Imine resveratrol analogs (IRAs): The strong antioxidant that can protect lymphocytes from oxidative damage

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Abstract

Imine resveratrol analogs (IRAs) are promising new agents that can have higher positive effects and, simultaneously, lower negative properties than resveratrol. In this study, three imine hydroxy derivatives (2-((4-hydroxyphenylimino)methyl)phenol [IRA1], 3-((4-hydroxyphenylimino)methyl)phenol [IRA2], and 4-((4-hydroxyphenylimino)methyl)phenol [IRA3]) were prepared and tested in several biological assays. They performed superior to resveratrol in several antioxidant and biological assays, showing high antioxidant capacity and low genotoxicity. Ferric reducing antioxidant power assay (FRAP) and hydroxyl radicals scavenging assay revealed good Fe3+ to Fe2+ reduction and strong inhibition of hydroxyl radical formation, respectively. High dosage (1 mmol/dm3) of IRA2 and IRA3 did not cause genotoxicity in human lymphocytes. Moreover, lymphocytes pretreated with all three IRAs accumulated only very few DNA breaks induced by H2O2 than lymphocytes pretreated with resveratrol. Additionally, the number of detected DNA breaks appearing after removal of damaged DNA bases, 8-oxo-7,8-dihydroguanine (8-oxoG), did not dramatically increase in lymphocytes treated with IRA2. Thus, we concluded that IRAs, especially IRA2, are strong antioxidants with the ability to protect lymphocytes from oxidative damage.

KEYWORDS
antioxidants, Comet assay, DNA repair, imine resveratrol analogs (IRAs), human lymphocytes

1 | INTRODUCTION

The combination of science, Mediterranean cuisine, and traditional medicine shows a great potential of natural compounds in the prevention or treatment of many diseases. The stability of natural plant extracts and their bioavailability for the human organism is still an immense problem. However, these compounds may be a paradigm for the synthesis of precisely chemically defined substances that chemical and biological properties can be easily tested. Additionally, modifications, improving their beneficial features, can be introduced as well.
One of the thoroughly studied natural compounds is resveratrol. This phytoalexin occurs mainly in grapes, peanuts, berries, and red wine. A plethora of studies showed its biological significance as an antioxidant, anticancer, chemopreventive, and anti-inflammatory agent. Resveratrol has its role in immunomodulation, cardiovascular and neurodegenerative protection. It can serve as an antiviral and antimicrobial compound. The activity of resveratrol is structure-dependent. To improve resveratrol's biological properties, different resveratrol analogs have been produced by several groups.

Recently, resveratrol analogs drew attention due to their antioxidant properties. It was shown that some resveratrol analogs were able to reduce the production of reactive oxygen species (ROS) in breast epithelial cells. Sueishi et al. found that resveratrol analogs, for example, piceatannol, can scavenge ROS [HO·, O2·−, RO·, 1O2] and methyl radical. Moreover, piceatannol exhibited 11 times higher scavenging ability against peroxy radical than resveratrol.

Imine resveratrol analogs (IRAs) are another interesting group of resveratrol analogs. Šeršňová et al. showed that hydroxy-substituted aromatic bisimines, 4,4′-bis (dihydroxybenzylanilideneamino) diphenylmethanes or diphenyl ethers, with two OH groups in positions 2 and 5 are better scavengers of 2,2-diphenyl-1-pircylylhydrazyl (DPPH) radicals than resveratrol. The ones with OH groups in positions 2 and 4 did not scavenge DPPH. On the other hand, studied bisimines did not scavenge hydroxyl radicals. Lu et al. tested 25 IRAs to scavenge DPPH and their quenching capacity of 1O2. The authors found out that scavenging property strongly depends on the OH group position on both aromatic rings. In some cases, better scavenging activity compared to resveratrol was observed. In our previous work, 21 IRAs were examined for their ability to scavenge DPPH, ABTS, and galvinoxyl radicals. Some IRAs were better scavengers of DPPH and galvinoxyl radicals as parent resveratrol, but resveratrol was the better scavenger of ABTS radicals.

Besides the improved antioxidant performance, resveratrol analogs are important for their biological functions. IRAs with different functional groups displayed anticancer activity against HeLa cells and low cytotoxicity against normal HEK293 cells. IRAs derived from 2-[[2-hydroxyphenyl]methylene]amino]phenol showed an anti-proliferative effect against the colorectal carcinoma cell line HCT-116wt carrying wild-type p53. Anticolitis effects were demonstrated for 3,4,5,4-tetramethoxystilbene. Zimerman-Franco et al. studied 6 IRAs that were more effective DPPH scavengers than resveratrol and showed low cytotoxicity. They exhibited anti-inflammatory and immunomodulatory activity. No less interesting findings showed the interaction of IRAs with sirtuins, thus exhibiting antiaging activity. Others have antileishmanial activity against promastigotes and amastigotes.

This study aimed to evaluate the antioxidant capacity, potential genotoxicity, and DNA protective effects of three resveratrol imine analogs:

- 2-((4-hydroxyphenylimino)methyl)phenol (IRA1); 3-((4-hydroxyphenylimino)methyl)phenol (IRA2); and 4-((4-hydroxyphenylimino)methyl)phenol (IRA3) (Figure 1) and detect their ability to modulate cell response to mutagen-induced damage. The genotoxic effect was tested as a capability to induce direct DNA breaks. Additionally, we employed formamidopyrimidine DNA glycosylase (FPG) to uncover the potential genotoxic effect of IRAs introduced via modification of DNA nitrogenous bases. FPG is one of the first components of complex repair mechanisms called base excision repair (BER). The enzyme recognizes and cuts out oxidatively damaged guanosine form, 8-oxo-7,8-dihydroguanine (8-oxoG) and thus introducing ssDNA breaks. The ssDNA breaks are later repaired via other enzymes involved in BER.

We tested the hypothesis that changes in the original resveratrol structure can deliver strong antioxidants with little or no genotoxicity at all towards human lymphocytes. Investigation of these analogs can bring new insights into the relationship between the structure and biological properties, including adverse effects.

## MATERIALS AND METHODS

### 2.1 General information

Imine analogs of resveratrol were prepared similarly as described in our previous work by Kotora et al. Briefly, equimolar amounts of corresponding aldehyde and 4-hydroxybenzamine were stirred on a magnetic stirrer at room temperature in distilled water for 0.5–4 h. The precipitated product was filtered off, washed with cold water, and dried at 45°C. The yield was between 38% and 84%. All spectral
characteristics were in good agreement with previously published results.[22]

List of used chemicals: Hepes and Triton-X100 (AppliChem GmbH, BRD); plasmid pBR322 (BioLabs); K2HPO4 Puriss p.a. grade (Centralchem); FeSO4·7H2O Puriss p.a. grade, NaH2PO4 Puriss p.a. grade, dimethyl sulfoxide (DMSO) Puriss p.a. grade and thiourea Puriss p.a. grade (Lachema Czech Republic); redgel (EUROLAB LAMBDA, a.s.); KH2PO4; Na2HPO4·12H2O Puriss p.a. grade, KCl Puriss p.a. grade, NaCl Puriss p.a. grade, NaOH Puriss p.a. grade, EDTA Na2·H2O Puriss p.a. grade and CuSO4·7H2O Puriss p.a. grade (LPP); Ethidium bromide (MP Biomedicals, Inc.); Agarose Low Melting Point and Agarose Normal Melting Point (Carl Roth GmbH, BRD); FeCl3 Puriss p.a. grade (Reanal Labor); l-ascorbic acid cryst. research-grade and cytochrome c (SERVA Electrophoresis GmbH, BRD); sodium citrate tribasic dihydrate, Histopaque-1077; H2O2 30%, glycerol 99%, BSA 96%, K3Fe(CN)6, trichloroacetic acid (TCA) resveratrol, 99%, gallic acid (GA), FPG 90%, Histopaque-1077, bovine serum albumin (BSA) (Sigma-Aldrich GmbH, BRD); ethanol 99% (Slavus).

List of used instruments: spectral scanning multimode reader Varioskan Flash (Thermo Fisher Scientific); Centrifuge Eppendorf 5403 (Eppendorf Czech & Slovakia s.r.o.); Olympus BX 51 Fluorescent Microscope (Olympus Deutschland GmbH).

In all experiments, the 1 M and 10 mM stock solutions of resveratrol and its analogs, respectively, dissolved in 100% DMSO, were used. To obtain a working concentration, the stock solution was diluted in 1×phosphate-buffered saline (PBS) depending on the used assay.

2.2 | Ferric reducing antioxidant potential (FRAP) assay

The reduction power of studied compounds was estimated based on the ability to reduce Fe3+ ferricyanide complex into Fe2+ ferrocyanide complex according to the work of Zhao.[23] The method is based on a color change from yellow Fe3+ ferricyanide to blue-green Fe3+ ferrocyanide complexes after the electron-donating action of antioxidants. The reduction was monitored by measuring the change of absorbance at 700 nm. All solutions were freshly prepared before the use and mixed as follows: 200 µl of a studied compound at a different concentration, or GA as a positive control the (10 mM ethanol stock solution), was added to 500 µl of 0.2 M potassium phosphate buffer (pH = 6.6) and 500 µl of potassium ferricyanide (1% [w/v] dH2O solution) and mixed. Prepared samples were incubated for 20 min at 50°C. Afterward, 500 µl of trichloroacetic acid (TCA) (10% [w/v] dH2O solution) was added to each tube and centrifuged at 3000 rpm, for 10 min at room temperature (RT). Subsequently, 500 µl of the upper layer of supernatant was mixed with 500 µl of dH2O and 100 µl of FeCl3 (0.1% [w/v] dH2O solution). The absorbance of the samples was measured at 700 nm.

All measurements were calculated from the value obtained from triplicate assays.

2.3 | Hydroxyl radicals scavenging assay

The scavenging of hydroxyl radicals was carried out as described in Liu et al.[24] Briefly, *OH radicals were generated in Cu2+/ascorbic acid solution as a result of Cu2+ reduction. The generated *OH radicals oxidize cytochrome C resulting in a changed absorbance of the solution at 550 nm. The scavenging activity was calculated according to the relationship:

\[
\text{% of scavenging } *\text{OH radicals} = \left[\frac{(T - T_2)}{(T - T_1)}\right] \times 100
\]

T, the transmittance of the radical generating system without studied compounds; T1, the transmittance of the system with thiourea preventing radical formation; T2, the transmittance of studied compounds in the system. The reaction mixture for (T) contained: phosphate buffer (0.15 mM, pH = 7.4), 100 µM l-ascorbic acid, 100 µM CuSO4, 12 µM cytochrome c; for (T1): individual components of (T) added to 6.6 mM thiourea (T1); for (T2): (T) reaction mixture with studied compounds of different concentrations.

2.4 | DNA topology assay

Plasmid DNA (pBR22 0.1 µg µl−1) was added into a reaction mixture containing 0.05% FeSO4·7H2O in 1×PBS together with resveratrol or its analogs. First, different resveratrol concentrations (50–1000 µM) were applied, without the addition of Fe2+, to test their genotoxic potential on plasmid DNA. Next, the selected concentrations (1–200 µM) were used in combination with Fe2+. Based on resveratrol results the particular concentrations of analogs were used. Similarly, the highest used concentration of analogs without adding Fe2+ was 1000 µM. Further, to detect a protective effect, 20, 50, and 10 µM were used. The reaction mixture was prepared as follows: dDH2O, 1×PBS, resveratrol or analogs, Fe2+, plasmid DNA and incubated for 20 min at 37°C. After the incubation, samples were detected using agarose gel electrophoresis.

2.5 | Comet assay

The comet assay was carried out following the protocol of Collins et al.[25]

2.5.1 | Lymphocytes isolation

Using fingerpick methods 40–60 µl of blood was collected into a tube with 1 ml of 1×PBS on ice and mixed. The tubes were incubated on ice for 30 min. Afterward, 100 µl of Histopaque-1077 was layered onto a blood specimen. Tubes were centrifugated at low speed, 1500 rpm for 5 min at 4°C. Lymphocytes visible as a milky cloud on the top layer, approximately 300 µl, were collected into new tubes.
The pellets containing the rest of the blood particles were discarded. Tubes with lymphocyte layer were again centrifuged at 1500 RPM for 5 min at 4°C. The supernatant was discarded and the pellet containing isolated lymphocytes was immediately used for the next experiments.

### 2.5.2 | Lymphocytes treatment I

Before embedding into agarose, isolated lymphocytes were dissolved in 1 ml of a tested compound in 1×PBS and incubated for 60 min at 37°C. The negative control was dissolved in 1×PBS only. Next, the samples were centrifuged at 1500 RPM for 5 min at 4°C. The supernatant was removed and the pellet with lymphocytes was used for embedding into agarose.

### 2.5.3 | Embedding into agarose

The pellet containing isolated lymphocytes from the previous step was combined with 120 µl of 1% low melting point agarose, mixed well, and dropped on preprepared slides covered with 1% normal melting point agarose. The drop was covered with a coverslip, and so prepared slides were incubated for several minutes at 4°C to assure agarose gel full solidification. Afterward, coverslips were carefully removed, and slides with lymphocytes were further treated.

### 2.5.4 | Lymphocytes treatment II

Slides with embedded lymphocytes were immersed into a cuvette with 100 µM hydrogen peroxide for 5 min at 4°C.

### 2.5.5 | Lysis

To remove membranes and cytosolic soluble proteins and keep nuclei only,[24] slides with embedded lymphocytes were immersed into cold lysis solution (2.5 M NaCl; 0.1 M EDTA; 10 mM Tris base, pH 10) with 1% Triton-X 100 and incubated, at least, for 1 h but maximum 24 h at 4°C.

### 2.5.6 | Alkaline electrophoresis and neutralization

After the lysis, slides with nuclei were placed into an electrophoretic tank filled with a cold electrophoresis buffer (300 mM NaOH; 1 mM EDTA; pH <13). The empty places in the tank were filled with blank slides. Nuclei were left in the solution to unwind for 20 min at 4°C followed by electrophoresis at constant 25 V and current 280–330 mA for 30 min. The current was adjusted by lowering or increasing the volume of the electrophoretic buffer. Next, slides were neutralized once in 1×PBS for 10 min at 4°C and once in ddH2O for another 10 min at 4°C. Finally, slides were air-dried on RT, stained with ethidium bromide, and subjected to microscopic observation.

### 2.5.7 | Nuclei scoring

Evaluation of nuclei was performed manually using Comet visual software. One hundred randomly chosen nuclei per slide were scored and assigned into four distinct levels, depending on the comet tail. Per each experiment, at least three independent experiments were performed.

### 2.6 | Modified comet assay using repair enzyme

The procedure was performed based on reference.[27]

#### 2.6.1 | Calibration of FPG enzyme

The commercially available formamidopyrimidine DNA glycosylase—FPG was 100× diluted in buffer F (40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 0.1 M KCl; 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin added before use, pH 8.0 adjusted with 6 M KOH) with 10% glycerol and stored at −80°C. Subsequently, dilutions as followed: 1: 10,000; 1:50,000; 1:100,000; 1:200,000, from the 100× diluted aliquots, were applied on nontreated isolated lymphocytes (see 4.5.1; 4.5.3) to test nonspecific DNA damage (breaks). After the lysis step (see Section 2.5.5), 50 µl enzyme was applied onto slides and cover with parafilm. Slides were incubated in a humid chamber for 30 min at 37°C. Next, electrophoresis was carried out (see Section 2.5.6).

#### 2.6.2 | Comet assay with FPG

The lymphocyte isolation, treatment with resveratrol and IRAs, embedding, and lysis were performed as in standard comet assay protocol (see Sections 2.5.1–2.5.5). As a positive control, isolated lymphocytes were treated with 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), to induce base modification, as described in Horváthová et al.[28] Briefly, lymphocytes were incubated with 50 mM of DMNQ in DMSO for 30 min at 37°C, embedded onto slides, and place into lysis solution. After the lysis (1h), slides were washed two times with buffer F, 5 min each, and once with buffer F + bovine serum albumin (BSA). Next, 50 µl of the selected concentration of FPG, recognizing and cutting out the specific DNA base modifications, was applied onto slides and covered with parafilm. Slides were incubated in a humid chamber for 30 min at 37°C. The negative control was treated with buffer F only. After the incubation electrophoresis and scoring were carried out (see Sections 2.5.6 and 2.5.7).
2.7 Cellular repair assay

The method is based on a classical alkaline comet assay. The experiment was performed following the protocol of.\[29\] Briefly, isolated lymphocytes were treated with 100 µM H₂O₂ for 5 min at 4°C. Next, the microtubes were centrifuged at 1500 rpm for 5 min at 4°C, the supernatant was discarded and the pellet with lymphocytes was resuspended in resveratrol or its analogs of tested concentration. So prepared samples were incubated at 37°C for 0, 5, 10, 15, and 60 min. After the indicated incubation time, tubes were centrifuged (1500 rpm, 5 min at 4°C), the pellet was mixed with LMP agarose, and embedded on a slide. The following steps were executed as described in lysis, alkaline electrophoresis and neutralization, and nuclei scoring paragraphs.

2.8 Statistical analysis

Results are presented as a mean of values with error bars displaying standard deviations. The statistical analysis was performed using F-test for equality of variances followed by t-test with the two-tailed distribution.

3 RESULTS AND DISCUSSION

3.1 FRAP

FRAP showed that all tested compounds (resveratrol and IRAs) are able to reduce Fe³⁺ to Fe²⁺ less effectively than GA. GA is a potent antioxidant and more stable than ascorbic acid when exposed to heat.\[30\] While the reduction capability improved with increased concentration, the maximal reduction of Fe³⁺ by IRA1 and IRA2 at the highest concentration (2400 µM) was approximately 30% less than reduction by GA. IRA3 displayed half of the GA reduction and resveratrol even less than half of GA reduction (Figure 2). Interestingly, IRA3 seemed to be less potent in Fe³⁺ reduction while it was the most active compound in the DPPH assay.\[22\] Nevertheless, IRA1 and IRA2 can reduce Fe³⁺ to Fe²⁺ more efficiently than IRA3 or resveratrol itself. Although no significant differences were detected among the three independent experiments (2400 µM), due to the higher standard deviations, the trend remained the same. Other concentrations displayed differences at \( p \leq 0.05 \). It was found that studied IRAs exhibited very good reductive properties, even better as resveratrol. It is caused by the donor ability of IRAs, which are Schiff’s bases\[31\] with good reduction properties.

3.2 Scavenging of hydroxyl radicals

Next, resveratrol and IRAs were tested for the scavenging/eliminating ability of hydroxyl radicals (•OH) (Figure 3). Resveratrol demonstrated ineffective scavenging of •OH even at high concentration (800 µM). On the other hand, all three tested IRAs showed similar ability, increasing with the concentration, to eliminate •OH. Resveratrol eliminated approximately three times less •OH than any IRA at any tested concentration. Interestingly, IRAs exhibited over 100% efficiency of •OH scavenging at a concentration of ≥200 µM. We assume that IRAs as Schiff’s bases can form complexes with Cu²⁺\[31\] and thus prevent the generation of •OH even more than thiourea (because even 200 µM of IRAs reduce the production of •OH more than 6600 µM of thiourea). This assumption is also confirmed by Zhu et al.\[32\] who showed that thiourea prevents the copper-induced formation of hydroxyl radicals through the chelation of copper and the formation of a redox-inactive complex.\[32\] These findings are also...
supported by our previous work,\textsuperscript{10} that bis-imine analogs of resveratrol did not scavenge hydroxyl radicals. So, in contrast to resveratrol, IRAs do not scavenge hydroxyl radicals but rather hinder their formation.

3.3 | DNA topology assay

To test the putative protective effect of resveratrol, and its imine analogs, on DNA topology exposed to Fe\textsuperscript{2+}, a DNA topology assay was performed. Ferrous ions (Fe\textsuperscript{2+}) oxidize in reaction creating ferric ions (Fe\textsuperscript{3+}) that can react with plasmid DNA and alter its topology, introducing breaks. The changes in DNA topology are detected as a reduced DNA motion in the electric field. The native supercoil form moves fastest. The breaks give rise to a slower linear form with dsDNA breaks or a circular one with ssDNA breaks, which is the slowest form of plasmid DNA. If the tested compounds can chelate ferrous ions in the reaction, no DNA oxidation occurs later.\textsuperscript{33–35}

To investigate the effect of resveratrol and IRAs on the pBR322 plasmid DNA structure, a topological assay was applied. First, plasmid DNA was exposed to the different concentrations of resveratrol (50–1000 µM) without the addition of Fe\textsuperscript{2+} to determine the possible genotoxic effect of resveratrol alone (Figure 4). No changes in plasmid topology were observed, similarly to a negative control (NC), that is, plasmid without treatment with Fe\textsuperscript{2+}. Next, lower resveratrol concentrations (1–200 µM) were selected to monitor the putative protective effect on DNA when exposed to Fe\textsuperscript{2+}. The 50 µM and lower concentrations showed a less protective effect than 10 and 200 µM. Nevertheless, the cleavage pattern of the plasmid was never as strong as in the case of positive control (PC), that is, plasmid treated with Fe\textsuperscript{2+} only (Figure 4, top). This finding is also supported by the ability of the resorcinol group, present in resveratrol, to chelate metal ions and thus constrain plasmid DNA cleavage.\textsuperscript{36,37}

Based on resveratrol results, only the highest (1000 µM) concentration of IRAs was tested for potential genotoxic effect. None of the IRAs caused changes in plasmid topology. The lower (200, 50, and 10 µM) concentrations were used in protective effect monitoring (Figure 4). No changes in plasmid topology were observed, similarly to NC (Figure 4, bottom). The clear protective effect of all IRAs was detected only for a 200 µM concentration. The IRA1 was able to reduce the DNA cleavage partially even when 50 µM was used. Similarly, 50 and 10 µM of IRA3 reduced the DNA cleavage, in contrast to IRA2. This analog showed the weakest protective properties in DNA topology assay.

3.4 | Comet assay

The genotoxic effects of resveratrol and its imine analogs on human lymphocytes were tested using a comet assay approach. This method is routinely used to monitor DNA damage. In principle, the direct DNA breaks induced by the genotoxic agents can be detected in bare nuclei. Additionally, modifications of DNA nitrogenous bases lead to their removal and further DNA breaks. The mobility of the DNA molecule is influenced by the breaks which can be seen as a comet tail. The bigger tail is, the more serious the damage.\textsuperscript{38–40}

The high concentrations of resveratrol (200–1000 µM) caused significant DNA damage compared to the negative control (NC—lymphocytes incubated with 1×PBS only) but less than the positive control (H\textsubscript{2}O\textsubscript{2}—lymphocytes treated with 100 µM hydrogen peroxide to induce DNA breaks). The high genotoxic effect of resveratrol might be a result of its pro-oxidant properties. Hadi et al.\textsuperscript{41} showed that resveratrol can reduce Cu\textsuperscript{2+} to Cu\textsuperscript{+}, and reoxidation of Cu\textsuperscript{+} back to Cu\textsuperscript{2+} state produces oxidative stress. The other studies reviewed in Shaito et al.\textsuperscript{42} showed the pro-oxidant effect of high-dosage resveratrol. The higher concentrations (400–1000 µM) of IRA1 showed a strong genotoxic effect, whereas IRA2 and IRA3 did not generate DNA damage. In several cases, lower DNA damage was observed than in NC (Figure 5).

To monitor the ability of resveratrol and IRAs to protect cells from oxidative damage lymphocytes were pretreated with compounds before exposure to H\textsubscript{2}O\textsubscript{2}. One-hour pretreatment of lymphocytes with 50 µM resveratrol resulted in a similar amount of DNA damage as the positive control (H\textsubscript{2}O\textsubscript{2}—lymphocytes treated with 100 µM hydrogen peroxide to induce DNA breaks). The high genotoxic effect of resveratrol was reduced by IRA1, IRA2, and IRA3 at 200 µM. The IRA1 showed the strongest protective effect, whereas IRA2 and IRA3 did not reduce DNA damage in higher concentrations (400–1000 µM). The IRA2 showed a genotoxic effect at 1000 µM, but at 200 µM, it reduced DNA damage, as observed in the comet assay. The IRA3 showed the weakest protective effect in lymphocytes. In contrast to resveratrol, the highest concentration of IRA3 (1000 µM) did not change the plasmid topology in the presence of Fe\textsuperscript{2+} (Figure 4, bottom). The clear protective effect of all IRAs was detected only for a 200 µM concentration. The IRA1 was able to reduce the DNA cleavage partially even when 50 µM was used. Similarly, 50 and 10 µM of IRA3 reduced the DNA cleavage, in contrast to IRA2. This analog showed the weakest protective properties in DNA topology assay.
DNA breaks. NC (grey-blue—negative control, lymphocytes incubated with 1×PBS only; H2O2 (yellow)—positive control lymphocytes treated with 100 µM hydrogen peroxide to induce DNA breaks. Data are presented as the mean of three independent experiments +STDEV. Error bars display the maximal detected DNA damage in lymphocytes. Differences between the negative control and tested compounds *p < 0.05; **p < 0.01; ***p < 0.001; b = p < 0.05 lower DNA damage. The statistical analysis was performed using F-test and t-test. PBS, phosphate-buffered saline

breaks as in the H2O2 treated control. The higher resveratrol concentration, 100 µM, made lymphocytes more susceptible to DNA damage (Figure 6). On the other hand, pretreatment of lymphocytes with all studied IRAs, in both used concentrations, significantly reduced the level of DNA damage after exposition to H2O2 (Figure 6). Thus, imine analogs showed a potential to alter cell response to oxidative damage. These properties of IRAs could be of advantage to protect healthy cells from the toxic effect of drugs used in cancer therapy. The study of Becker et al. showed that the natural herbal extract, containing rocaaglamide, used in Chinese medicine, could reduce DNA damage in non-cancer cells. Also, resveratrol and quercetin reduced cardiotoxicity introduced by the antitumor drug. Indeed, this would require another set of experiments, like testing the effects on cancer cells and possible drug contraindications, but this potential, to diminish the drug side-effects, should not stay overlooked.

3.5 | Cellular repair assay

Using cellular repair assay, we investigated the role of resveratrol and IRAs in the DNA repair kinetics after hydrogen peroxide pretreatment. Over the course of posttreatment incubation with both 50 µM and 100 µM concentrations of resveratrol, no significant differences in the amount of detected DNA breaks were observed in the first 15 min (Figure 7). On the other hand, while DNA breaks in H2O2 control decreased about 15% after 60 min of regeneration (time provided to cells to regenerate after DNA damage), the sample incubated with resveratrol (50 µM) sustained still a high level of DNA breaks. Interestingly, this value (50%) was higher than in 5 and 10 min after regeneration started. In the case of IRAs, the reduction of DNA breaks was detected in the first 10 min. Afterward, further incubation with IRA2 reduced DNA breaks up until 30% after 60 min. IRA1 and IRA3 displayed similar behavior as resveratrol, thus showing an increasing trend in DNA breaks after 15 min incubation. Notably, due to the limitations in the procedure, the residual H2O2 after pretreatment could react with the tested compounds and thus promote oxidative stress. It was shown that resveratrol can degrade in the presence of sodium hydrogen carbonate in a cultivation medium. Therefore, it is likely that some of the IRAs can undergo degradation as well and thus enhance the level of DNA breaks.

3.6 | Modified comet assay using FPG repair enzyme

Although we showed that IRAs induce less direct DNA breaks than resveratrol, we were interested in whether they can cause hidden oxidative damage by modifying DNA nitrogenous bases. These modifications are recognized and cut by specialized enzymes, leaving a break in the DNA sequence. Thus, arisen ssDNA breaks can be detected using the comet assay. We employed a comet assay with DNA-glycosylases FPG to test the IRAs’ ability to modify guanosine.

Firstly, the FPG enzyme was calibrated, so the used concentration would actively cut 8-oxoG without introducing nonspecifically...
DNA breaks (Figure S1). To modify guanosine, isolated lymphocytes were treated with 2,3-dimethoxy-1,4-naphthoquinone (DMNQ). A 1:50,000 dilution of FPG was more active than 1:100,000, still without any nonspecific activity (Figure S1). To investigate the potential of resveratrol and IRAs to cause oxidative damage, FPG was added on slides with lymphocytes pretreated with resveratrol or IRAs (Figure 8). A little elevation of DNA breaks was observed in the case of lymphocytes pretreated with 50 µM concentration of resveratrol when incubated with FPG compared to non-FPG samples. A significantly higher amount of DNA breaks was detected in the case of IRA1 and IRA3 (Figure 8A). Whereas no changes were observed for 50 µM IRA2 with or without FPG (Figure 8A), a higher 400 µM concentration of IRA2 showed significantly more DNA breaks after FPG application (Figure 8B). On the other hand, this raise of DNA breaks was around 10% what is less than in the case of 50 µM IRA1 and IRA3.

Although trends in DNA breaks development tended to remain the same in all comet assay related experiments, we often observed inconsistencies in the volume of DNA breaks in untreated control cells despite that the blood sample originated from one donor. One factor that could play a role is year seasons. Experiments were performed from October through March, and several studies reported so-called seasonal variations in lymphocyte sensitivity to DNA damage.[46–49] Giovannelli et al.[49] showed that air temperature changes impact this phenomenon more than other factors like solar radiance.

FIGURE 7 Cellular repair assay on lymphocytes exposed to H2O2 and followed by the incubation with 50 µM resveratrol or its imine analogs. Lymphocytes exposed to 100 µM H2O2 for 5 min and incubated in the presence of 50 µM resveratrol (blue), IRA1 (black), IRA2 (green), or IRA3 (red). All tested compounds showed reduce DNA damage after 5 min incubation compared to H2O2 control, incubated with 1× PBS only (yellow). Longer incubation (60 min) with resveratrol, IRA1, and IRA3 led to higher DNA damage than detected in control, whereas IRA2 slightly reduced detectable DNA damage. Three independent experiments were performed, and the same trend of DNA damage changes was detected. Data are presented as the mean of three independent experiments. The observed differences: resveratrol, after 60 min of incubation at p ≤ 0.05; IRA1 after 5 and 15 min at p ≤ 0.05, 60 min at p ≤ 0.01; IRA2 after 5 min at p ≤ 0.05; IRA3 after 5, 15, and 60 min at p ≤ 0.05, compared to H2O2 sample. The statistical analysis was performed using F test and t test. PBS, phosphate-buffered saline

4 | CONCLUSIONS

Employing several antioxidant and genotoxic assays we showed that IRAs display different characteristics than resveratrol. Based on the performed antioxidant assays, we concluded that IRA2 possesses the best antioxidant properties out of all tested compounds. The high dosage of IRA2 reduced Fe3+ to Fe2+ twice as good as resveratrol. Moreover, it displayed high activity, increasing with concentration, in hydroxyl radicals scavenging assay. Notably, all studied IRAs are more effective in preventing *OH radicals production compared to thiourea. Therefore, their ability to scavenge *OH radicals cannot be detected by the method described in Section 2.3. On the other hand,
resveratrol was able to scavenge hydroxyl radicals, which is in accordance with the results of our previous work.\textsuperscript{[10]}

Our data show that resveratrol is genotoxic for human lymphocytes at a high concentration: 200 µM and higher, while IRA2 and IRA3 do not cause DNA breaks even at a 1000 µM concentration. The superiority of IRAs is clearly visible when cells pretreated with IRAs displayed minimum DNA breaks after exposure to hydrogen peroxide, while cells pretreated with resveratrol accumulated DNA breaks. Interestingly, the other way around, when lymphocytes were first exposed to H₂O₂ and then incubated with compounds, instead of slow DNA repair, typical for cells incubated with H₂O₂ only, resveratrol, IRA1, and IRA3 accumulated DNA breaks. The decrease in DNA breaks was visible in IRA2 pretreated cells. Although IRAs did not introduce DNA breaks directly, still, they could generate oxidative damage through the modification of DNA nitrogenous bases. Using the DNA repair enzyme, FPG, which can recognize and cut out 8-oxoG, we uncovered that cells treated with 50 µM IRA1 and IRA3 displayed more DNA breaks. This implies that some of the IRAs can have a potentially hidden genotoxic effect. Noteworthy, the same experiment did not reveal a dramatic increase in DNA breaks even when cells were treated with 400 µM IRA2.

Taken together, all studied IRAs exhibit better antioxidative properties and low genotoxicity to human lymphocytes than resveratrol. We conclude that 3-((4-hydroxyphenylimino)methyl)phenol (IRA2) has optimal properties from the investigated substances.

To conclude, we wish to bring IRAs into the awareness of the research community not only due to their antioxidant properties but mainly due to their performance in genotoxic assays; particularly, their ability to protect DNA from oxidative damage.

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**CONFLICT OF INTERESTS**

The authors declare that there are no conflict of interests.

**ETHICS STATEMENT**

The blood donor voluntarily participated in this study with informed consent.

**DATA AVAILABILITY STATEMENT**

Data available on request from the authors.

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