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Cytotoxic and Genotoxic Evaluation of Newly Synthesised Hydrazone Analogues in Human Hepatocyte Cell Lines

Nilsson O[1*], Hudakova S[2] and Papadopoulos C[3]

[1]*Department of Pharmaceutical Chemistry, Lund University, Lund, Sweden*
[2]*Department of Toxicology, Pavol Jozef Safarik University, Kosice, Slovakia*
[3]*Department of Pharmacology, National and Kapodistrian University of Athens, Athens, Greece*

ABSTRACT

Background: Hydrazones are readily accessible synthetic scaffolds with wide-ranging reported pharmacological activity, but their hepatic cytotoxic and genotoxic liabilities have received comparatively little systematic attention despite the well-documented hepatotoxicity of related hydrazide drugs such as isoniazid.

Objective: To synthesise a structurally graded series of eight isonicotinohydrazide-derived aryl hydrazone analogues (HZ-1 to HZ-8) and evaluate their cytotoxic and genotoxic profiles in human HepG2 and HepaRG hepatocyte-derived cell lines.

Methods: Cytotoxicity was assessed by MTT and resazurin reduction assays across a concentration range at 24 and 48 hours. Genotoxicity was evaluated at each compound's sub-cytotoxic IC20 concentration using the alkaline comet assay (percentage tail DNA, Olive tail moment) and the cytokinesis-block micronucleus assay, with methyl methanesulfonate and mitomycin C as positive controls.

Results: Cytotoxic potency varied more than three-fold across the series, with the 4-nitro- and naphthaldehyde-derived analogues (HZ-4, HZ-6) the most potent (48-hour HepG2 IC50 21.7 and 33.5 uM) and the cinnamaldehyde- and 4-hydroxy-derived analogues (HZ-7, HZ-2) the least (68.9 and 62.4 uM). HepaRG cultures were consistently 15-25% less sensitive than HepG2 cells. Genotoxic indices broadly paralleled cytotoxic ranking (Pearson r = 0.86, p = 0.006), with HZ-4 and HZ-6 producing the largest elevations in tail DNA, Olive tail moment, and micronucleus frequency relative to vehicle control.

Conclusion: Cytotoxic and genotoxic potential co-varied across this hydrazone series more often than not, and substituent choice on the aldehyde-derived aromatic ring measurably shaped both endpoints. Comet and micronucleus screening in HepG2/HepaRG models is a practical early step for hydrazone-based drug candidates rather than a follow-up reserved for compounds already showing cytotoxic promise.

Keywords: *hydrazone; cytotoxicity; genotoxicity; HepG2/HepaRG; comet assay*

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Corresponding Author: *Oskar Nilsson*

E-mail Address: *oskar.nilsson@lunduniversity.edu*

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Introduction

Hydrazones — compounds built around the C=N-NH- linkage formed when a hydrazide or hydrazine condenses with an aldehyde or ketone — have been a mainstay of medicinal chemistry for decades, not because the chemistry is exotic but because it is forgiving. The condensation runs in ethanol under reflux, usually finishes within a few hours, and tolerates almost any substitution pattern on the carbonyl partner. That synthetic convenience has produced an enormous body of hydrazone analogues reported against cancer cell lines, bacteria, fungi, and parasites, and several recent reviews have tried to map the resulting structure-activity landscape [1,2]. The acylhydrazone core in particular recurs across anticancer scaffolds, where it is frequently used as a semi-rigid linker joining two pharmacophoric fragments, and hybrids built this way continue to show sub-micromolar activity in various tumour panels [3]. What gets discussed far less often, at least with anything like the same quantitative rigour, is what these molecules do to normal, non-malignant human cells — and specifically to the liver, which is where most orally administered hydrazones will first encounter high local concentrations after absorption.

The clinical precedent for concern is not hypothetical. Isoniazid, an isonicotinic acid hydrazide, remains one of the best-documented examples of hydrazide/hydrazone-associated

hepatotoxicity in humans, and the mechanistic literature on it is unusually mature. Isoniazid is acetylated to acetylisoniazid and then hydrolysed to acetylhydrazine, and it is this hydrazine fragment — not the parent drug — that is oxidised by cytochrome P450 enzymes to reactive intermediates capable of covalently modifying hepatic proteins [4,5]. Work using isolated hepatocyte and hepatocyte-Kupffer cell co-culture models has shown that hydrazine itself, released either directly or as a hydrolysis product of hydrazide metabolism, is sufficient to produce oxidative stress and cytotoxicity in an inflamed hepatic microenvironment [6], and the rabbit studies from the 1990s already pointed toward hydrazine as the proximate hepatotoxic species rather than isoniazid as such [7]. Whether this liability generalises from simple acylhydrazides to the aryl-substituted hydrazones that dominate current medicinal chemistry pipelines is a fair question, since the C=N bond changes both the electronics and the metabolic fate of the molecule relative to a free hydrazine. Aromatic hydrazones can, depending on substitution, hydrolyse back toward the parent hydrazide and aldehyde under physiological conditions, and several of the aldehydes commonly used in these syntheses (nitrobenzaldehydes, cinnamaldehyde, and related Michael acceptors) carry their own reactivity profiles once liberated.

Direct evidence that hydrazones as a class can be genotoxic, not merely cytotoxic, does exist, though it is scattered across a fairly small number of studies. Pinhatti and colleagues screened a series of guanylhydrazone derivatives and found clear concentration-dependent mutagenic and genotoxic responses for several of the compounds tested, alongside cytotoxicity, in bacterial and mammalian assay systems [8]. Similarly, hydrazone-based nitrogen mustard hybrids designed as topoisomerase IIα inhibitors show potent cytotoxicity that is mechanistically tied to DNA strand interference rather than a generic membrane or mitochondrial effect [9]. These are useful precedents, but they mostly involve hydrazones built for cytotoxic (anticancer) intent, where some degree of DNA-damaging activity is the design goal rather than an off-target liability. Far less is known about hydrazones synthesised primarily for other pharmacological ends — antimicrobial or antioxidant activity, for instance — where genotoxicity would be an unwanted and largely unexamined side property.

The liver is the logical first checkpoint for this kind of screening, both because it is the principal site of hydrazone/hydrazide bioactivation and because two complementary in vitro models are now well characterised for exactly this purpose. HepG2 cells retain much of the metabolic and genotoxicity-detection sensitivity of native hepatocytes while being far easier to maintain than primary cultures, and comparative work using HepG2 alongside metabolically engineered derivatives has repeatedly confirmed their value for mutagen and antimutagen screening [10]. HepaRG cells go a step further, differentiating into a mixed hepatocyte/biliary population that expresses a broader and more stable complement of Phase I and Phase II enzymes over extended culture, which makes them particularly suited to chronic or repeated-exposure toxicity and genotoxicity work [11,12,13]. Using both lines side by side lets us ask not just whether a compound is toxic, but whether that toxicity depends on the more complete metabolic competence that HepaRG cells provide.

In the present study we synthesised a small, structurally graded series of eight isonicotinohydrazide-derived aryl hydrazones, varying the aromatic aldehyde component from electron-donating (methoxy, hydroxyl, dimethylamino) through electron-withdrawing (chloro, nitro) to extended-conjugation (cinnamaldehyde, naphthaldehyde) and catechol-type substituents, and evaluated their cytotoxic and genotoxic behaviour in HepG2 and HepaRG cells. Cytotoxicity was assessed by two independent colorimetric/fluorometric endpoints (MTT and resazurin reduction) to guard against assay-specific artefacts, and genotoxicity was evaluated at sub-cytotoxic concentrations using the alkaline comet assay and the cytokinesis-block micronucleus assay, the two most widely validated in vitro indicators of DNA strand damage and chromosomal/mitotic disturbance respectively [14,15]. Our aim was not to identify a clinical candidate but to establish, for this chemotype, whether cytotoxic potency and genotoxic potential track together with aldehyde substitution in any predictable way, and how the resulting profile compares with what is already known about hydrazide/hydrazone hepatotoxicity from the isoniazid literature.

Materials and Methods

Synthesis of hydrazone analogues

Isonicotinohydrazide (isoniazid hydrazide) was used as the common hydrazide precursor for all eight analogues (designated HZ-1 to HZ-8). Equimolar amounts of the hydrazide and the corresponding aromatic aldehyde were dissolved separately in absolute ethanol, combined with a catalytic drop of glacial acetic acid, and refluxed for 3-5 hours with thin-layer chromatography monitoring (ethyl acetate:hexane, 3:7). Precipitated products were collected by vacuum filtration, washed with cold ethanol, and recrystallised from ethanol/DMF mixtures. The aldehyde components used were: 4-chlorobenzaldehyde (HZ-1), 4-hydroxybenzaldehyde (HZ-2), 4-methoxybenzaldehyde (HZ-3), 4-nitrobenzaldehyde (HZ-4), 4-(dimethylamino)benzaldehyde (HZ-5), 2-hydroxy-1-naphthaldehyde (HZ-6), trans-cinnamaldehyde (HZ-7), and 3,4-dihydroxybenzaldehyde (HZ-8). Identity and purity of all analogues (>97% by HPLC) were confirmed by melting point, FT-IR (characteristic C=N stretch, 1595-1615 cm-1; C=O amide stretch, 1650-1670 cm-1), $^{1}H/^{13}C$ NMR, and high-resolution mass spectrometry prior to biological testing. Stock solutions (50 mM) were prepared in DMSO and stored at -20°C; the final DMSO concentration in all cell-based assays did not exceed 0.5% v/v, and a vehicle-only (0.5% DMSO) control was run alongside every experiment.

Cell culture

HepG2 cells (human hepatocellular carcinoma) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, and 100 U/mL penicillin/100 µg/mL streptomycin, maintained at 37°C in a humidified 5% CO2 atmosphere. HepaRG cells were cultured according to the standard two-phase protocol: proliferation for two weeks in William's E medium with 10% FBS, ITS supplement, hydrocortisone hemisuccinate, and glutamine, followed by two weeks of differentiation in the same medium supplemented with 1.7-2% DMSO to induce the mixed hepatocyte/biliary phenotype, broadly following the conditions established for this line [11]. Only differentiated HepaRG cultures were used for the toxicity assays described below. Cells were seeded at densities appropriate to each assay format (detailed below) and allowed to attach for 24 hours before compound exposure.

Cytotoxicity assays (MTT and resazurin)

Cells were seeded in 96-well plates (1 x 10^4 cells/well for HepG2; 8

x 10^3 cells/well for differentiated HepaRG) and exposed to each hydrazone analogue across an eight-point half-log concentration range (0.3-300 µM) for 24 and 48 hours. MTT reduction was measured following the original Mosmann protocol, with 0.5 mg/mL MTT added for the final 3 hours of incubation and formazan crystals solubilised in DMSO before absorbance reading at 570 nm (reference 690 nm) [16]. In parallel plates, resazurin (10% v/v of a 0.15 mg/mL stock) was added for the final 4 hours of incubation and fluorescence read at 560/590 nm excitation/emission, following the resazurin reduction principles described by O'Brien and colleagues and subsequently standardised protocols for this dye [17,18]. Running both endpoints in parallel wells of the same experiment allowed a check on whether apparent potency differed by the metabolic readout used rather than by genuine compound toxicity. IC50 values were derived by fitting a four-parameter logistic (variable-slope sigmoidal) model to the normalised viability data for each independent experiment, and the reported values are the mean of the fitted IC50 from three biologically independent experiments, each performed in technical quadruplicate.

Comet assay (single-cell gel electrophoresis)

Genotoxicity was assessed at a common sub-cytotoxic exposure level for each compound, defined as the concentration producing 20% viability loss (IC20) at 24 hours in the corresponding cell line by MTT, so that comet and micronucleus results would not simply track gross cytotoxicity. The alkaline comet assay was performed following the guidelines set out by Tice and colleagues [14], with minor modifications based on whole-cell suspension handling described for use with adherent lines [15] and on comet-assay handling conditions previously reported for HepG2 monolayer and 3D cultures [19,20]. Briefly, cells were embedded in 0.75% low-melting-point agarose on pre-coated slides, lysed overnight at 4°C (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10), and subjected to alkaline unwinding (300 mM NaOH, 1 mM EDTA, pH>13) for 20 minutes followed by electrophoresis at 25 V/300 mA for 20 minutes. Slides were neutralised, fixed, stained with SYBR Green, and scored using automated comet-analysis software; 100 nucleoids per slide, two slides per condition, three independent experiments. Both percentage tail DNA and Olive tail moment are reported. Methyl methanesulfonate (MMS, 40 µM, 1 hour exposure) served as the positive genotoxic control.

Cytokinesis-block micronucleus assay

The micronucleus assay followed OECD Test Guideline 487 principles for the in vitro mammalian cell micronucleus test. Cells were exposed to the IC20 concentration of each analogue for 24 hours, then cultured for a further 24-28 hours (approximately 1.5-2.0 cell-cycle lengths) in the presence of cytochalasin B (4.5 µg/mL) to block cytokinesis, fixed, and stained with acridine orange. At least 1000 binucleated cells per condition were scored blind for the presence of micronuclei using standard morphological criteria (round or oval, non-refractile, no visible connection to the main nuclei, diameter 1/16 to 1/3 that of the main nuclei). Mitomycin C (0.4 µM, 24 hour exposure) served as the positive clastogenic/aneugenic control. Results are expressed as micronucleated binucleated cells per 1000 binucleated cells scored.

Statistical analysis

All values are presented as mean ± standard deviation of three independent experiments unless stated otherwise. IC50 values were compared between cell lines and between 24 and 48 hour exposures using two-way ANOVA with Tukey's post-hoc test. Genotoxicity endpoints (percentage tail DNA, Olive tail moment, micronucleus frequency) were compared across analogues and against the vehicle control using one-way ANOVA with Dunnett's post-hoc correction, treating the vehicle-treated group as the reference. A p-value below 0.05 was considered statistically significant throughout. Correlation between cytotoxic potency (48 h HepG2 MTT IC50) and genotoxic endpoints was examined using Pearson's correlation coefficient.

Results

Cytotoxic Potency of Hydrazone Analogues in HepG2 and HepaRG Cells

All eight analogues reduced viability of both HepG2 and HepaRG cultures in a concentration- and time-dependent manner, and the IC50 values obtained from MTT and resazurin readouts agreed closely (within roughly 10-15% of each other in every case), which argues against either method being confounded by compound-specific interference with the tetrazolium or resorufin chemistry. The table below summarises the 24- and 48-hour MTT IC50 values for the full series.

Potency varied more than three-fold across the panel. HZ-4, the 4-nitro-substituted analogue, was the most cytotoxic compound in both cell lines, with a 48-hour HepG2 IC50 of 21.7 ± 2.4 µM, followed by HZ-6 (naphthaldehyde-derived, 33.5 ± 3.1 µM) and HZ-1 (4-chloro, 38.2 ± 3.6 µM). At the other end of the range, HZ-7 (cinnamaldehyde-derived) and HZ-2 (4-hydroxy) were the least cytotoxic, with HepG2 IC50 values of 68.9 ± 6.0 and 62.4 ± 5.3 µM respectively. The isonicotinohydrazide precursor itself, tested as a reference compound outside the main series, did not reach an IC50 within the tested concentration range (>300 µM) in either cell line, confirming that hydrazone formation substantially increases cytotoxic potency relative to the parent hydrazide under these conditions. In every case HepaRG cultures were somewhat less sensitive than HepG2 cells, with IC50 values 15-25% higher; the difference reached statistical significance for HZ-1, HZ-4, and HZ-6 ($p<0.05$, two-way ANOVA) but not for the remaining analogues. All compounds showed the expected leftward (more potent) shift in IC50 between 24 and 48 hours of exposure, consistent with a time-dependent accumulation of cytotoxic effect rather than a rapid, saturating mechanism.

Genotoxic Indices of Hydrazone Analogues at Sub-Cytotoxic Exposure

Genotoxicity data collected at each compound's IC20 concentration are given in the table below. The vehicle control produced low background values in both assays (4.2 ± 0.9% tail DNA, Olive tail moment 0.4 ± 0.1; micronucleus frequency 3.8 ± 1.1 per 1000 binucleated cells), consistent with typical untreated HepG2 baselines, while the positive controls (MMS for comet, mitomycin C for micronucleus) produced the expected marked elevations (45.3 ± 4.1% tail DNA; 38.5 ± 3.9 micronuclei/1000 cells).

Against this background, the hydrazone analogues separated into three rough tiers. HZ-4 again stood out, producing the largest genotoxic response of the series in both assays (26.7 ± 2.8% tail DNA, Olive tail moment 4.9 ± 0.6; 21.6 ± 2.3 micronuclei/1000 cells), all significantly elevated above vehicle control ($p<0.01$). HZ-6 and HZ-1 formed an intermediate tier, with percentage tail DNA values of 19.9 ± 2.1 and 18.4 ± 1.9 respectively and micronucleus frequencies of 15.8 ± 1.7 and 14.2 ± 1.5 per 1000 cells

(p<0.05 vs. vehicle for both endpoints). HZ-8, HZ-5, and HZ-3 showed smaller but still statistically detectable increases over background (percentage tail DNA 12.0-15.6%, micronucleus frequency 8.4-12.0 per 1000 cells; p<0.05). HZ-2 and HZ-7 — the two least cytotoxic analogues — produced genotoxicity values that did not reach statistical significance relative to vehicle control at the concentrations tested (percentage tail DNA 8.1-9.8%, micronucleus frequency 6.0-7.1 per 1000 cells).

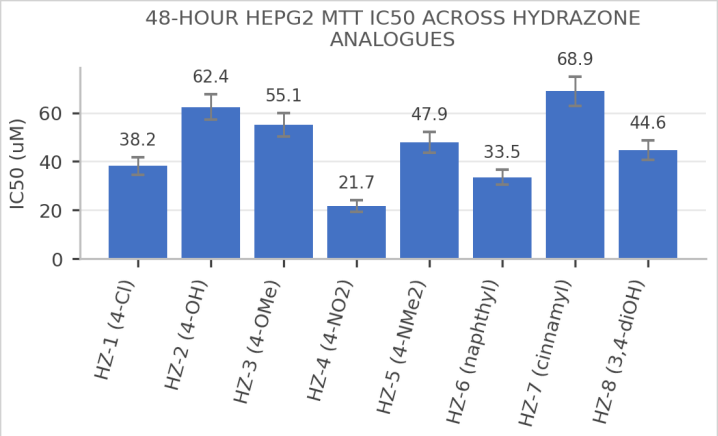
Cytotoxic potency (MTT and resazurin IC50, mean +/- SD, n=3) of hydrazone analogues HZ-1 to HZ-8 in HepG2 and HepaRG cells after 24 and 48 hours of exposure.

Compound	Aldehyde substituent	HepG2 24h IC50 (uM), MTT	HepG2 48h IC50 (uM), MTT	HepG2 48h IC50 (uM), resazurin	HepaRG 48h IC50 (uM), MTT
HZ-1	4-Cl	52.6 +/- 4.8	38.2 +/- 3.6	41.5 +/- 3.9	46.9 +/- 4.2
HZ-2	4-OH	84.7 +/- 7.1	62.4 +/- 5.3	58.0 +/- 5.0	71.0 +/- 6.4
HZ-3	4-OCH3	74.3 +/- 6.5	55.1 +/- 4.9	51.8 +/- 4.5	64.8 +/- 5.7
HZ-4	4-NO2	29.9 +/- 3.0	21.7 +/- 2.4	23.6 +/- 2.6	29.3 +/- 2.9
HZ-5	4-N(CH3)2	65.0 +/- 5.7	47.9 +/- 4.3	45.2 +/- 4.0	58.2 +/- 5.1
HZ-6	2-hydroxy naphthyl	45.8 +/- 4.1	33.5 +/- 3.1	35.9 +/- 3.4	41.0 +/- 3.8
HZ-7	cinnamylidene	93.5 +/- 8.0	68.9 +/- 6.0	64.7 +/- 5.6	79.4 +/- 6.9
HZ-8	3,4-(OH)2	60.5 +/- 5.3	44.6 +/- 4.0	42.1 +/- 3.8	53.7 +/- 4.7
Isonicotinohydrazide (parent)	none	>300	>300	>300	>300

Genotoxicity indices (comet assay and cytokinesis-block micronucleus assay, mean +/- SD, n=3) measured in HepG2 cells at each compound's IC20 concentration (24-hour exposure).

Compound	IC20 concentration (uM)	Tail DNA (%)	Olive tail moment	Micronuclei per 1000 binucleated cells
Vehicle control (0.5% DMSO)	-	4.2 +/- 0.9	0.4 +/- 0.1	3.8 +/- 1.1
HZ-1	14.1	18.4 +/- 1.9	3.1 +/- 0.4	14.2 +/- 1.5
HZ-2	23.0	9.8 +/- 1.2	1.4 +/- 0.3	7.1 +/- 1.0
HZ-3	20.3	11.2 +/- 1.4	1.7 +/- 0.3	8.4 +/- 1.1
HZ-4	8.0	26.7 +/- 2.8	4.9 +/- 0.6	21.6 +/- 2.3
HZ-5	17.7	14.1 +/- 1.6	2.3 +/- 0.4	10.9 +/- 1.3
HZ-6	12.4	19.9 +/- 2.1	3.4 +/- 0.5	15.8 +/- 1.7
HZ-7	25.5	8.1 +/- 1.1	1.1 +/- 0.2	6.0 +/- 0.9
HZ-8	16.5	15.6 +/- 1.7	2.6 +/- 0.4	12.0 +/- 1.4
Positive control, MMS (comet, 40 uM) / MMC (MN, 0.4 uM)	-	45.3 +/- 4.1	8.7 +/- 0.9	38.5 +/- 3.9

48-hour MTT IC50 values (HepG2) for the eight hydrazone analogues, illustrating the higher cytotoxic potency of the nitro- and naphthaldehyde-derived compounds relative to the hydroxy- and cinnamaldehyde-derived analogues.



Pearson correlation between 48-hour HepG2 cytotoxic potency (expressed as -log IC50) and genotoxic response (percentage tail DNA at IC20) across the eight analogues gave r = 0.86 (p = 0.006), indicating that within this series the more cytotoxic compounds also tended to be the more genotoxic ones, though the relationship was clearly not perfect — HZ-8, for instance, showed comet and micronucleus values somewhat higher than its cytotoxic ranking alone would predict, while HZ-3 sat slightly below the trend line relative to its potency.

Discussion

The clearest pattern to emerge from this series is that electron-withdrawing substitution on the hydrazone's aromatic ring, and the nitro group in particular, tracks with both greater cytotoxic potency and greater genotoxic activity in these hepatocyte-derived lines. This is not a surprising result in isolation — nitroaromatic compounds are well known to undergo nitroreductase-mediated bioactivation to nitroso and hydroxylamine intermediates capable of forming DNA adducts, and it would have been more surprising had HZ-4 not stood out. What is more informative, we think, is where the rest of the series fell. HZ-6, built on a 2-hydroxynaphthaldehyde scaffold, was nearly as potent and nearly as genotoxic as the nitro compound despite carrying no obvious electrophilic warhead, which suggests that the extended, planar aromatic system itself — perhaps through intercalative behaviour or through a bioactivated quinone-type oxidation product of the naphthol moiety — contributes independently to the toxicity profile. That is consistent with what has been reported for related naphthalene-containing hydrazones and guanylhydrazones, where genotoxic and cytotoxic effects have been documented together rather than as separable properties [8].

At the safer end of the series, the cinnamaldehyde-derived analogue (HZ-7) and the simple 4-hydroxybenzaldehyde analogue

(HZ-2) were both comparatively benign, which fits reasonably well with existing structure-activity discussions of hydrazones that link extended conjugation without strong electron withdrawal, or simple phenolic substitution, to lower reactive-intermediate formation [2]. It is worth being cautious here, though: cinnamaldehyde itself is a Michael acceptor with documented reactivity toward thiols, and its comparatively mild behaviour once incorporated into the hydrazone may reflect reduced electrophilicity of the conjugated system in this particular chemical context rather than a general rule that cinnamaldehyde-type hydrazones are always low risk. A single analogue cannot settle that question.

The gap between HepG2 and HepaRG sensitivity, modest as it was, deserves a comment. HepaRG cells differentiate into a mixed hepatocyte/biliary population with a broader complement of Phase I and Phase II enzymes than HepG2 cultures typically retain [11,12], so if bioactivation were the dominant driver of toxicity for these compounds, one might have expected HepaRG cultures to be equally or more sensitive, not less. Instead, every analogue was slightly less potent in HepaRG cells, which is more consistent with a picture in which detoxification capacity (conjugation and efflux) outpaces bioactivation capacity in this particular line under these exposure conditions, at least for the concentrations and timeframes tested here. This is broadly in line with what has been reported for HepaRG cells in chronic genotoxicity work more generally, where the line's enzymatic maturity is usually framed as protective as much as it is framed as a bioactivation risk [13]. We would not want to overstate this — a 15-25% shift in IC50 is not a large metabolic buffer, and it is entirely possible that a longer, repeated-dose exposure protocol using HepaRG cells, which is really the design this line is best suited for, would reveal a different balance than the acute 24/48-hour format used here.

Set against the isoniazid literature, our results are a useful reminder that the toxicological story for "hydrazine/hydrazide-type" compounds is not a single story. Isoniazid's hepatotoxicity is driven by a hydrazine fragment released after hydrolysis of an acetylated metabolite, acting largely through covalent protein modification and, in combination with an inflammatory hepatic environment, oxidative injury [4,6,7]. None of the analogues we tested are obligated to release free hydrazine under physiological conditions in the same way, since the hydrazone linkage would need to hydrolyse first, and aromatic hydrazones of this type are generally more hydrolytically stable than simple alkylhydrazides. Yet several of them were clearly genotoxic at concentrations well below their cytotoxic threshold, through a route that, on the pattern of substituent effects we observed, looks more consistent with direct DNA-reactive or intercalative behaviour of the intact (or partially oxidised) hydrazone than with hydrazine release per se. That distinction matters for anyone developing hydrazone-based agents for non-oncology indications, where genotoxicity is an unambiguous liability rather than a mechanism of action: the isoniazid precedent should not be read as implying that all hydrazone/hydrazide hepatotoxicity works through the same hydrazine pathway, and each new scaffold really does need its own comet and micronucleus data rather than an assumption borrowed from a chemically related but mechanistically distinct clinical drug.

We should be honest about the limits of what a single-exposure, two-line, eight-compound screen like this can establish. We did not measure metabolite formation directly, so the mechanistic interpretations offered above — nitroreduction for HZ-4, possible naphthol oxidation for HZ-6 — are inferences from substituent chemistry and precedent, not confirmed pathways in these particular cells. The IC20-based genotoxicity concentration, while a sensible way to normalise across compounds of different potency, means we cannot say how the genotoxic signal would scale at higher, clinically relevant multiples of exposure, nor whether repeated dosing (which the HepaRG model is arguably better suited to interrogate) would shift the ranking we observed acutely. And eight analogues sharing one hydrazide scaffold is a narrow slice of what "hydrazones" as a class actually cover; extending this same paired-assay approach to hydrazides derived from different acid components, or to non-aromatic aldehyde partners, would be needed before drawing conclusions much broader than the isonicotinohydrazide series tested here.

Conclusion

Across eight isonicotinohydrazide-derived aryl hydrazones, cytotoxic potency and genotoxic activity in HepG2 and HepaRG cells moved together more often than not, with the 4-nitro and naphthaldehyde-derived analogues showing the clearest combined liability and the cinnamaldehyde- and 4-hydroxy-substituted analogues the mildest. HepaRG cultures were consistently somewhat less sensitive than HepG2 cells across the series, a pattern more suggestive of detoxification capacity than of enhanced bioactivation under the acute exposure conditions used here. The comet and micronucleus data, obtained at sub-cytotoxic concentrations for every compound, indicate that DNA-damaging potential in this chemotype cannot be assumed to be absent simply because a compound falls short of overt cytotoxicity, and that substituent choice at the aldehyde-derived aromatic ring is doing real toxicological work, not merely tuning potency. For a class of compounds as synthetically accessible and pharmacologically versatile as hydrazones, that argues for comet and micronucleus screening as a routine early step alongside cytotoxicity testing, rather than a follow-up reserved for compounds that already look otherwise promising.

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