Hypochlorous acid/N-chloramines are naturally produced DNA repair inhibitors

Ronald W.Pero$^{1,2,3}$, Yezhou Sheng$^1$, Anders Olsson$^1$, Carl Bryngelsson$^1$ and Margaretha Lund-Pero$^{1,2}$

$^1$Department of Molecular Ecogenetics, Wallenberg Laboratory, University of Lund, Box 7031, S-220 07 Lund, Sweden and $^2$NYU Medical Center, Department of Environmental Medicine, 550 First Avenue, New York, NY 10016, USA

$^3$To whom correspondence should be addressed at: Department of Molecular Ecogenetics, Wallenberg Laboratory, University of Lund, Box 7031, S-220 07 Lund, Sweden

Human mononuclear leukocytes (HML) respond to oxidative DNA damage by activation of ADP ribosylation and initiation of DNA repair synthesis (i.e. unscheduled DNA synthesis, UDS), whereas neutrophils do not. When neutrophils are added to HML cultures in ratios up to 4:1 ADP ribosylation becomes inhibited to -50–60%. The ability of neutrophils to inhibit HML ADP ribosylation was shown to depend on H$_2$O$_2$, chloride ions and myeloperoxidase, which in turn are factors known to govern HOCl and N-chloramine production by phagocytic cells. HOCl and a model N-chloramine, chloramine T, were shown to give a dose-dependent inhibition of DNA repair using four independent estimates, namely ADP ribosylation, UDS and the repair of DNA strand breaks estimated by nucleoid sedimentation and alkaline elution profiles. All the DNA repair measurements used on HML were inhibited ~70–80% by 100 μM doses of HOCl or chloramine T, which was considered a biologically relevant dose because: (i) viable neutrophils equal in concentration to those found in blood could easily produce 100 μM levels in short-term culture; (ii) 100 μM doses of these agents were not acutely cytotoxic judged by trypan blue stained cells after 30–60 min exposure and under the conditions used for assay, but yet they abolished 86–95% of the growth response of HML to phytohemagglutinin.

Introduction

DNA repair has been repeatedly implicated as a cellular event important to the carcinogenic process (for reviews see 1,2). The evidence has come mainly from autosomal recessive chromosome instability syndromes such as xeroderma pigmentosum, Bloom’s syndrome and ataxia telangiectasia, which are both DNA repair-deficient and cancer prone disorders (3,4). However, this laboratory and others (5–13) have provided data that dominantly inherited cancers or a genetic predisposition for their occurrence also have reduced DNA repair capacity, which has emphasized that not all DNA repair deficiencies can be explained by our current knowledge of inherited defective DNA repair genes. In an effort to explain this apparent anomaly we have sought to identify a mechanism that might regulate DNA repair by a post-transcriptional alteration of the DNA repair genes.

It is generally well recognized that phagocytes are a rich source of endogenously produced reactive oxygen species such as H$_2$O$_2$, O$_2$*, *OH, HOCl and N-chloramines (14). Reactive oxygen species are thought to be involved in both the initiation and promotion phases of carcinogenesis (15,16). This laboratory (12,17) and others (reviewed in 15,16) have shown that H$_2$O$_2$, O$_2$*, *OH can induce cellular DNA damage and activate the DNA repair process, but comparable data for HOCl and N-chloramines, although very limited (14,18,19), have not confirmed that they act like H$_2$O$_2$, O$_2$* or *OH in cellular systems.

One feature of HOCl and N-chloramines that has been established in the literature is their ability to react with sulfhydryls, a mechanism identified for this type of prooxidant as explaining their high degree of cytotoxicity (14,19). Poly-(ADP-ribose) transferase (ADPRT) is a nuclear enzyme that becomes activated by DNA strand breaks, ADP-ribosylates chromatin proteins and, as such, participates in the DNA repair process (20,21). The ADPRT enzyme has been cloned, sequenced and three regions identified; i.e. a DNA binding domain, a catalytic domain and a NAD binding domain (22). There are two zinc fingers in the DNA binding domain and each one contains three cysteine residues where the zinc binds (23), which enables this enzyme to bind to DNA and to participate in DNA repair. Hence, DNA repair that involves ADPRT is sensitive to cellular reduction/oxidation balance. This laboratory has confirmed the sensitivity of DNA repair to the presence of sulfhydryls by up- and down-regulating this process by exposure to reduced and oxidized glutathione (24).

Here we have examined in depth the hypothesis that DNA excision repair can be inhibited by phagocyte-produced HOCl and N-chloramines at physiologically relevant doses.

Materials and methods

Materials

N-Acetoxy-2-acetylaminofluorene (NA-AAF) was supplied by Chemsyn Science Laboratories. Radiolabeled [2,8-3H-adenine]NAD and [3H]Tdr (24 and 5 Ci/mmol) came from DuPont and Amersham respectively. Fisher Scientific supplied sodium hypochlorite. Sigma was the commercial source for 3-amino-1,2,4-triazole, A'-chloro-p-toluene sulfonamide (sodium salt, chloramine T), phorbol-12-myristate-13-acetate (PMA), H$_2$O$_2$, hydroxyurea and sodium azide. Neutrophil isolation medium and mononuclear leukocyte isolation medium were from Cardinal Associates Inc. (Sante Fe, NM) and Organon Teknika Corporation (Durham, NC) respectively.

Blood cell preparation

Peripheral blood samples from apparently healthy volunteers were obtained by venous puncture and collected into heparinized vacutainers (143 USP U/10 ml tube). When only human mononuclear leukocytes (HML) were desired the blood was layered on top of a commercially available density cushion (1.077 gm/ml; Organon Teknika) and the cells were isolated and washed using RPMI 1640 medium in the conventional manner (25). When both HML and neutrophils were needed the cell fractions were simultaneously isolated and washed using RPMI 1640 medium in the conventional manner (25).
isolated by layering the blood sample on top of neutrophil isolation medium (Cardinal Associates) and carrying out all steps in the density gradient isolation using Krebs–Ringer phosphate buffer with glucose, pH 7.4 (KRPG; 26), according to the procedure of Nathan (27).

Estimation of viability by trypan blue exclusion

Regardless of the isolation method used for blood cell fractionation, HML were always resuspended in 10-20% serum- or plasma-supplemented RPMI 1640 medium, pelleted and then resuspended again in either physiological saline or KRPG buffer for treatment with either HOCl or chloramine T. HOCl concentration was determined from the $E_{252} = 100/M/cm$. HML viability was monitored by cellular exclusion of trypan blue (0.2% isotonic solution + 5% serum) after 15 min incubation with the dye at 37°C. The stained (non-viable) and unstained (viable) HML were recorded 30-60 min after each treatment as a percent viability (i.e. unstained HML).

ADPRT assay

The procedure was adapted from the permeabilized cell technique of Berger (28) with modifications as previously described (11). Duplicate samples of $10^6$ HML in the presence of 0-4X$10^6$ neutrophils were cultured in 1 ml KRPG buffer for 30 min at 37°C in the presence of PMA (25 ng/ml). After this co-incubation the HML + neutrophil mixtures were harvested by centrifugation, permeabilized and ADPRT activity determined by a radio-monometric procedure as described in detail elsewhere (11). In other experiments duplicate HML samples of $10^6$ per ml KRPG buffer were directly treated with 0-100 $\mu$M dose ranges of HOCl or chloramine T for 30 min at 37°C, which was then followed immediately by treatment with standardized doses of either H$2$O$_2$ (100 $\mu$M) or PMA (25 ng/ml) for another 30 min before analysis of ADPRT activity as already outlined above (11).

 Unscheduled DNA synthesis (UDS) assay

Approximately $5X10^6$ HML were exposed to a standardized 10 $\mu$M dose of NA-AAF for 1 h at 37°C in 5 ml 20% autologous plasma-supplemented RPMI 1640 medium. The NA-AAF-treated HML were harvested, resuspended in physiological saline and next immediately exposed to a dose range of HOCl or chloramine T from 0 to 100 $\mu$M for 30 min at 37°C. The NA-AAF + HOCl- or + chloramine T-treated HML were again harvested by centrifugation and then incubated overnight at 37°C in fresh 20% autologous plasma-supplemented RPMI 1640 medium containing 10 $\mu$M hydroxyurea and 10 $\mu$M sodium azide (10 $\mu$M sodium azide is added to the medium at a final concentration of 6 $\mu$g/ml and the cells incubated for 48 h at 37°C in a 5% CO$_2$ atmosphere before pulsing for an additional 48 h with $[3H]$FdR (5 $\mu$Ci/ml, 2 $\mu$M/ml; Amersham).

Nucleoid sedimentation assay

The repair of H$2$O$_2$-induced DNA damage in the presence of chloramine T was evaluated using nucleoid sedimentation. HML (5X10$^6$/ml) were cultured in physiological saline in the presence or absence of 10 $\mu$M H$2$O$_2$ for 30 min at 0°C. Next $5X10^6$ Mchlonine T was added at 0°C, the culture continued for another 120 min at 37°C and 50 $\mu$l aliquots (250 000 HML) taken at various time points during this period for the measurement of DNA damage and repair. Nucleoid bodies were prepared and centrifuged at 25 000 r.p.m. in a Beckman SW 50.1 rotor for 30 min at 4°C after laying on top of 15-30% sucrose gradients as described (24). The sedimentation of nucleoids was recorded as a percentage of the untreated control nucleoid sedimentation distance.

Alkaline elution assay for single-strand DNA breaks

HMLs were prepared and exposed to 100 $\mu$M H$2$O$_2$ $\pm$ 100 $\mu$M chloramine T as described in the method for nucleoid sedimentation. The alkaline elution assay was carried out as described by Kohn and co-workers (29), with modifications to measure the unlabeled DNA by microfluorometry (30). Briefly, 2X$10^6$ cells in ice-cold phosphate-buffered saline were layered onto 2 pore size 25 mm diameter polycarbonate filters (Millipore), lysed with 2 M NaCl, 0.04 M EDTA, 0.2% sarkosyl, 0.5 mg/ml proteinase K, pH 10.0, washed with 2.5 ml 0.02 M EDTA, pH 10.0, and eluted in the dark with 0.01 M sodium EDTA, pH 12.3, at a flow rate of 0.038 ml/min. Fractions were collected every 90 min for 9 h and they represented the single-strand modifications to measure the labeled DNA by microfluorometry (30).

HOCI measurement

Mixed cultures of HML + neutrophils were assayed for the production of HOCl in the extracellular conditioned medium by removal of the cells by centrifugation following the incubation period and immediate trapping of the produced HOCl with tauroine (20 mM). Tauroine chloramine was then quantified spectrophotometrically by using the conversion of $1'$ to $1''$ ($\epsilon = 2.29X10^3/M/cm$). Details of this procedure have been described by Weiss et al. (31).

Measurement of lymphocyte response to phytohemagglutinin (PHA)

HML initially suspended in 10% autologous plasma-supplemented RPMI 1640 medium were first exposed to 0-100 $\mu$M doses of chloramine T or HOCl in physiological saline for 30 min at 37°C at a cell density of 1.2X10$^6$/ml. The cells were then harvested by centrifugation and placed in RPMI 1640 medium fortified with 10% autologous plasma. Next the HML were subjected to a standard 96-well microculture technique where 200 000 cells/200 $\mu$l well were used. Highly purified PHA (Flow Laboratories, catalogue no. 16-935-60) was added at a final concentration of 6 $\mu$g/ml and the cells incubated for 48 h at 37°C in a 5% CO$_2$ atmosphere before pulsing for an additional 48 h with $[3H]$TdR (5 $\mu$Ci/ml, 2 $\mu$M/ml; Amersham).

Results

Neutrophil modulation of ADP ribosylation in HML

When H$2$O$_2$, O$_2$ or *OH generating systems are used to damage DNA in HML there is activation of ADP ribosylation (12,17). Contrarily, terminally differentiated neutrophils do not respond to the induction of DNA damage by activation of ADP ribosylation (32). For example, in our laboratory when 4X$10^6$ human purified neutrophils were exposed to a standardized dose of 100 $\mu$M H$2$O$_2$ in KRPG buffer for 30 min there was no measurable activation of ADP ribosylation when determined as reported in Materials and methods. These earlier results could be explained by the possibility that neutrophils may produce inhibitors of ADPRT which prevent their activation. In order to test this possibility we have combined, under conditions that permit viable cell culturing, a standardized amount of HML (1X10$^6$) together with increasing amounts of neutrophils from 0-4X10$^6$ cells per culture. Next these combined cultures were exposed to PMA to activate ADP ribosylation in HML and to induce the production of oxy radicals by neutrophils. The data in Figure 1 show that when HML + neutrophil ratios reached 1:2 (X10$^6$ cells/ml), which is comparable with the proportion and concentration in blood, HML ADP ribosylation began to become severely inhibited. The respiratory burst induced by PMA exposure of neutrophils was monitored by HOCl production using the taurine trapping technique (31). It was concluded that either the presence of 2X$10^6$ neutrophils or the production of ~80 $\mu$M HOCl or
The cells were simultaneously treated with 100 \( \mu \text{M} \) H\(_2\text{O}_2\) and with the indicated concentrations of 3-amino-1,2,4 triazole (3-AT) or sodium azide in KRPG buffer amounts of NaF for 1 h at 37°C.

The cells were first washed in 0.1 M phosphate buffer, 1.5 mM MgSO\(_4\), pH 7.4, \( \text{NaF} \), and myeloperoxidase activity could be measured by trypan blue exclusion.

The data presented in Table I show that: (i) addition to HML + neutrophil cultures of 3-aminotriazole or sodium azide, which are well-established inhibitors of myeloperoxidase, also activates HML ADPRT activity and thus blocks the ability of neutrophils to inhibit ADPRT. Taken together these data strongly suggest that HOCl, and not some other neutrophil factor, is responsible for inhibition of HML ADPRT activity.

### Table I. Dependence of ADPRT activity in HML on \( \text{H}_2\text{O}_2\), chloride ions and myeloperoxidase activity

<table>
<thead>
<tr>
<th>HML + neutrophils</th>
<th>( \text{H}_2\text{O}_2) (( \mu \text{M} ))</th>
<th>NaF (mM)</th>
<th>Myeloperoxidase inhibitor</th>
<th>ADPRT activity (% control ± SD)</th>
</tr>
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<tbody>
<tr>
<td>1+2°</td>
<td>Control</td>
<td>100+14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+2°</td>
<td>10 ( \mu \text{M} )</td>
<td>77±8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+2°</td>
<td>20 ( \mu \text{M} )</td>
<td>41±5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+2°</td>
<td>30 ( \mu \text{M} )</td>
<td>42±6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+2°</td>
<td>40</td>
<td>26±3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+2°</td>
<td>100</td>
<td>30±4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+1+2°</td>
<td>100 Control</td>
<td>100±17</td>
<td></td>
<td></td>
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<td>100 10</td>
<td>114±12</td>
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<tr>
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<td>100 100</td>
<td>174±23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+2°</td>
<td>100 Control</td>
<td>100±9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+2°</td>
<td>100 10 mM 3-AT</td>
<td>139±13</td>
<td></td>
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</tr>
<tr>
<td>1+2°</td>
<td>100 50 mM 3-AT</td>
<td>220±37</td>
<td></td>
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<tr>
<td>1+2°</td>
<td>100 10 ( \mu \text{M} ) azide</td>
<td>650±57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+2°</td>
<td>100 100 ( \mu \text{M} ) azide</td>
<td>710±71</td>
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</table>

*The cells were treated with 0–100 \( \mu \text{M} \) H\(_2\text{O}_2\) in KRPG buffer for 1 h at 37°C.

The per cent of control determinations was calculated by dividing the ADPRT activity present in HML + neutrophil cultures by the ADPRT activity present in HML cultures only. There is no ADPRT activity in neutrophils. The ADPRT activities for HML + neutrophils (1 + 2 x 10\(^9\)) treated with 100 \( \mu \text{M} \) H\(_2\text{O}_2\) ranged from 414 to 616 c.p.m. (9.1–13.6 pmol) TCA-precipitable radioactive NAD.

*The cells were first washed in 0.1 M phosphate buffer, 1.5 mM MgSO\(_4\), pH 7.4, treated with 100 \( \mu \text{M} \) H\(_2\text{O}_2\) and then incubated in the presence of increasing amounts of NaF for 1 h at 37°C.

*The cells were simultaneously treated with 100 \( \mu \text{M} \) H\(_2\text{O}_2\) and with the indicated concentrations of 3-aminotriazole (3-AT) or sodium azide in KRPG buffer for 1 h at 37°C.

**Physical effects on DNA repair and cell function induced by these prooxidants are not related to acute cytotoxicity (i.e. trypan blue staining within 30–60 min after exposure). We have observed that HML are very sensitive to HOCl and HOCI are the only endogenous source of HOCl production (14,33). In an effort to distinguish between whether the HML ADPRT inhibition induced by neutrophils was due to HOCl production or to the presence of some other inhibiting factor(s) coming from neutrophils we have determined if HML ADPRT was dependent on myeloperoxidase activity. There are three factors known to govern HOCI production by neutrophils, namely \( \text{H}_2\text{O}_2\), chloride ions and myeloperoxidase.

**Hypochlorous acid/N-chloramines are DNA repair inhibitors**

### Fig. 2. Effect of chloramine T and HOCl on \( \text{H}_2\text{O}_2\)-activated ADPRT activity in HML. HML were first treated with 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2\) and then immediately exposed to the indicated doses of chloramine T or HOCl. Data points and bars represent mean ± SD.

### Fig. 3. Effect of chloramine T and HOCl on PMA-activated ADPRT activity in HML. HML were first treated with 25 ng/ml PMA and then immediately exposed to the indicated doses of chloramine T or HOCl. Data points and bars represent mean ± SD. Reproduced from *Biochimie, 77, 390 (1995).*
was assessed by addition of these agents to HML cultures for 30 min as described in Materials and methods. Both chloramine T and HOCl gave a dose-dependent inhibition of ADP ribosylation regardless of whether it was induced by \( \text{H}_2\text{O}_2 \) or PMA (Figures 2 and 3). About 70–80% inhibition of ADPRT activity was observed at doses of 100 \( \mu \text{M} \) HOCl or chloramine T. These doses and the effects on HML ADPRT activity were comparable with those produced physiologically by neutrophils (Figure 1).

**Effects of HOCl and chloramine T on other estimates of DNA repair**

DNA excision repair is a cellular process involving several enzymatic events (1–3,20,21) and it is possible that although ADPRT is implicated in DNA repair (20,21), the inhibitory effects of HOCl or chloramine T on ADPRT might not be reflected in other quantitative estimates of DNA repair. We have confirmed that this is not the case using three other independent estimates of DNA repair, namely UDS and the repair of DNA strand breaks estimated by nucleoid sedimentation and alkaline elution profiles. NA-AAF-induced UDS was dose dependently inhibited by 67 and 75% at doses of 100 \( \mu \text{M} \) chloramine T and HOCl respectively (calculated from Figure 4). This inhibition was apparently irreversible, since the cells were washed free of these two prooxidants before estimating UDS over an 18 h incubation period. Similar results were obtained when the repair of \( \text{H}_2\text{O}_2 \)-induced DNA strand breaks measured up to 2 h after \( \text{H}_2\text{O}_2 \) treatment by either nucleoid sedimentation (Figure 5) or alkaline elution (Figure 6) was inhibited by exposure to 100 \( \mu \text{M} \) chloramine T. Furthermore, the effects of HOCl and chloramine T on DNA repair appear to be quite independent of any direct effects on DNA damage induction, because neither agent could be shown to induce significant DNA damage in HML in short-term culture (Figures 5–7).

**Effects of HOCl and chloramine T on HML PHA responsiveness**

The effect of DNA repair inhibition caused by HOCl or chloramine T exposure on the function of HML was examined by quantifying the growth induced by the polyclonal mitogenic stimulus PHA. The data in Table II clearly demonstrate that there was a dose-dependent inhibition of the growth response to PHA after exposure to 50–100 \( \mu \text{M} \) doses of HOCl or chloramine T and that the level of inhibition was nearly complete, being 86–95% compared with the control levels at doses of 100 \( \mu \text{M} \). These data support the hypothesis that DNA repair inhibition by HOCl/\( \text{N} \)-chloramine type products results in a proportionate loss of immune cell (i.e. HML) responsiveness without directly inducing DNA damage (Figure 7), but by permitting its accumulation (Figures 5 and 6).
This study presents evidence that neutrophil production of chloramine T and HOCl compromises DNA repair and immune cell (i.e. HML) responsiveness. Four independent measures of DNA repair (i.e. ADPRT activity, UDS and the repair of DNA strand breaks estimated by nucleoid sedimentation and alkaline elution profiles) all gave dose–response curves for DNA repair inhibition by HOCl and the model N-chloramine, chloramine T, where ~70–80% inhibition occurred at 100 μM doses (Figures 2–6). Moreover, 100–200 μM doses of HOCl or chloramine T also resulted in ~60% inhibition of DNA repair when it was produced naturally by cultured neutrophils (Figure 1) or ~86–95% inhibition of growth response when it was added directly to HML cultures activated by the mitogen PHA (Table II). The strong correspondence of these data, combined with the potential for irreversibility (Figure 4) and selectivity (Figure 7) of the effects on DNA repair, have emphasized a consistency and specificity in the data that is convincing of an important physiological role for HOCl and N-chloramines as DNA repair inhibitors.

Moreover, there are two equally compelling explanations for the data reported in this study. One is that HOCl/N-chloramines are very broad spectrum biologically reactive oxidants affecting separate mechanisms in at least three biochemical processes we have studied, namely ADPRT activity, UDS and PHA mitogenic stimulation of HML. This interpretation is supported by the fact that the dose–response curves for inhibition by HOCl/N-chloramines were slightly different for the ADPRT and UDS measures (Figures 2–4), as well as that the observed effects on PHA HML responsiveness (Table II) could have been explained by interference in PHA binding to receptors and not related to DNA damage and repair. The other possibility is that these biochemical processes have a common controlling mechanism that affects all three assay procedures. There are data available to support this contention. Although it is true that the repair of single-strand breaks is not ADPRT dependent (24), the repair of bulky NA-AAF DNA lesions that induce large patch UDS is ADPRT dependent and inhibited by H2O2 (24). Likewise, the PHA response of HML is inhibited by ADPRT inhibitors, which demonstrates the dependence on ADPRT (34,35).

In addition, our data are further supported by biochemical considerations. HOCl and N-chloramines are prooxidants expected to be reactive with sulfhydryl amino acid-containing proteins and both ADPRT activity (24) and UDS (24), as well as the responsiveness of HML (36,37), have been shown to be reduction/oxidation-sensitive biochemical processes.

A role for DNA repair in cancer and ageing has previously been based on the concept that there are inherent or acquired defects in the genes that carry out DNA repair (1–4) or on the concept that there is a regulation of the level of mutations found in oncogenes. Although there is ample evidence for the occurrence of DNA repair gene defects (4), their association with rare genetic events such as chromosome instability syndromes makes it difficult to postulate a more generalized role for DNA repair in cancer of the breast, colon and lung, even if heterozygote populations are considered (17). The data reported on here offer a new approach where post-transcriptional regulation of DNA repair is by production of endogenous DNA repair inhibitors (HOCl and N-chloramines), which occur as by-products of the inflammatory response via normal phagocytic function (Figures 1–5). This concept of DNA repair regulation would predict that modulation of the inflammatory response, either by inheritance or by acquisition, for example from dietary factors, would up- or down-regulate DNA repair and, as a consequence, affect immune responsiveness, cancer susceptibility and ageing. Support for this hypothesis comes from: (i) overactive inflammatory responses, such as the case with ulcerative colitis, have increased cancer risk (15,33,38) and suppressed HML ADPRT activity (13); (ii) dietary factors, such as a reduced calorific intake, can enhance DNA repair (39), inhibit spontaneous cancer development (15) and extend longevity (15); (iii) immunosuppressive corticosteroids also suppress ADPRT in vivo (40) and immunosuppression in turn has been linked with cancer and ageing (15,41); (iv) tamoxifen inhibits the production of H2O2 and thus HOCl by neutrophils (42), enhances ADPRT in vivo (43) and can prevent cancer (44); (v) ADPRT activity and UDS correlate with the lifespan of mammals (43,44, and references therein) and this correlation was not related to the amount of ADPRT present but rather to its activity (46). These available data are considered ample justification for other investigators to further validate this hypothesis.

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