Contribution of hydrazines-derived alkyl radicals to cytotoxicity and transformation induced in normal c-myc-overexpressing mouse fibroblasts

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Several hydrazine derivatives (HD) tested so far have pharmacological activities, but many also have toxic side effects, including carcinogenesis. Their toxicity has been ascribed to carbocations (via formation of azoxy intermediates), alkyl radicals or reactive oxygen species. Cytotoxicity and transformation in Balb/c 3T3 fibroblasts by carbocations is widely accepted, but the role of alkyl radicals is still questioned.

We have investigated the cytotoxicity of HD to mouse fibroblasts in three activation systems in which enhanced alkyl radical formation is demonstrated by electron spin resonance/spin-trapping. Cytotoxicity was assayed by inhibition of [3 H-methyl]thymidine uptake into DNA of Balb/c 3T3 and/or Myc 9E fibroblasts (normal Balb/c 3T3 cells over-expressing the c-myc proto-oncogene). Based on the results obtained in the cytotoxicity assays we also investigated the transforming potential of procarbazine (PCZ) and methylhydrazine (MeH) activated by horseradish peroxidase (HRP) using the Myc 9E cell line, which aims at the activation of a second cooperating oncogene. Our results show that: (i) cytotoxicity of HD to mouse fibroblasts is increased by HRP activation of MeH, phenelzine and PCZ, which displayed enhanced alkyl radical formation, but not of 1,2-dimethylhydrazine (DMH), which did not produce increased alkyl radical formation under these conditions; (ii) cytotoxicity of neutrophil-activated MeH (producing a 10-fold higher concentration of methyl radicals), is more pronounced than DMH; (iii) MeH and DMH activated by prolonged auto-oxidation in 24-h incubations have comparable cytotoxicity and alkyl radical formation; and (iv) PCZ and MeH activation by HRP to alkyl radicals increased the transformation induced in Myc 9E cells. Taken together, our results strongly support a role for hydrazine-derived alkyl radicals in HD-induced cytotoxicity and cell transformation.

Introduction

Hydrazine derivatives constitute an important class of compounds to which the human population is often exposed, as they are natural components of mushrooms and tobacco, and are constituents of herbicides, rocket fuel and drugs. Several hydrazines displaying pharmacological activities are currently in use. Phenelzine (PEH*) is a monoamine oxidase inhibitor clinically employed as an antidepressant; hydralazine is used as an anti-hypertensive agent; procarbazine (N-isopropyl-α-(2-methyl-hydrazine)-p-toluamide hydrochloride) (PCZ) is part of a chemotherapeutic cocktail used in the treatment of Hodgkin’s disease, melanoma and bronchogenic carcinoma; and isoniazide is one of the basic antibiotics employed in the treatment of tuberculosis (1,2). 1,2-Dimethylhydrazine (DMH) is a potent rodent carcinogen used as a model in the study of colon carcinogenesis. DMH and methylhydrazine (MeH) have been used as models to study the metabolism and toxicity of hydrazine derivatives.

However, in spite of their pharmacological applications as therapeutic agents in important pathologies, their use is limited by toxic side effects, such as hepatotoxicity, induction of systemic lupus erythematosus and carcinogenicity (3–5). All hydrazines tested so far have carcinogenic properties in experimental animals (6,7), although they have been shown to be weak mutagens in current bacterial tests (7–9). Due to their widespread pharmaceutical applications the metabolism of hydrazine derivatives has been intensely investigated and they have been shown to be extensively metabolized both in vitro and in vivo.

Mono-substituted derivatives have been shown, by spin-trapping, to be metabolically oxidized to carbon-centered radicals by several enzymatic systems, such as horseradish peroxidase (HRP), cytochrome P 450, microsomes, hepatocytes, oxyhemoglobin (HbO₂), neutrophils (PMNs) and myeloperoxidase (10–13). The metabolism of di-substituted hydrazines is more controversial. Several studies suggest the importance of the azoxy derivatives in the metabolism and toxicity of DMH and PCZ (14–16), which would lead to alkylation processes through a carbocation intermediate. On the other hand, spin-trapping studies have established the formation of carbon-centered radicals in vivo has been shown, by spin-trapping, after administration of PCZ to rats (19).

In brief, there is evidence for the formation of two reactive alkylation species in the metabolism of di-substituted hydrazine derivatives: carbonium ions (carbocations) and alkyl radicals (carbon-centered radicals) (Scheme 1, paths a and b respectively), which would be produced by distinct pathways that derive after azo compounds (R–N=N–R’) oxidation. The relative yields of these two pathways are likely to vary in different organs, but this has not been assessed as yet. Toxicity of hydrazine derivatives has been ascribed to these metabolism-generated reactive intermediates, namely, carbocations (20–22) and carbon-centered radicals (23–26) and also to reactive oxygen species (27,28).
Cytotoxicity of some hydrazine derivatives has been investigated in vitro, in fibroblasts, hepatocytes, leukemia cell lines and also in vivo (4,21,22,29,30). Most of these studies implicated, directly or indirectly, formation of carbonium ions in the observed toxic processes. However, due to the experimental design, they do not exclude participation of alkyl radicals in the cytotoxicity of the hydrazine derivatives. Hemolysis due to phenylhydrazine has been shown to be mediated by phenyl radicals (23,25). Nonetheless, cytotoxicity of alkyl radicals generated in the metabolism of hydrazine derivatives to mouse fibroblasts has not been clearly demonstrated yet.

DNA alkylation is considered to have a role in chemical carcinogenesis. Alkylation of DNA by carbocations arising from the metabolism of di-substituted hydrazines via azoxy derivatives (as with structurally related nitrosamines and nitroso compounds) is widely accepted (15,22), but the role of alkyl radicals in the induction of carcinogenic processes is still controversial. Even though the likelihood of in vivo DNA alkylation by carbon-centered radicals has been questioned (31), several lines of evidence have suggested a role for alkyl radical-induced DNA damage in the induction of carcinogenic processes (32). The capacity of hydrazine-derived alkyl radicals to interact with DNA in vitro has been demonstrated by the induction of DNA strand breaks and alkylation by alkyl radicals produced during PEH and DMH enzymatic oxidation, with a preference for guanine residues (24,33). DNA damage by alkyl radicals produced in the oxidation of several hydrazines has recently been confirmed (26).

The analysis of DNA alkylation products has been used as a tool to investigate the mechanism of DNA damage by hydrazines, which is an analogy to the use of 8-oxodeoxyguanosine as a marker for hydroxyl radical attack (34). It is assumed that carbocation ions produce N2- and O6-alkylguanine (20) and it has been demonstrated that carbon-centered radicals produce mostly C6-alkylguanine in vitro (35,36). Treatment of DNA with the MeH/HRP system or administration of DMH to rats leads to the formation of N2-methylguanine and C8- methylguanine (37,38). These results further support DNA alkylation in vitro and in vivo by alkyl radicals. DNA strand scission and/or alkylation products from alkyl radical attack may have a role in the carcinogenic properties induced by hydrazine derivatives (32).

Mutations in K-ras have been demonstrated in the colon tumors of rats treated with DMH, which were characterized as G→A transitions in the second nucleotide of codons 12 or 13 and the first nucleotide of codon 59 (39). It is known that O6-methylguanine may mispair with thymidine inducing G:C→A:T transitions, this adduct being formed upon methylated hydrazine derivative administration (15). These results also suggest that guanine alkylation by these drugs may be an important mechanism in the carcinogenic process, but do not differentiate between possible reactive species, since both carbocations and alkyl radicals have been shown to produce these adducts (15,36). The mutagenic potential of C8-methylguanine has not been investigated yet.

Malignant transformation involves alterations in cell-cycle control and differentiation (40). Chemical carcinogenesis has been shown to proceed through multiple stages, including initiation, promotion and progression; however, the molecular mechanisms involved are still obscure. Nevertheless, there is substantial indirect evidence that free radicals may have a role in all three stages of malignant transformation (41–43). Although many studies are available on the carcinogenicity and mutagenicity of hydrazine derivatives, very few studies focus on the transforming properties of these compounds in cell culture, systems which would be highly convenient to investigate the mechanistical pathways of cell transformation. One conceivable explanation for this deficiency in the literature is the notion that hydrazine derivatives would constitute weak complete carcinogens and, therefore, cell transformation in culture would be difficult to achieve. It has recently been shown that the administration of DMH to rats leads to activation of K-ras oncogenes in the induced colon tumors (39). On the other hand, it is well established that ras and myc oncogenes cooperate to lead to complete cell transformation (44). Oncogene cooperation can be used as a tool to investigate the mechanisms involved in cell transformation induced by metabolism-generated reactive species (45). Based on these results and in view of the oncogene cooperation concept, we used Myc 9E cells, a normal cell line produced by transfection of Balb/c-3T3 cells with a c-myc proto- oncogene (46), to investigate the transforming properties of hydrazine derivatives metabolically activated to alkyl radicals, aiming at the activation of a second cooperating oncogene.

Here we studied the cytotoxic effect of mono- and di- substituted derivatives, on cultured mouse fibroblasts in three systems for which hydrazine-derived carbon-centered radical formation is demonstrated: (i) HRP-activation in 1-h incubations; (ii) metabolism by activated PMNs; and (iii) prolonged auto-oxidation in 24-h incubations. The following derivatives were investigated: the anticancer drug, PCZ, the potent colon carcinogen, DMH, the anti-depressant drug, PEH, and, the methylated mono-substituted model derivative, MeH.
Metabolism of hydrazine derivatives to alkyl radicals was demonstrated by electron spin resonance/spin-trapping and cytotoxicity was assayed by the inhibition of [3H-methyl]-thymidine incorporation into DNA. The transforming potential of PCZ and MeH activated by HRP was also investigated using the Myc 9E cell line, based on the results obtained in the cytotoxicity assays. The results described herein strongly support the contribution of alkyl radicals to the cytotoxic and transforming properties of metabolically activated mono- and di-substituted hydrazine derivatives.

Materials and methods

Chemicals

The following reagents were obtained from commercial sources: HRP (Type VI), DMH, PEH, Dulbecco’s modified Eagles medium (DMEM), Hanks balanced salt solution (HBSS), diethyltetraminepenta acetic acid (DTPA); hydrocortisone, phosphor-b-12-lyristate-13-acetate (PMA), eth-(4-ethylpyridyl)-1-oxide)-N-tert-butylintrone (POBN), superoxide dismutase (SOD) (S5959), catalase (C6665), Zymosan and Tempol (4-hydroxy-2,2,6,6-tetramethyl-piper- idine-N-oxyl) were from Sigma Chemical Co. (St Louis, MD). [3H-methyl]thymi dinide was from Amersham International (Buckinghamshire, UK). Fetal calf serum was from CultiLab (Campinas, Sao Paulo, Brasil). MeH was from Aldrich Chemical Co (Milwaukee, WI). MeH was precipitated with H2SO4 to form MeH2SO4 and recrystallized from methanol. PCZ was kindly supplied by the Drug Synthesis and Chemistry Branch from the National Cancer Institute (Bethesda, MD). Hydrazine derivative stock solutions were prepared immediately before use in DMEM containing 0.1 mM DTPA, pH 7.6-7.5 and maintained on ice until used. Water grade Milli-Q (Millipore) was used throughout.

Cells and culture conditions

Balb/c-3T3 cells (clone A31) and Myc 9E cells were used for the cytotoxicity and transformation assays. Myc 9E cells are a Balb/c-3T3 derived cell line produced by co-transfection with a plasmid containing two coding exons of the mouse c-myc proto-oncogene under the control of MMTV (mouse mammary tumor virus) promoter, which is inducible by hydrocortisone, and a plasmid containing the neo gene under the control of an HSV (Harvey sarcoma virus) promoter, which confers resistance to the antibiotic geneticin (G418) (46). Constitutive expression of c-myc mRNA in this cell line is comparable to platelet derived growth factor-stimulated cells and hydrocortisone further increases c-myc mRNA levels ~5-fold (46). Cells were routinely cultured from stock cells stored in liquid nitrogen. Cultures were maintained in tissue culture dishes in DMEM supplemented with 10% fetal calf serum for 10 days. This was done in order to select cells with higher expression of the c-myc proto-oncogene, as the cell line was produced by selection with 0.2 mg/ml geneticin in DMEM supplemented with 10% fetal calf serum for 10 days. This was done in order to select cells with higher expression of the c-myc proto-oncogene, as the cell line was produced by selection with 0.2 mg/ml geneticin (46); without re-selection no transformation was observed. Cells were then replated and maintained in culture for 1 more week to obtain enough cells for the assays for formation of transformed colonies. These experiments were repeated twice and produced similar results.

PMN preparation

PMNs were isolated from female Wistar rat peritoneal exudates were obtained as previously described (47), by injection of 0.8% glycogen dissolved in saline, 12 h before they were killed with diethyllether. Cells were collected by peritoneal washing with 10% heparin in HBSS, centrifuged and washed twice with HBSS plus 5 mM glucose, before final cell suspension was adjusted to 2~3X10^7 cells/ml. This procedure regularly produces a population with 98% viability, determined by trypan blue exclusion, 90% of the cells were PMNs. The cellular suspension was maintained at the most for 4 h at 0–4°C.

Treatment of mouse fibroblasts with activated hydrazine derivatives in the cytotoxicity assays

The cells were seeded at 2.5X10^3 cells/well in 24-well trays, 24 h before treatment and maintained in DMEM with 10% FCS. The cells were then subjected to one of the three treatments: (i) HRP-activation: increasing concentrations of the hydrazine derivatives in 0.3 ml of DMEM containing 0.1 mM DTPA, in the presence or absence of 20 µM HRP, incubated for 1 h; (ii) PMN-activation: hydrazine derivatives in the presence of PMNs (5X10^3 cells/ml) and Zymosan (0.2 mg/ml opsonized with rat serum) in DMEM containing 0.1 mM DTPA, incubated for 1 h; or (iii) prolonged auto-oxidation: 24 h incubations with increasing concentrations of the hydrazine derivatives in 0.3 ml of DMEM containing 0.1 mM DTPA. Control cells were treated with DMEM containing 0.1 mM DTPA and all treatments were incubated at 37°C in a 5% CO2 atmosphere.

Cytotoxicity assay

The cells were seeded 24 h before treatment at 2.5X10^3 cells/well in 24-well trays. Following the treatments with the hydrazine derivatives, the reaction mixtures were immediately removed, the cells rinsed with PBS and incubated with DMEM containing 10% FCS and 0.5 µCi of [3H-methyl]thymidine, at 37°C for 24 h in a 5% CO2 atmosphere. The radioactive medium was then removed and, after rinsing with PBS, the cells were lysed with 0.5 N NaOH at 65°C for 15 min. The supernatants were transferred to pieces of thick filter paper which were dried to a constant weight, and dissolved in 1 ml of scintillation fluid (POBN-radical adducts were calculated by double integration using Tempol as standard). The oxidative metabolism of hydrazine derivatives to alkyl radicals with DME containing 10% FCS and 1 µCi of [3H-methyl]thymidine, after which they were trypsinized and seeded into 24-well trays (2X10^5 cells/well). Following treatment with the activated hydrazines, 300 µl of lysis buffer (10 mM Tris, 0.1 mM EDTA, 0.05 N NaOH and 2% SDS, pH 12.4) and 300 µl of 120 mM KCl were added to each well. The trays were then maintained at 65°C for 10 min after which 400 µl of the lysates were transferred to microcentrifuge tubes and maintained for 15 min in an ice bath. A DNA-protein–K–SDS complex is formed in this process, which precipitates at low temperature (49). Low molecular weight single-stranded DNA is released from the complex and separated by centrifugation at 3500 r.p.m. for 10 min at 10°C. Both the supernatants and the precipitates (dissolved in 100 µl of water at 65°C for 15 min) were transferred to pieces of thick filter paper and processed as described for the cytotoxicity assay for determination of radioactivity in a scintillation counter. The experiments were done in quintuplicate.

Treatment of Myc 9E cells with hydrazine derivatives/HRP in the transformation studies

Myc 9E cells were plated at 1X10^5 cells/well in 12-well plates. After 24 h, cells were subjected to a 1-h treatment with one of the hydrazine derivatives (2.0 mM MeH or 0.5 mM PCZ), in the presence or absence of 20 µM HRP, in 0.5 ml of DMEM (without serum or iron chelators) at 37°C in a 5% CO2 incubator. The reaction mixture was then removed and cells were maintained in culture for 1–2 days for recovery before replating for subsequent treatment. Following six consecutive treatments, the remaining cells were re-selected in 6-well plates at sub-confluence with a 0.5 mg/ml effective dose of genetinicin in DMEM supplemented with 10% fetal calf serum for 10 days. This was done in order to select cells with higher expression of the c-myc proto-oncogene, as the cell line was produced by selection with 0.2 mg/ml geneticin (46); without re-selection no transformation was observed. Cells were then replated and maintained in culture for 1 more week to obtain enough cells for the assays for formation of transformed colonies. These experiments were repeated twice and produced similar results.

Electron spin resonance (ESR) studies

Standard reaction mixtures containing the hydrazine derivatives in the presence or absence of an activation system, 0.1 mM DTPA and 100 mM POBN in DMEM were incubated at 37°C in a humidified 5% CO2 atmosphere for the indicated periods of time. Aliquots (100–200 µl) taken from the incubation mixtures were transferred to flat quartz cells. Spectra were recorded at room temperature on a Bruker ER 200D-SRC spectrometer. The concentration of POBN-radical adducts were calculated by double integration using Tempol as standard.

Results

Formation of hydrazine-derived alkyl radicals determined by ESR/spin-trapping

The oxidative metabolism of hydrazine derivatives to alkyl radicals was demonstrated by ESR, using POBN, an effective spin-trap for carbon-centered radicals (24). Analysis of alkyl-POBN radical adduct formation in different activation systems confirmed the oxidation of hydrazine derivatives to the expected alkyl radicals (50), which were the methyl radical.
for MeH, DMH and PCZ, and the 2-phenylethyl radical for PEH, with hyperfine splitting constants of $a_N = 15.25$ and $a_H = 2.75$ (Figure 1).

Incubation of hydrazines for 1 h in DMEM in the presence of DTPA and the spin-trap, POBN, leads to the formation of alkyl-POBN radical adducts in low yields (Table I, control). The metabolism of hydrazine derivatives to alkyl radicals catalyzed by HRP/H$_2$O$_2$ has been previously demonstrated by spin-trapping (51), however we chose to activate the hydrazine derivatives by HRP because this methodology is sufficient to trigger catalysis by HRP (our unpublished results). Here we used HRP-catalysis of hydrazine oxidation to form H$_2$O$_2$, which slows down auto-oxidation and, consequently, formation of alkyl radicals. Under these conditions, activation of hydrazine derivatives by HRP leads to increased alkyl radical formation from MeH, PEH and PCZ (over 5-fold), but not from DMH (Table I, Figure 1). We have previously demonstrated the formation of alkyl radicals in the metabolism of these hydrazine derivatives by activated neutrophils (13). Auto-oxidation of the derivatives for 24 h increases radical adduct formation by ~5- to 10-fold (Table I). Inclusion of trypsinized whole cells in this system, does not increase radical-adduct formation, but rather, inhibits it, probably due to competition of cell components with the spin-trap. Auto-oxidation of the hydrazines to alkyl radicals in the cells does not surpass auto-oxidation in solution or competition with cellular components. These results are in accord with the general notion that fibroblasts do not have efficient metabolizing systems (52), as, for example, hepatocytes (12).

### Cytotoxicity of HRP-activated hydrazine derivatives

Cytotoxicity of activated hydrazine derivatives to mouse fibroblasts was evaluated by the inhibition of $[^3$H-methyl]thymidine incorporation into DNA. Treatment of Myc 9E cells with non-activated hydrazine derivatives for 1 h shows that MeH and DMH display low cytotoxicity up to 12 mM, whereas at 2 mM, cytotoxicity of PEH and PCZ is evident (Figure 2). Cytotoxicity induced by the hydrazines alone cannot be ascribed to alkyl radical formation, but, rather, is probably due to either, the nucleophlicity of hydrazines per se (which could react with important carbonilic cellular constituents) or to the formation of reactive species such as azoxy derivatives and hydrazones (22,53). Activation by HRP significantly increases the cytotoxicity of MeH, PEH and PCZ (Student’s t-test, $P < 0.05$), but not that of DMH (Figure 2). In parallel, MeH, PEH and PCZ, but not DMH, show increased alkyl radical formation upon activation by HRP (Table I). This increase in the cytotoxicity only in the systems in which HRP activation increases alkyl radical formation, strongly suggests their contribution to hydrazine derivatives’ cytotoxicity.

### Cytotoxicity of PMN-activated hydrazine derivatives

Metabolism of mono- and di-substituted hydrazines by PMNs activated by opsonized Zymosan (opsZym), produces increased formation of alkyl radicals (13). As determined above, non-activated MeH or DMH display low cytotoxicity for Myc 9E cells between 1 and 5 mM (Figure 3). PMN/opsZym alone exhibited some cytotoxicity for Myc 9E cells above 1×10$^6$ PMN/ml (Figure 3). SOD and catalase partially inhibit this cytotoxicity (not shown) and were included to prevent reactive oxygen species-induced cytotoxicity. MeH or DMH activation by PMN/opsZym leads to a dose-dependent inhibition of $[^3$H-methyl]thymidine uptake into DNA (Figure 3). MeH is more cytotoxic than DMH in this system at the same concentration (2 mM), which correlates with the 5-fold greater formation of methyl radical, as previously determined by spin-trapping with

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**Table I. Metabolism of hydrazine derivatives by different activation systems: Formation of alkyl–POBN radical adducts**

<table>
<thead>
<tr>
<th>System hydrazine</th>
<th>Alkyl–POBN radical adduct concentration$^a$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control$^b$ (1 h)</td>
</tr>
<tr>
<td>MeH</td>
<td>3.6</td>
</tr>
<tr>
<td>PEH</td>
<td>1.7</td>
</tr>
<tr>
<td>DMH</td>
<td>1.8</td>
</tr>
<tr>
<td>PCZ</td>
<td>2.0</td>
</tr>
</tbody>
</table>

$^a$Values are the average of two independent determinations. The standard reaction mixtures containing the hydrazine derivatives (5 mM), DTPA (0.1 mM) and POBN (100 mM) were incubated in DMEM (without FCS) with the appropriate activation system at 37°C in a 5% CO$_2$ atmosphere. Radical-adduct concentration was determined as described in Materials and methods.

$^b$1 h incubation with no activation.

$^c$Activation by HRP (20 µM) in 1 h incubations.

$^d$Activation by auto-oxidation for 24 h.

$^e$Activation by auto-oxidation for 24 h in the presence of trypsinized whole cells (Myc 9E, 8×10$^3$/ml).
Cytotoxicity and transformation by hydrazine derivatives

Fig. 2. Cytotoxicity of hydrazine derivatives activated by HRP. Subconfluent Myc 9E cells were incubated with increasing concentrations of the hydrazine derivatives in the presence or absence of 20 \( \mu \text{M} \) HRP in serum-free DMEM with 0.1 mM DTPA for 1 h at 37°C in a 5% CO\(_2\) atmosphere. Cytotoxicity was assayed immediately after the treatments by incorporation of [\( ^{3} \text{H-methyl} \)]thymidine as described in Materials and methods. Values are the means ± SD of one representative experiment done in quadruplicate. *Values of derivatives in the presence and absence of HRP are statistically different (Student’s \( t \)-test, \( P < 0.05 \)).

POBN (21). Hydrazines’ concentrations used were the highest possible before inducing toxicity to PMNs (13). On the other hand, SOD and catalase partially inhibited alkyl radical formation in PMN-activated systems (13). MeH and DMH in the presence of non-activated PMNs display no cytotoxicity to Myc 9E cells at these concentrations (not shown). Also in this system, activation of the hydrazine derivatives to alkyl radicals, correlates with the observed increase in cytotoxicity.

Cytotoxicity of hydrazine derivatives activated by prolonged auto-oxidation

Activation of hydrazine derivatives, by prolonged auto-oxidation in 24-h incubations, was performed in the presence of the iron-chelator, DTPA, to slow down extracellular auto-oxidation and the consequent free radical formation, thus permitting diffusion of the derivatives into the cells before undergoing oxidation. Incubation of Myc 9E cells with increasing concentrations of MeH or DMH for 24 h shows an initial cytotoxic effect at very low concentrations (below 0.5 mM), followed by an enhancement of [\( ^{3} \text{H-methyl} \)]thymidine incorporation between 0.5 and 2.0 mM, and a more intense cytotoxic effect at higher concentrations (above 1 or 2 mM) (Figure 4). Extensive repetition of these experiments have shown the same trend, some of which were statistically significant (Student’s \( t \)-test, \( P < 0.1 \)). These results suggest the occurrence of a proliferative effect of hydrazine derivative auto-oxidation at low concentrations, which interferes with the determination of cytotoxicity induced by these systems. This proliferative phenomenon superimposed on hydrazines’ cytotoxicity has been confirmed, and the involvement of reactive species was characterized using an assay specific for proliferation (54). At concentrations in which the proliferative phenomenon is less important, MeH auto-oxidation in prolonged incubations induces 50% inhibition of [\( ^{3} \text{H-methyl} \)]thymidine uptake at around 3 mM, whereas for DMH this inhibition is observed at ~6 mM (Figure 4). In parallel, a higher concentration of methyl radical-adduct is produced in prolonged auto-oxidations with MeH than with DMH (Table I). Cytotoxicity of DMH and MeH to the parental Balb/c-3T3 cells displayed similar patterns (results not shown), showing that enhanced expression of the \( c-my c \) proto-oncogene is not responsible for the occurrence of the observed proliferative effect nor does it alter hydrazines’ cytotoxicity to mouse fibroblasts. Cell survival assays, although with higher deviations, displayed similar patterns (not shown). Once more a correlation can be established between the degree of cytotoxicity and the formation of alkyl radicals in the metabolism of the hydrazine derivatives.

DNA strand breaks in cells treated with activated hydrazine derivatives

DNA damage was evaluated by precipitation of DNA–protein–K\(^+\)–SDS complex (49), following treatment with the activated
Fig. 3. Cytotoxicity of hydrazine derivatives metabolized by activated PMNs. Subconfluent Myc 9E cells were incubated with hydrazine derivatives in the presence or absence of PMNs (5×10^6 cells/ml) and opsonized Zymosan (0.2 mg/ml) in serum-free DMEM with 0.1 mM DTPA, SOD (25 µg/ml) and catalase (0.2 µg/ml) for 1 h at 37°C in a 5% CO2 atmosphere. Cytotoxicity was assayed immediately after the treatments by incorporation of [3H-methyl]thymidine as described in Materials and methods. Values are the means ± SD of one representative experiment in sextuplicate. *Values statistically different from control with activated PMNs (Student’s t-test, P < 0.05). **Values statistically different from 2 mM DMH/PMNopsZym (Student’s t-test, P < 0.05).

Fig. 4. Cytotoxicity of DMH and MeH activated by auto-oxidation in 24 h incubations. Subconfluent Myc 9E cells were incubated with increasing concentrations of DMH or MeH in serum-free DMEM with 0.1 mM DTPA for 24 h at 37°C in a 5% CO2 atmosphere. Cytotoxicity was assayed immediately after the treatments by incorporation of [3H-methyl]thymidine as described in Materials and methods. Values are the means ± SD of one representative experiment in sextuplicate.

Fig. 5. Cell transformation induced by PCZ/HRP and MeH/HRP treatments. Myc9E cells were subjected to six consecutive treatments with PCZ (0.5 mM) or MeH (2 mM) in the presence or absence of HRP (20 µM) as indicated in the figure and plated at 200 cells/10-cm dish as described in Materials and methods. Cell culture during the assay for formation of transformed colonies was performed in DMEM with 10% FCS and the following additions: (A) none; (B) hydrocortisone (100 ng/ml) and PMA (2×10^-8 M).

Cell transformation induced by PCZ/HRP and MeH/HRP treatment

It can be taken from the cytotoxicity assays that the most convenient system to investigate the contribution of alkyl radicals to the transforming properties of hydrazine derivatives would be with HRP-activation. PMN-activation is a more complex system and has been shown to display transforming properties per se. In addition, activation by prolonged auto-oxidation is complicated by the occurrence of the proliferative effect induced by oxygen radicals generated during the extended incubation periods (54). Since DMH is not activated to alkyl radicals by HRP under these conditions, the transformation assays were carried out with the di-substituted derivative, PCZ, and its mono-substituted pair, MeH, both of which demonstrated methyl radical formation (Figure 1, Table I). The concentrations used (0.5 mM PCZ and 2.0 mM MeH), were chosen due to the very slow recovery of the cells at higher concentrations. Myc 9E cells subjected to six consecutive treatments with PCZ, formed dark-stained colonies of higher cell density than that of control cells (Figure 5A). Six consecutive treatments were performed due to the low transforming effect observed upon one or three treatments (not shown). Transformation induced by PCZ alone can be ascribed to spontaneous formation of reactive intermediates such as azoxy hydrazines. Cells incubated with hydrazine derivatives activated (or not) by HRP for 1 h, show essentially no strand breaks in the concentration range tested, i.e. up to 12 mM MeH and DMH. MeH activation by auto-oxidation in the 24-h incubations displayed no DNA damage up to 10 mM, but at this concentration DMH induces an ~30% increase in DNA solubilization in relation to the untreated controls. However, at 10 mM, MeH or DMH-treated cells did not recover from treatment, as determined by cell survival assays (not shown). Incubation with 200 µM H2O2, used as a positive control, induced 75% solubilization of DNA from Myc 9E cells (not shown). Although DNA strand breaks do not seem to be important for the observed cytotoxicity of activated hydrazine derivatives, other forms of DNA alterations, such as alkylative processes cannot be excluded.
derivatives and hydrazones (16,53). Enzymatic activation by HRP greatly increases this transforming effect (Figure 5A). Microscopic analysis of the colonies formed in control and PCZ/HRP-treated cultures, shows that while the former consists of a normal monolayer, the latter displays clear foci of piled-up transformed cells (not shown). Figure 5A also shows that the methylated mono-substituted derivative, MeH, induced a subtle increase in cell density. Albeit with a lower efficiency as compared with PCZ, this effect was more pronounced in the presence of HRP. As expected, HRP alone displayed no transforming properties (not shown). Parallel treatment of the parental Balb/c-3T3 cells with these systems did not lead to formation of colonies of higher cell density (not shown), which suggests that the effect of hydrazine derivatives are due to cooperation with the c-myc oncogene product.

The glucocorticoid, hydrocortisone, has been shown to enhance (~5-fold) the expression of the transfected c-myc proto-oncogene (46) and the phorbol ester, PMA, cooperates with the ras oncogene, but not with myc (55). Addition of both hydrocortisone and PMA to the culture medium during the assay (but not during the treatments) greatly increases Myc 9E cells’ transformation by metabolically activated MeH or PCZ, but had no significant effect in the absence of HRP (Figure 5B). Hydrocortisone alone uniformly enhanced the plating efficiency of treated and control cells, and led to larger and slightly denser colonies (not shown). PMA alone induces the formation of smaller and denser colonies and enhanced the effect of MeH or PCZ in the presence of HRP, but not in its absence (not shown). These results show that there is an increase in cell transformation induced in Myc 9E cells by PCZ and MeH, in systems in which increased alkyl radical formation is demonstrated, and this strongly suggests their contribution to cell transformation.

Discussion

Since many pharmacologically active hydrazine derivatives currently in use display toxic side effects, it is important to characterize the ultimate cytotoxic and carcinogenic species. Two pathways of metabolic activation have been demonstrated for di-substituted derivatives: one via formation of azoxy derivatives leading to generation of carbocations and the other leading to alkyl radical formation (Scheme 1). Considerable emphasis has been given to the azoxy derivatives and subsequent carbocations formation upon metabolism of DMH and PCZ (20–22). However, the methyl radical has been implicated in the covalent binding of PCZ metabolites to cellular macromolecules, by establishing a correlation with methane formation (56). Furthermore, methyl radical formation was demonstrated by spin-trapping in the metabolism of DMH and PCZ (18,19,51). Nevertheless, cytotoxicity of alkyl radicals generated in hydrazines’ metabolism has not been clearly established yet.

Here we demonstrate that DMH has increased cytotoxicity to mouse fibroblasts upon PMN-catalyzed activation (Figure 3) or prolonged auto-oxidation (Figure 4), both of which metabolism to alkyl radicals is demonstrated, but not upon HRP-catalyzed activation (Figure 2), when increased alkyl radical formation is not observed (Table I). PCZ-induced cytotoxicity is also increased by HRP-catalyzed activation (Figure 2), which leads to enhanced alkyl radical formation (Table I). Although the non-enzymatic formation of trace azoxy derivatives of PCZ in solution has been demonstrated (53), metabolism by HRP should not alter azoxy derivatives formation, which is considered to be catalyzed by cytochrome P-450 (21). Cytotoxicity due to hydrazines per se or to other reactive metabolites such as azoxy derivatives and hydrazones, would be accounted for in the non-activated systems, emphasizing the role of alkyl radicals in the observed increase in cytotoxicity upon hydrazine activation.

Formation of alkyl radicals in the metabolism of mono-substituted hydrazine derivatives by several enzymatic systems has been detected by spin-trapping (10–13,24) and the formation of carbocations is not likely. We have previously determined that the metabolism of PEH by HbO2 (which should follow similar mechanisms as HRP) produced several oxygenated products, all of which were inhibited by the spin-trap, POBN, showing that the alkyl radical was the main reactive species produced (24). Our results demonstrate that MeH and PEH activation by HRP, in which enhanced alkyl radical formation is demonstrated (Table I), leads to increased cytotoxicity to mouse fibroblasts (Figure 2). MeH metabolism by activated PMNs increases its cytotoxicity and this system, which produces a 5-fold higher concentration of methyl radicals (21), is more cytotoxic to mouse fibroblasts than DMH metabolized by activated PMNs (Figure 2). Prolonged auto-oxidation of MeH causes a 10-fold increase in alkyl radical formation (Table I) and substantially increased its cytotoxicity (Figures 2 and 4).

DMH is a potent carcinogen in experimental animals (14) and DNA alkylation in vivo has been demonstrated (15). However, it displays low mutagenicity in the Ames’ test in Salmonella auxotrophs when the drug is activated by S9 mixture (7–9). Cytotoxicity of DMH to human fibroblasts, assessed by the DNA synthesis inhibition test, has also given negative results (44). Sequestration of reactive intermediates by components of the S9 mixture may account for the inhibition of mutagenicity and cytotoxicity. Here we demonstrate cytotoxicity of DMH to mouse fibroblasts upon oxidation by activated PMNs (Figure 3) or when activated by prolonged auto-oxidation (Figure 4), both of which display increased alkyl radical formation [Table I, (13)].

The cytotoxicity of these compounds in the absence of metabolic activation (Figure 1) shows that PEH and PCZ display greater cytotoxicity than MeH and DMH, since a significant decrease in [3H-methyl]thymidine uptake is observed at 1–2 mM for the former pair, but not below 10 mM for the latter pair. These results do not correlate with the low yields of alkyl radical formation (Table I), nor with hydrazine substitution (which could implicate analogous metabolic pathways and common reactive intermediates), nor with their carcinogenic potential (DMH and PCZ are more potent carcinogens). However, the observed cytotoxicity may correlate with the compounds’ hydrophobicity due to aromatic substitutions, which would point to the importance of intracellular drug distribution.

Based on the results obtained in the cytotoxicity assays, we investigated the transforming potential of HRP-activated PCZ and MeH to the normal c-myc-overexpressing cell line, Myc 9E. Cells were subjected to treatments with PCZ or MeH, activated or not by HRP. Cell transformation was evaluated by formation of colonies with higher cell density, confirmed by microscopic analysis. PCZ alone displayed greater transformation potential than MeH (Figure 5), which is in accord with their carcinogenic potency (7). Metabolic activation by HRP increased the transforming potential of PCZ and MeH,
which are conditions that demonstrated increased alkyl radical formation (Figure 5, Table I). Although HRP-activated MeH yields higher levels of alkyl radicals than PCZ in solution, the presence of whole cells levels off radical-adduct formation (Table I). We have demonstrated metabolism of these derivatives to alkyl radicals by HRP, but the general stepwise catalytic reactions either by HRP, the cells themselves or by the combination of both, is not known. The apparent paradox in our results, if one considers that consumption of the derivatives by HRP would produce the reactive species outside the cells (and, therefore, far from the target DNA), is counterbalanced by the possibility of initial oxidative steps occurring outside the cells with internalization of more stable intermediates and final oxidative steps occurring closer to DNA. This would explain why metabolic activation outside the cells increases the transformation efficiency. This is proposed as a mechanism for the toxicity of PCZ to leukemia L1210 cells, since these cells do not metabolize PCZ to toxic species (21). On the other hand, greater production of alkyl radicals in the activation of MeH outside the cells may decrease formation of alkyl radicals close to the target DNA, which could account for the greater transformation levels observed for activated PCZ.

In addition to being involved in the response to oxidative stress, hydrocortisone, which increases the expression of the transfected c-myc proto-oncogene in Myc 9E cells, cooperates with PMA in increasing transformation of these cells upon treatment with HRP-activated hydrazine derivatives. PMA, a phorbol ester known to induce protein kinase C activity (58) and to cooperate with the ras oncogene (55); increased cell transformation particularly in HRP-activated hydrazine derivatives. Activation of a second oncogene, possibly from the ras family, which may be in cooperation with c-myc, is under investigation.

Our results demonstrate transformation of Balb/c-3T3 mouse fibroblasts (Myc 9E transfectant cell line) by mono- and di-substituted hydrazine derivatives under conditions in which increased alkyl radicals formation is demonstrated. Formation of azoxy intermediates, necessary for carbocation production, was not assessed, but these cells do not have significant cytochrome P450 activity (52) (demonstrated to be responsible for azoxy formation) and azoxy derivatives are not proposed as intermediates in mono-substituted hydrazine metabolism. Transformation by the hydrazines per se, or spontaneously formed reactive intermediates such as azoxy derivatives or hydrazones, are accounted for in the treatments with the hydrazines alone, and the role of alkyl radicals becomes evident in the increased transformation observed in the HRP-activated systems. Altogether these results support a role for carbon-centered radicals in the carcinogenic properties of hydrazine derivatives.

In summary, our results show that activation of hydrazine derivatives by HRP increased their cytotoxicity and trans- formation potential in Myc 9E cells only for systems in which increased alkyl radical formation is demonstrated. Taken together, our results strongly support a role for hydrazine-derived alkyl radicals in the cytotoxicity and cell transformation induced by hydrazine derivatives.

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References
Cytotoxicity and transformation by hydrazine derivatives


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