-16 ng/mL of free HQ) found in the blood of workers of a rubber product manufacturing and finishing facility located near Shanghai, China (Kerzic *et al.*, 2010), exposed to airborne benzene levels ranging from 1 mg/m^3 (around 0,3 ppm) up to a maximum of 80 mg/m^3 (around 25 ppm). Interestingly, the highest blood levels of total HQ were

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observed even at very low levels of air benzene exposure (≤1ppm), levels that have been shown to cause hematotoxicity (Lan *et al.*, 2004). Moreover, when the ratio of bound to unbound metabolites were compared in subsets of exposed workers, the increase in blood metabolite concentration was nearly all due to an increase in the protein-bound molecule. These results suggest that it is very difficult to define a tight correlation between the level of benzene exposure and the concentration of HQ, especially the free form, in the blood. Considering that HQ has a half life of at least 20hr (IPCS, INCHEM, SIDS 2002), that the molecule undergoes rapid redox reactions and that 10% FBS present in the medium contains many HQ-binding proteins, the amount of free HQ may vary considerably during the experiments, making it difficult to accurately measure the final concentration of free HQ. Altogether, the 110 ng/mL HQ used for the long-term experiments is close to the maximum total levels detected in the blood of the workers (120 ng/mL), in the effort to mimic *in vitro* the conditions of a strong occupational exposure, although we could not define the exact working concentration range of free HQ.

We focused our attention on alteration in the epigenetic machinery occurring in each setting at the times and doses where cell viability was retained and variations in cell cycle/apoptosis were absent or not significant, to avoid non-specific effects, analysing both histone modifications and DNA methylation. Aberrant expression of DNMTs, accompanied by hyper-methylation of tumour suppressor genes and hypo-methylation of unique genes or repetitive sequences, are the main types of aberration in the DNA methylation machinery occurring in AML and other cancer types (Brait and Sidransky, 2011; Hamilton, 2011).

Given the variations observed in our study in HMTs proteins (as well as UHRF1), we primarily focused our attention on chromatin, performing a ChIP experiment on LINE-1 5'UTR (the region containing the promoter sequence of this retrotransposable element): our aim was to evaluate for the first time some of the histone modification in these regions, in particular H3K9me3, H3K9me2, H3K27me3 and H3K4me3, as well as UHRF1 binding. We applied stringent PCR condition in order to prevalently evaluate conserved sequences (present in intronic regions, where the interspersed LINE-1 are better preserved) that can give origin to chimeric transcript or to the production of proteins responsible for the insertional activity of LINE-1 sequences (Slotkin and Martienssen, 2007).

At first analysis, in treated cells we observed the instauration of various degrees of H3K4 tri-methylation, absent in basal condition, which evidenced the tendency toward a poised chromatin conformation typical of embryonic cells and normally lost during development. The persistence of H3K27me3, coupled with H3K4me3 (two histone marks associated with opposite transcriptional status and actually widely recognized as a bivalent mark) clearly shows the transition in these sequences towards a more permissive conformation following exposure to HQ, as illustrated in the hypothetical model in Figure 5. In this figure we represented what we experimentally observed (5A and 5B) and the two hypothetical fates: if the exposure is removed (Fig 5C, left) we could observe restoration of the initial condition, with loss of tri-methylation on K4 and persistence of K27me3; if the exposure becomes chronic (Fig 5C, right) it might end up with the loss of repressive marks (K27me3) and activation of transcription in the presence of K4me3. The plasticity of this fundamental chromatin bivalent mark (H3K27me3/H3K4me3), firstly described in embryonic cells, appears to play a role also in poising non-active genes for aberrant transcription in cancer (Bernstein *et al.*, 2006; Voigt *et al.*, 2013). Several studies actually suggest that loss of this bivalent chromatin mark at promoters is accompanied by gene activation (when H3K27me3 is lost) or gene repression (when H3K4me3 is lost) during development, and this event is likely to be a critical step also in cancer pathogenesis, responsible for the activation of crucial genes leading to progression and invasiveness, as demonstrated in colon and ovarian cancer (Chapman-Rothe *et al.*, 2013; Hahn *et al.*, 2014).

These variations, however, seems not persistent in time: analysis at longer intervals after the exposure to single and repeated treatments showed a recovery in LINE-1 chromatin modifications, meaning the ability of HL60 to revert to the original chromatin configuration in the absence of HQ stimulus. This reversibility could be interpreted as a cellular mechanism of defence against environmental insults that can produce permanent modifications in chromatin, ultimately leading to alteration in the transcriptional levels of targeted genes/sequences. In case of environmental chronic exposure, in fact, the persistence of the agent might lead to the fixation of the alteration at later times: permanent gain of H3K4me3, possibly followed by loss of H3K27me3, could determine the reactivation of the studied sequences, as previously demonstrated for gene promoters in other cancer types (Hahn *et al.*, 2014).

However, even long-term administration in these *in vitro* conditions were not enough to stabilize these alterations, as the concentration used determined toxicity before we could assess if these

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modifications could be fixed in time. We speculated that the responses we obtained in ChIP experiments are hydroquinone specific and not only 'simply' stress related, as the epigenetic effects were visible since the first week of treatment, and both with single and repeated treatments. It is known that HQ treatment produces cellular stress, contributing to the general stress load that leads to cytotoxicity and alteration in the cellular life span even at doses as low as 1 µM. Further experiments will be needed to investigate better the nature of the 5th week collapse, and to understand which phenotype is generated (cell arrest/senescence/death or other) and which mechanism is responsible for it.

Given the alterations observed in chromatin, as well as in UHRF1 and DNMTs proteins and the evidences in literature (Bollati *et al.*, 2007; Ji *et al.*, 2010; Liu *et al.*, 2012), we investigated the DNA methylation status on the same 5'UTR region of LINE-1 by pyrosequencing. Actually, the decrease in global methylation is largely studied at heavily methylated elements, such as satellite repeats (i.e. Sat2) and retrotransposons (i.e. LINE-1) (Yang *et al.*, 2004; Sahnane *et al.*, 2015). We could not detect a significant difference in all the settings, but a slight reduction in methylation was visible: these results indicate that global DNA hypo-methylation may only partially result from DNMTs inhibition induced by HQ exposure. Overall, we could not reproduce *in vitro* the alteration in LINE-1 methylation observed in epidemiologic studies (Bollati *et al.*, 2007), even after a long-term exposure to HQ: in fact, even prolonging the exposure was not sufficient to have statistically significant variations, clearly underlying the difficulties of a similar study in an *in vitro* setting.

The persistence of H3K27me3 observed by ChIP could be considered responsible for the absence of significant alteration in DNA methylation, even in the presence of H3K4me3, as well as for the absence of meaningful variations of LINE-1 transcriptional levels. As already stated, in case of environmental exposure, the persistence of the insult, more than the concentration of the pollutant, might determine the alteration of repressive and activating modifications, ultimately leading to DNA hypo-methylation and variation also in transcription, since histone and DNA modifications are strictly correlated and both determine the effect on promoter activation or repression.

Future investigations are thus needed to determine whether long-term exposed subjects develop stable epigenetic alterations on chromatin that can precede alteration in DNA methylation, and, in turn, whether such alterations could increase the risk of cancer after exposure to benzene and its

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metabolites. Given the technical difficulties in setting *in vitro* experiments reproducing occupational and environmental exposures, epidemiological and *in vivo* studies are probably the best alternatives. Additional studies are indeed required to investigate the mechanism by which benzene and its metabolites produce the alterations observed for the first time in this work, as well as the role of these modifications in AML development.

5. Conclusions

Exploring the epigenetic events occurring in chromatin, after *in vitro* treatment with concentrations of HQ representative of the levels produced by occupational exposures to airborne benzene, not able to alter cellular growth, we found the transient instauration in LINE-1 sequences of the combined signature H3K27me/H3K4me3, indicating the instauration of a poised chromatin conformation normally visible during development. The loss of these alterations after short-term treatments and the gradual increase in H3K4me3 observed after long-term treatment suggest the necessity of a prolonged exposure for the persistence of these epigenetic changes. This might explain the absence of statistically significant variations in DNA methylation in all settings, differently from what observed in epidemiologic studies, as well as in LINE-1 expression, even in the presence of decreased protein levels of UHRF1, DNMTs and HMTs.

Conflict of interest disclosure

The authors declare no conflict of interest.

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Supplementary material

Supplementary data associated with this article includes Figures 1, 2 and Table 1.

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Figure legends

Fig. 1. Survival curve and flow cytometry analysis of HL60 cells treated with HQ. (**A**, **B**, **C**) Cell counting was performed to evaluate the effect of HQ on cell growth: concentration of 25 μM in the single treatment (**A**) and of 15 μM in the repeated treatment (**B**) resulted highly toxic and were thus excluded in the following experiments, while in the long-term treatment even a very low concentration (1 μM) resulted toxic after four weeks (**C**). (**D**, **E**, **F**) The effect on cell cycle distribution and apoptosis levels was evaluated through flow cytometry: at the selected concentrations no significant alteration in the percentage of cycling and apoptotic cells was observed, with the exception of the long-term treatment, where we could detect a slight increase in apoptosis after the 4th week of treatment and a decrease in G0/G1 cells starting from the 2nd week of exposure. All results are shown as mean + SD of four independent biological replicates. * P ≤ 0.05; **P ≤ 0.005; ***P ≤ 0.0001.

Fig. 2. Evaluation of factors involved in epigenetic modifications after different treatments with HQ. (**A**, **B**, **C**) DNMTs, UHRF1 and HMTs protein levels were evaluated by Western Blot (single exposure in **A**, repeated in **B** and long-term in **C**). GAPDH levels were evaluated as internal control. Western Blot panels (on the left) are representative of the average variations observed in three independent biological replicates: DNMT3b is not shown because it was not detectable in any condition. The

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densitometry graphs (on the right) show the variations at the different times or concentrations, each compared to basal levels (NT): the results are shown as mean + SD of two independent biological replicates. * P ≤ 0.05.

Fig. 3. Evaluation of DNA methylation (**A**, **B**, **C**) and transcription (**D**, **E**, **F**) of LINE-1 after the different treatments with HQ. Four different cytosines in the 5'UTR region of LINE-1 were evaluated through Pyrosequencing analysis. Results are shown as mean + SD of the average of the four cytosines of three independent biological replicates. Transcriptional levels were evaluated through qPCR on the same region, harboring the promoter and transcribed: variation in expression are compared to basal levels (NT). The results are shown as mean + SD of three independent biological replicates. (**A, D**) Single treatment. (**B, E**) Repeated treatment. (**C, F**) Long-term treatment.

Fig. 4. Evaluation of histone modifications on chromatin after treatment with HQ. (**A**) Levels of H3K9me3, H3K9me2, H3K27me3 and H3K4me3, as well as UHRF1 binding, were evaluated in the 5'UTR region of LINE-1 through chromatin immunoprecipitation in basal conditions. (**B**, **C**, **D**) Analysis at the different end-points of the three treatment settings (single exposure in **B**, repeated in **C** and long-term in **D**) focused principally on H3K27me3 and H3K4me3. The chromatin status was evaluated also on a constitutively active gene (GAPDH). PCR panels are representative of the average variations observed; densitometry analysis of LINE-1 are shown as mean + SD of three independent biological replicates and are normalized versus IgG and Input. * P ≤ 0.05.

Fig. 5. Hypothetical model of chromatin alteration on LINE-1 5'UTR after treatment with HQ. **(A)** In basal condition, we observed the presence of repressive marks, such as H3K27 tri-methylation (as well as UHRF1 and H3K9me2, not reported in figure); H3K4me3 was generally absent, as expected in transcriptionally-repressed sequences. **(B)** After treatment with HQ we observed the appearance of H3K4 tri-methylation, coupled with retention of H3K27 tri-methylation, indicating a poised chromatin conformation. These variations, however, were not stable in time and were not sufficient to determine significant variations in LINE-1 5-UTR transcriptional levels, probably due to the maintenance of repressive markers (like H3K27me3 and methylation). **(C)** Following chronic exposure to HQ, LINE-1 sequence could hypothetically lose the repressive marks and activate transcription, while in the absence of the pollutant the original conformation of the chromatin could be restored.

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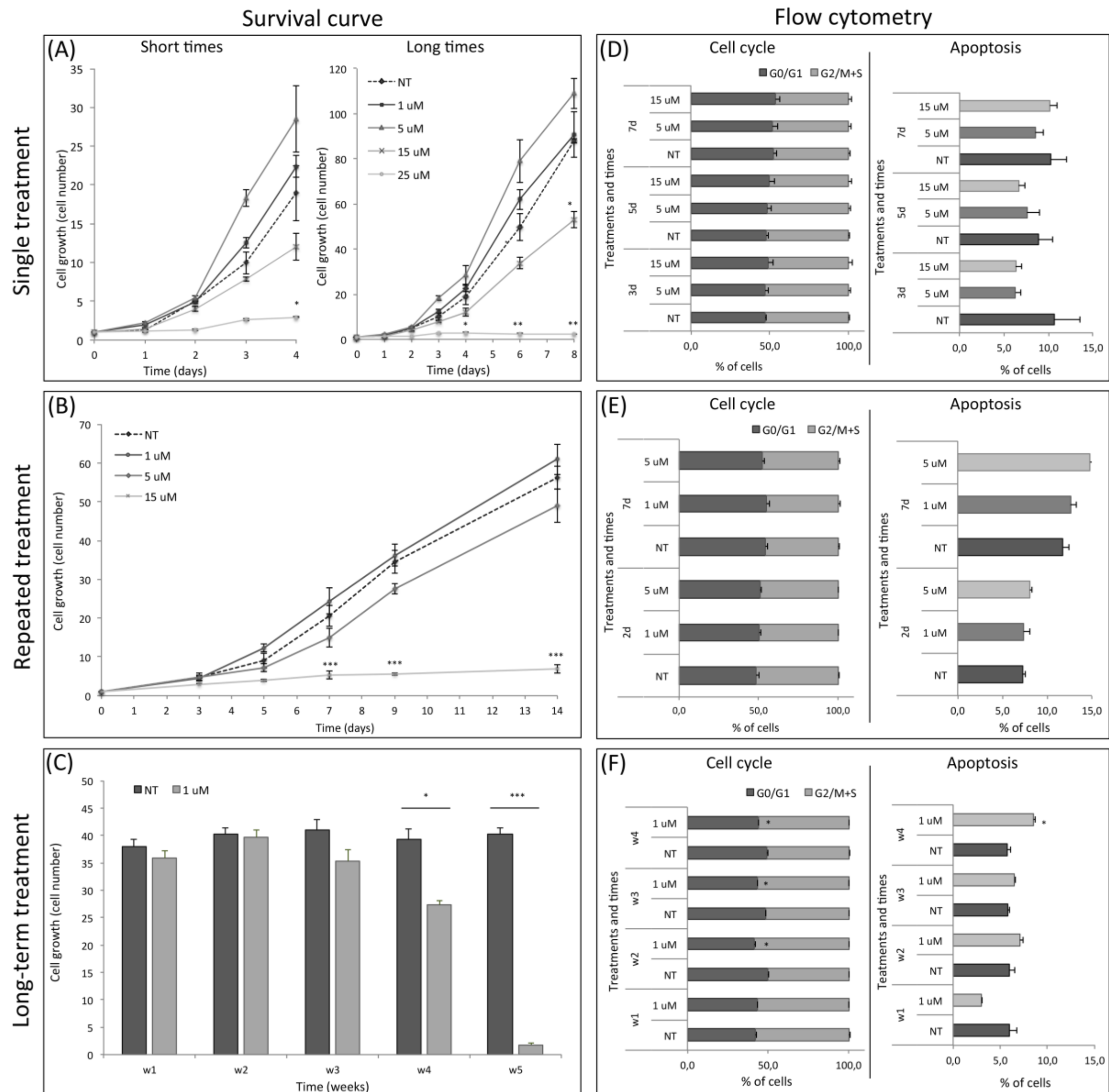

Fig. 1

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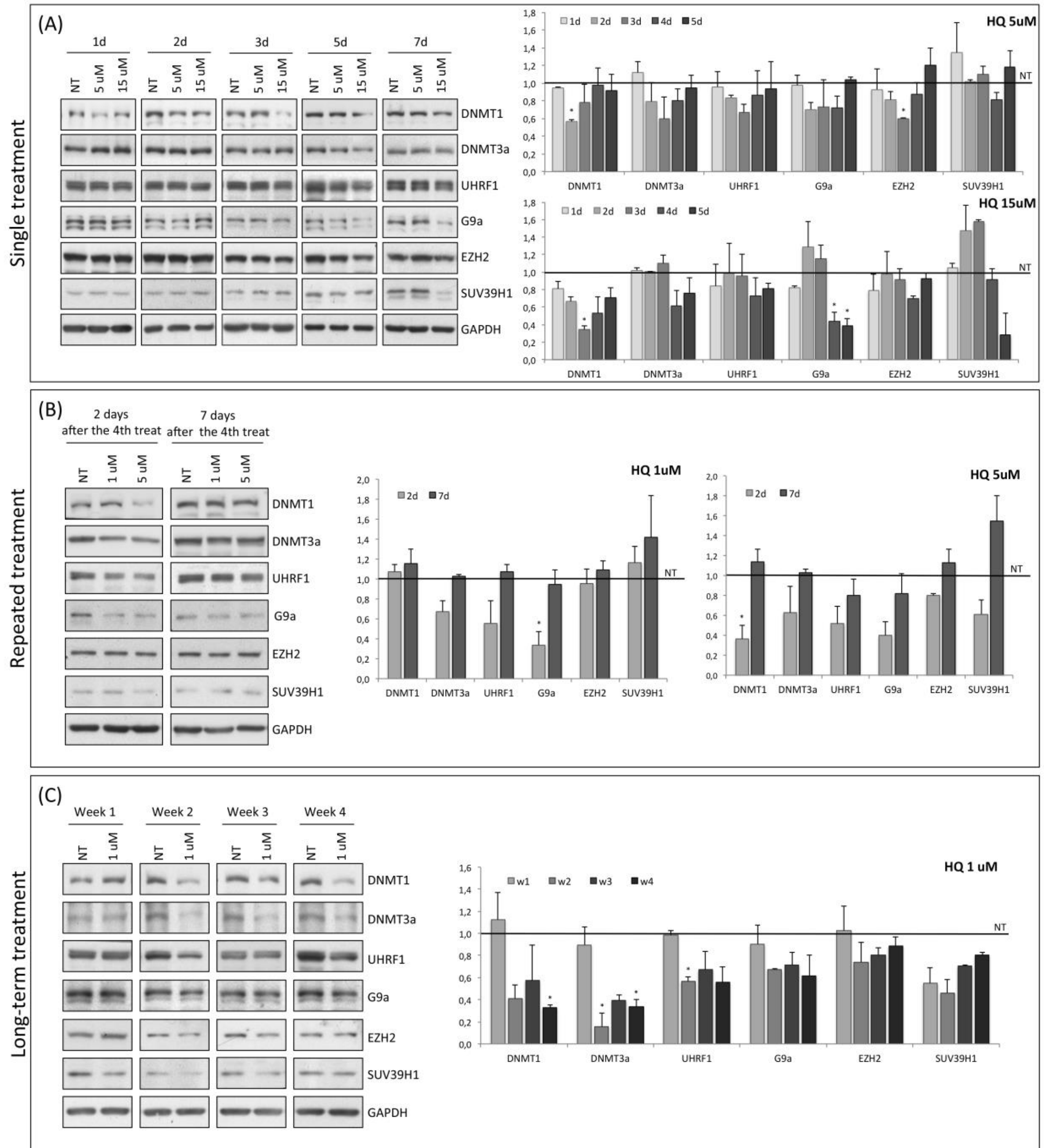

Fig. 2

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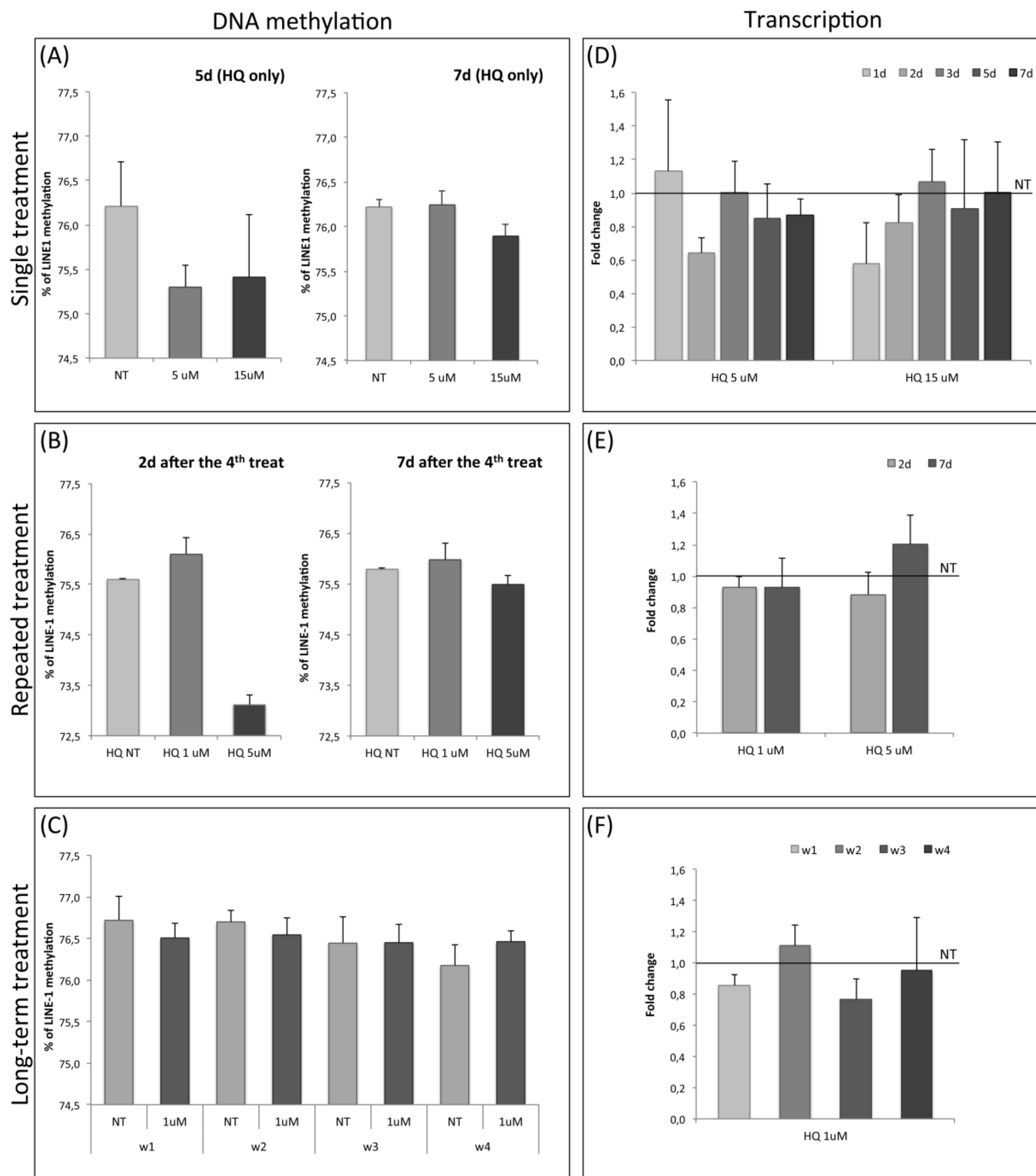

Fig. 3

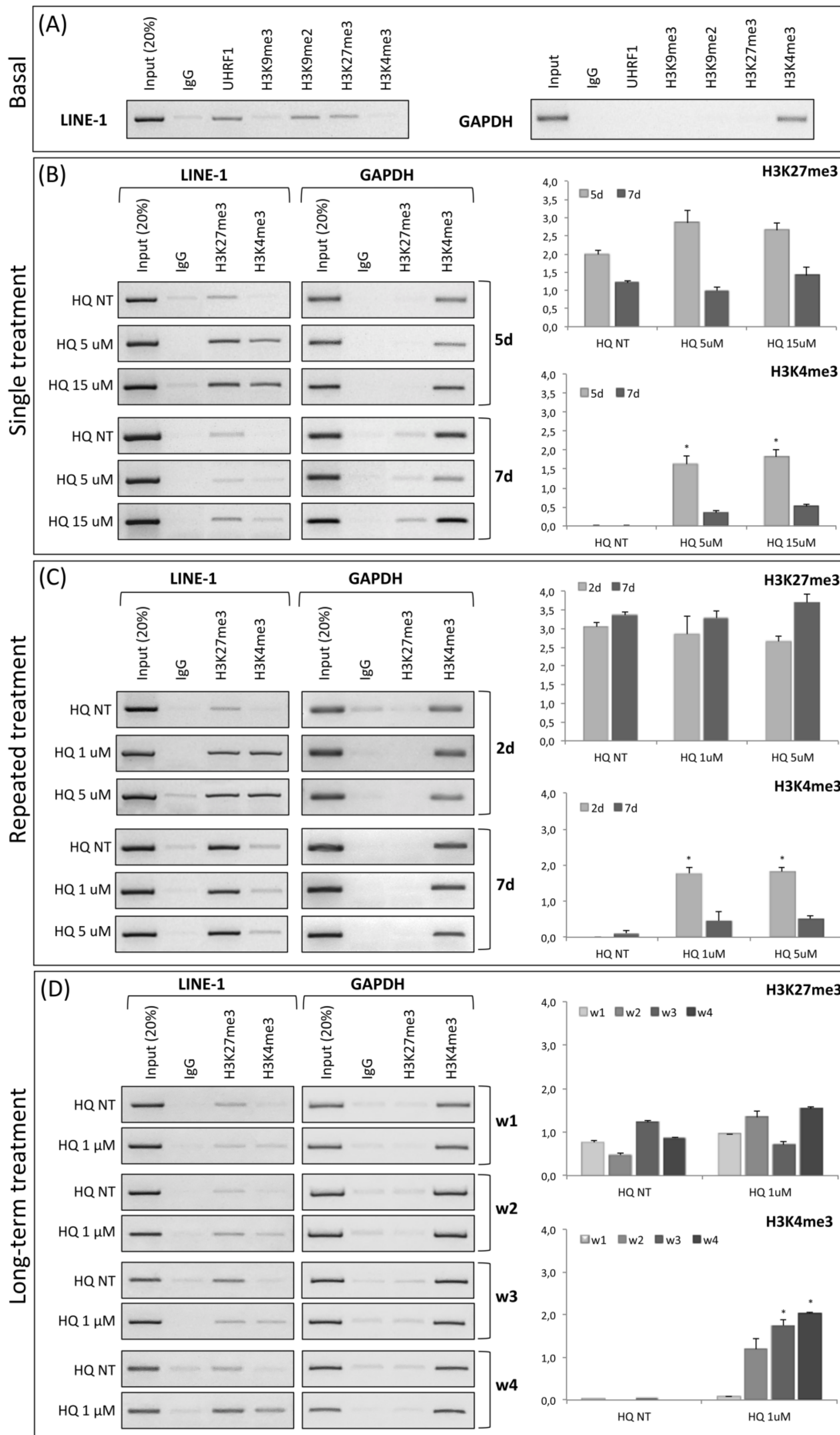

Fig. 4

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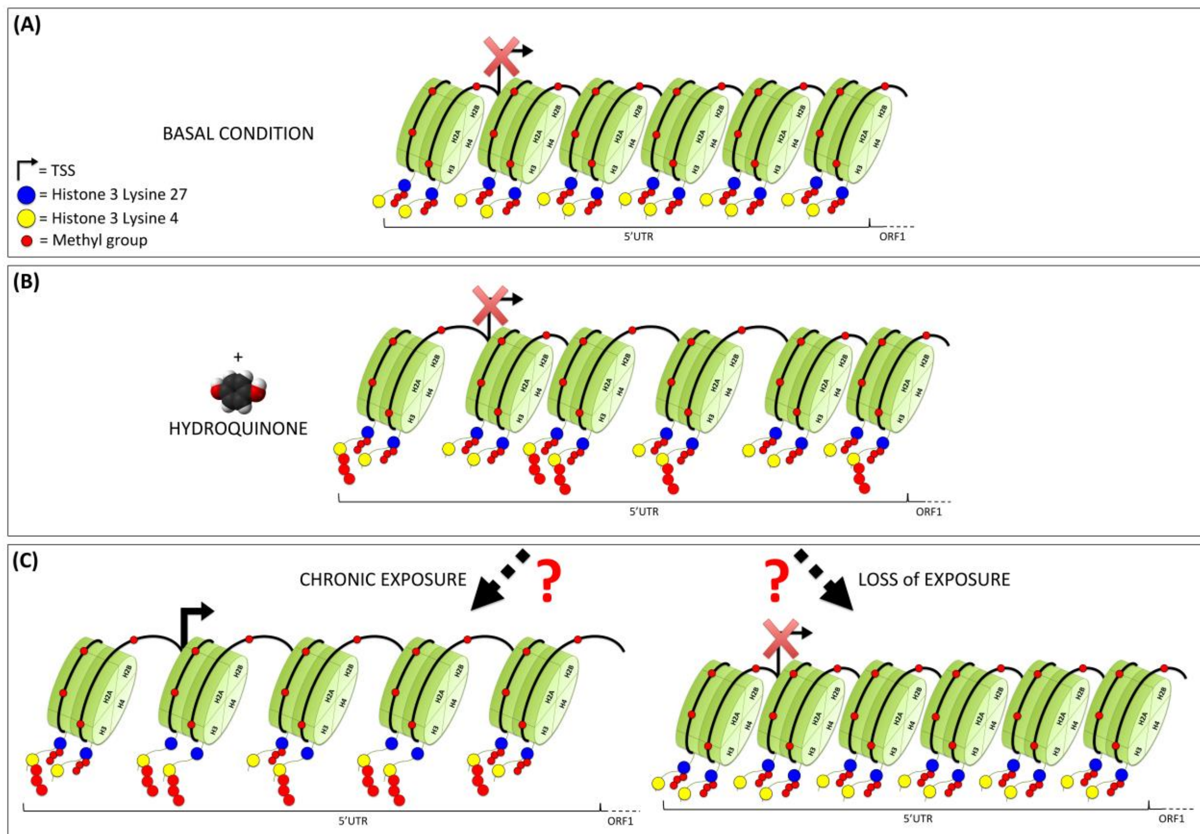

Fig. 5

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