Are Hydroethidine-Based Probes Reliable for Reactive Oxygen Species Detection?

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Abstract

Detection and quantification of the highly reactive and short-lived superoxide (O$_{2}^{\cdot-}$) can be challenging. Here, we present a new mass spectrometry (MS)-based method to detect and quantify O$_{2}^{\cdot-}$ using three fluorogenic hydroethidine probes: hydroethidine (HE), mito-hydroethidine (mito-HE), and hydropropidine (HPr$^+$), which measure cytosolic, mitochondrial, and extracellular O$_{2}^{\cdot-}$, respectively. The probes and their oxidation products were simultaneously quantified by applying multiple reaction monitoring (MRM) with MS that allowed the specific measurement of reactive oxygen species (ROS) distribution within the cell. The advantage of this liquid chromatography–tandem mass spectrometry (LC-MS/MS) method is that coeluting compounds can be precisely distinguished using specific precursor and fragment masses. This method overcomes limitations from spectral overlap of O$_{2}^{\cdot-}$-specific and nonspecific products in fluorescence spectra or the low specificity associated with chromatography-based approaches. However, our experiments showed that these HE probes can be prone to autoxidation during incubation at 37°C in Hank’s solution. Cell treatments with strong oxidants did not significantly increase levels of the O$_{2}^{\cdot-}$ radical. Thus, subtle changes in ROS levels in cell culture experiments might not be quantifiable. Our findings raise the question of whether HE-based probes can be used for the reliable detection of O$_{2}^{\cdot-}$ radicals in cell culture. Antioxid. Redox Signal. 31, 359–367.

Keywords: reactive oxygen species (ROS), superoxide (O$_{2}^{\cdot-}$), mass spectrometry, hydroethidine (HE), mito-hydroethidine (mitoSOX red or mito-HE), hydropropidine (HPr$^+$)

Introduction

Reactive oxygen species (ROS) are chemically reactive oxygen-containing molecules that include peroxides, superoxide (O$_{2}^{\cdot-}$), and hydroxyl radicals. ROS can be either natural byproducts of normal endogenous oxygen metabolism or generated by exogenous sources, such as ultraviolet radiation. ROS were long thought to be potentially damaging byproducts of cellular metabolism that can affect DNA, lipids, and proteins. However, more recent studies highlight the important role of ROS in cell signaling, homeostasis, cancer, and apoptosis (4). Thus, the ability to assess ROS status is valuable, and many different methods have been established that allow the direct or indirect measurement of redox states; the advantages and disadvantages of these methods were reviewed recently (1).

Innovation

Current methods to determine reactive oxygen species (ROS) levels within cells have limitations such as spectral overlap and low specificity in fluorescence- and high-performance liquid chromatography-based approaches, respectively. In this study, we showed that three hydroethidine (HE)-based probes and their oxidized products can be measured precisely, quantitatively, and simultaneously using specific precursor and fragment masses in a 10-min targeted liquid chromatography–tandem mass spectrometry method. However, our results also indicated that these fluorophores are prone to autoxidation during sample treatment. As such, results generated using these probes must include stability checks. Overall, our findings indicate that ROS determination using HE-based probes should be done with care and take into consideration how autoxidation could affect measured ROS values.

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The most commonly used fluorogenic probes for O$_{2}^-$ detection, and the current “gold standard,” are hydroethidine (HE; or dihydroethidium) and mito-hydroethidine (mito-HE or mitoSOX red) for intracellular and mitochondrial O$_{2}^-$ detection, respectively (3, 8). Moreover, membrane-impermeable hydropropidine (HPr$^+$) that can detect extracellular O$_{2}^-$ was recently developed (6). These three structurally analogous fluorogenic probes react with O$_{2}^-$ in a very similar way, wherein O$_{2}^-$-specific hydroxylated products (2-hydroxyethidium [2-OH-E$^+$], 2-hydroxy-mito-ethidium [2-OH-mito-E$^{2+}$], and 2-hydroxypropidium [2-OH-Pr$^{2+}$]) are formed as the main products (Fig. 1). However, many other oxidants are present in cells, including redox metal ions, heme proteins, and one- or two-electron oxidants, which can give rise to fluorescent ethidium and analogs (ethidium [E$^+$], mito-ethidium [mito-E$^{2+}$], and propidium [Pr$^{2+}$]) as well as weak or nonfluorescent dimeric products (diethidium [di-E$^+$], di-mito-ethidium [di-mito-E$^{2+}$], and dipropidium [di-Pr$^{2+}$]; Fig. 1) (3). The use of fluorescence microscopy or other fluorescence-based methods with these probes should be avoided, considering that the fluorescent characteristics of O$_{2}^-$-derived marker products and other nonspecific oxidized fluorescent products that are formed from these probes may involve spectral overlap (3).

The aim of this study was to develop a robust, rapid, and quantitative liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based method to measure O$_{2}^-$ levels in cells. Furthermore, the use of extracellular, intracellular, and mitochondria-targeted fluorogenic probes can allow spatial resolution of the ROS source. Thus, mass fingerprints for HE, mito-HE, and HPr$^+$ and their oxidation products were generated to uniquely identify and quantify the compounds. We established and optimized a single short LC-MS/MS method that involves applying multiple reaction monitoring (MRM) to simultaneously monitor all educts and their oxidation products in a cell system. The separation and determination of these probes and the corresponding oxidation products were previously achieved by high-performance liquid chromatography (HPLC)-based analyses (1, 3). Our LC-MS/MS method is superior to methods that rely solely on HPLC, as the identity of every peak can be verified in the mass spectrum, and coeluting peaks can be easily distinguished by mass differences in the unique fragment masses as part of a multiplexed single short LC-MS/MS run. DNA intercalations will not interfere with our method, as only metabolites and not DNA were extracted (3). By applying our LC-MS/MS method, we found that in Hank’s solution all three probes: HE, mito-HE, and HPr$^+$, were prone to autoxidation, which resulted in an increase in the levels of O$_{2}^-$-specific products. Thus, results obtained using HE, mito-HE, and HPr$^+$ as probes for O$_{2}^-$ detection should be interpreted with care due to the high autoxidation rate of these molecules. The observed autoxidation in Hank’s solution could compromise detection of endogenous O$_{2}^-$ production, and could confound the actual O$_{2}^-$ state in many experiments.

Results

Establishment of a MRM-based method to monitor HE, mito-HE, HPr$^+$, and their oxidation products

To identify and quantify relative differences in the three fluorogenic probes and their oxidation products in cell culture systems, we developed a targeted LC-MS/MS approach based on MRM. The mass spectrometry (MS) parameters for specific transitions of all monitored compounds were individually identified, optimized, and the three best transitions per metabolite were selected for the final method. The identity of fragments was verified by

![FIG. 1. Products formed from HE, mitoHE, and HPr$^+$. Adapted from Kalyanaraman et al. (3), 2-OH-E$^+$, 2-hydroxyethidium; 2-OH-Pr$^{2+}$, 2-hydroxypropidium; di-E$^+$, diethidium; di-mito-E$^{2+}$, di-mito-ethidium; di=Pr$^{2+}$, dipropidium; HE, hydroethidine; mito-HE, mito-hydroethidine; HPr$^+$, hydropropidine. Color images are available online.](image-url)
high-resolution MS (Fig. 2). To increase the robustness of the LC-MS/MS method, we monitored the correct retention time of all peaks, the peak shapes, and the ion ratios. The ion ratios were calculated by dividing all transitions by the largest peak area per compound, and the values need to match the ratios calculated for the pure probes (2). All selected transitions, including internal standards, were thus combined in a single LC-MS/MS method operating in a positive ionization mode (Fig. 3A, Table 1). Transitions for the three fluorogenic compounds and their $O_2^-\cdot$-specific products were additionally displayed individually (Fig. 3B–G).
Fluorogenic probe stability is a significant concern

A MS approach overcomes spectral overlap issues in fluorescence-based methods, as it relies on the exact masses of specific transitions. Given the natural tendency of the probes to undergo autoxidation, we first monitored probe stability. Several time-series measurements were performed under different light and temperature conditions to monitor the stability of educts and oxidation products in a cell-free environment. Regardless of the conditions, rapid autoxidation rates were observed in all experiments, which translated to an increase in the levels of O$_2^*$-specific products (Fig. 4).
A 150% increase within 60 min was observed for the O$_2^•$-specific products obtained from all three probes under the “25°C without light” condition at this time point (Fig. 4A–C). Under standard laboratory light conditions, the levels of the O$_2^•$-specific products increased by >200%, and after 2 h to >300% above initial values (Fig. 4D–F). Light conditions had a larger impact on O$_2^•$-specific products relative to that observed for temperature increases to 37°C (Fig. 4G–I). In contrast, the internal standard showed a standard deviation of 5.5% for all experiments, indicating normal variations of the LC-MS/MS runs and in turn robust measurement conditions.

**Effects of strong ROS stimulations in cells were smaller than those for autoxidation**

HepG2 cells were treated with strong ROS inducers, such as rotenone and hydrogen peroxide (H$_2$O$_2$), as well as the ROS quencher N-acetyl-cysteine (NAC) (5, 9), to identify maximum effects in a cell system. We observed whether these chemical treatments show stronger ROS signals than the autooxidation rates of the probes themselves.

Upon treatment with NAC, levels of all oxidation products did not change significantly, except for Pr$_2^+$. Indeed, the fold changes of these oxidation products were slightly reduced for HE and HPr$^+$, and unchanged for mito-HE (Fig. 5A). After incubating the cells with H$_2$O$_2$, levels of O$_2^•$-specific products were unchanged for mito-HE compared with controls, and even reduced for HE and HPr$^+$ (Fig. 5B). Rotenone treatment was associated with insignificant reductions in O$_2^•$-specific products for HE and HPr$^+$, and only 2-OH-mito-E$_2^+$ showed a significant increase of 50%. This result was perhaps predictable, given that mitochondria are sites of electron leakage (Fig. 5C). All harsh treatments did not induce significant changes in the amounts of O$_2^•$-specific products, most likely because the cell culture medium already...
induced a high oxidation rate of the three probes, which then diminished cell-specific changes.

Together, these results indicate that the autoxidation effects of the three probes were high upon incubation at 25°C or 37°C, especially under standard room light conditions. This autoxidation can compete with experimentally induced ROS changes to diminish or perhaps obscure the true ROS status upon harsh treatments with H₂O₂, rotenone, or NAC.

Discussion

Recently, fluorescence- and HPLC-based approaches have frequently been used with HE-based probes such as HE, mito-HE, or HPr⁺ to assess the ROS status in cells (3, 7, 8). However, such probes have several drawbacks in fluorescence or HPLC applications, particularly because of spectral overlap in fluorescence assays and low specificity in HPLC methods. In this study, we developed the first LC-MS/MS method that can simultaneously detect and quantify these three fluorogenic probes and their corresponding oxidation products. This method was rapid, sensitive, and specific to elucidate the ROS status in a cell system with spatial resolution. Three specific transitions for each of the educts and oxidized products as well as the ion ratios between the transitions and the correct retention time were monitored to ensure high selectivity. This method eliminates problematic fluorescence spectral overlap that is typical of fluorescence-based techniques and the low specificity associated with the use of only HPLC-based methods. However, we found that these HE-based probes were intrinsically prone to oxidation. Incubation at 25°C or 37°C in our stability test led to rapid probe autoxidation, which could complicate the results obtained when these probes are used for ROS detection. Accordingly, the harsh conditions used for ROS induction and inhibition treatments generated results for the oxidized fluorophores that lacked significance and reproducibility. Hence, subtle changes in ROS levels that can occur in biological systems and that can be used to achieve new insights into redox homeostasis may not be detectable.

In addition to the instability of HE-based probes, other inherent characteristics can impede ROS detection, including complex chemical reactions, intercalation with DNA, and interference with heme-containing enzymes (1). Heme proteins, such as hemoglobin and myoglobin, can react with HE and form fluorescent products. These chemical reactions can produce many other oxidation products that can compete with radical-specific products, and may influence the amount of detectable O₂⁻⁻⁻⁻specific products. In addition, formation of O₂⁻⁻⁻⁻specific products could be influenced by peroxidase reactions that can interfere with O₂⁻⁻⁻⁻ quantification (1). Meanwhile, mito-HE can be translocated to other intracellular organelles that have higher negative membrane potential (3).

Our targeted LC-MS/MS method allows the selective identification and quantification of the fluorogenic compounds HE, mito-HE, and HPr⁺ and their respective O₂⁻⁻⁻⁻specific products. However, we also detected a high autoxidation rate for the probes alone in Hank’s solution. These findings suggest that, despite the many recent methodological improvements in ROS quantification, direct measurements of ROS levels based on HE probes may be inaccurate due to artificial effects of autoxidation. Experiments performed using these probes should thus be interpreted with caution, and autoxidation rates should be monitored.

Notes

Chemicals

HE (D1168) and mito-HE (M36008) were purchased from Invitrogen (Carlsbad, CA). HPr⁺ was a kind gift from Prof. Jacek Zielonka, Medical College of Wisconsin. Potassium nitrosodisulfonate (NDS, 220930), chloranil (45374), potassium ferricyanide (455946), diethylthreiaminepentaaetic acid (D6518), bromodeoxyuridine (B9285), uridine (U3003), NAC (A9165), H₂O₂ (H1009), rotenone (R8805), and Triton X-100 (T9284) were all purchased from Sigma Aldrich (St. Louis, MO).

Synthesis of oxidation products derived from HE, mito-HE, and HPr⁺

The synthesis of oxidation products was done as previously described (8). In brief, 2-OH-E⁻ and 2-OH-mito-E⁻⁻⁻⁻ were synthesized by oxidizing HE and mito-HE with potassium NDS. E⁺ and mito-E⁻⁻⁻⁻ were synthesized by reacting HE
and mito-HE with chloranil, di-E+, and di-mito-E2+, and reacting HE and mito-HE with potassium ferricyanide. 2-OH-Pr2+, Pr2+, and di-Pr2+ were derived from HPr+, and synthesized using a procedure similar to that used to synthesize corresponding products from HE (6).

### Generation of MRM methods for fluorogenic probes, and derived oxidation products and LC-MS/MS conditions

Educts and oxidation products of the three probes were tuned individually to identify and optimize specific transitions for the QTrap 6500 mass spectrometer. About 100 ng/µL of all compounds was dissolved in methanol, and a constant flow of 7 µL/min was used. An automatic optimization procedure in the analyst software (v.1.6.2) was used. The 10 most intense fragment masses per compound were optimized for collision energy, declustering potential, and the collision cell exit potential (Table 1).

| Compound | Q1 mass (Da) | Q3 mass (Da) | RT (min) | DP (V) | CE (V) | CXP (V) | MRM ion ratio |
|----------|-------------|-------------|----------|--------|--------|---------|--------------|
| HE       | 316.2       | 210.1       | 6.75     | 76     | 43     | 24      | 1            |
| HE       | 316.2       | 287.1       | 6.75     | 76     | 29     | 28      | 0.71         |
| HE       | 316.2       | 271.1       | 6.75     | 76     | 41     | 14      | 0.16         |
| 2-OH-E+  | 330.2       | 301.1       | 4.95     | 101    | 37     | 8       | 1            |
| 2-OH-E+  | 330.2       | 300.1       | 4.95     | 101    | 55     | 28      | 0.93         |
| 2-OH-E+  | 330.2       | 255.1       | 4.95     | 101    | 69     | 12      | 0.84         |
| E+       | 314.1       | 284.1       | 5.15     | 1      | 51     | 24      | 1            |
| E+       | 314.1       | 286.1       | 5.15     | 1      | 39     | 16      | 0.80         |
| E+       | 314.1       | 285.1       | 5.15     | 1      | 37     | 18      | 0.69         |
| di-E+    | 313.2       | 285.1       | 5.22     | 141    | 35     | 26      | 1            |
| di-E+    | 313.2       | 284.1       | 5.22     | 141    | 41     | 26      | 0.77         |
| di-E+    | 313.2       | 299.1       | 5.22     | 141    | 31     | 10      | 0.56         |
| mito-HE  | 316.7       | 278.1       | 7.49     | 1      | 19     | 32      | 1            |
| mito-HE  | 316.7       | 209.1       | 7.49     | 1      | 43     | 22      | 0.28         |
| mito-HE  | 316.7       | 183.1       | 7.49     | 1      | 77     | 16      | 0.14         |
| 2-OH-mito-E2+ | 323.7 | 300.1 | 5.53 | 111 | 41 | 14 | 1 |
| 2-OH-mito-E2+ | 323.7 | 262.1 | 5.53 | 111 | 33 | 24 | 0.72 |
| 2-OH-mito-E2+ | 323.7 | 289.1 | 5.53 | 111 | 37 | 8 | 0.54 |
| mito-E2+ | 316.1       | 285.1       | 5.64     | 96     | 31     | 18      | 1            |
| mito-E2+ | 316.1       | 262.1       | 5.64     | 96     | 31     | 18      | 0.80         |
| mito-E2+ | 316.1       | 289.1       | 5.64     | 96     | 37     | 14      | 0.61         |
| di-mito-E2+ | 316.1 | 285.1 | 5.63 | 111 | 31 | 26 | 1 |
| di-mito-E2+ | 316.1 | 262.1 | 5.63 | 111 | 33 | 22 | 0.84 |
| di-mito-E2+ | 316.1 | 345.1 | 5.63 | 111 | 31 | 26 | 0.72 |
| HPr+     | 415.3       | 272.1       | 4.53     | 60     | 49     | 16      | 1            |
| HPr+     | 415.3       | 328.1       | 4.53     | 60     | 25     | 10      | 0.25         |
| HPr+     | 415.3       | 300.1       | 4.53     | 60     | 31     | 20      | 0.80         |
| 2-OH-Pr2+ | 215.1       | 266.1       | 4.42     | 60     | 25     | 24      | 1.00         |
| 2-OH-Pr2+ | 215.1       | 300.1       | 4.42     | 60     | 23     | 26      | 0.75         |
| 2-OH-Pr2+ | 215.1       | 72.0        | 4.42     | 60     | 25     | 10      | 0.63         |
| Pr2+     | 207.2       | 72.0        | 4.37     | 60     | 23     | 8       | 1            |
| Pr2+     | 207.2       | 250.0       | 4.37     | 60     | 25     | 24      | 0.64         |
| di-Pr2+  | 413.1       | 326.1       | 4.35     | 111    | 21     | 26      | 1            |
| di-Pr2+  | 413.1       | 331.1       | 4.35     | 111    | 21     | 18      | 0.02         |
| di-Pr2+  | 413.1       | 213.1       | 4.35     | 111    | 21     | 10      | 0.07         |
| IS       | 323.1       | 165.0       | 5.00     | 50     | 20     | 14      | 1.00         |
| IS       | 323.1       | 83.0        | 5.00     | 60     | 95     | 9       | 0.23         |

### Notes

2-OH-E+, 2-hydroxyethidium; 2-OH-mito-E2+, 2-hydroxy-mito-ethidium; 2-OH-Pr2+, 2-hydroxypropidium; CE, collision energy; CXP, collision cell exit potential; di-E+, diethidium; di-mito-E2+, di-mito-ethidium; di-Pr2+, dipropidium; DP, declustering potential; E+, ethidium; HE, hydroethidine; HPr+, hydropropidine; IS, internal standard chloramphenicol; mito-E2+, mito-ethidium; mito-HE, mito-hydroethidine; MRM, multiple reaction monitoring; MRM ion ratio, peak areas of all transitions were divided by the highest peak area per compound; Pr2+, propidium; RT, retention time.

TARGETED LC-MS/MS METHOD FOR ROS DETERMINATION

Three different LC columns and several different buffer conditions were used to identify the highest peak areas, optimal peak shapes, and retention times, as described previously (2). Finally, a Reprosil-PUR C18-AQ (1.9 µm, 120 Å, 50 × 2 mm ID; Dr. Maisch; Ammerbuch, Germany) was used for metabolite separation: A1: LC-MS grade water; 0.1% formic acid; B1: LC-MS grade acetonitrile; 0.1% formic acid. Gradients and flow conditions were as follows: the compounds were separated by a linear increase of B1 from 20% to 95% in 8 min and maintained at 95% B1 for 1 min. The concentration of B1 was then reduced to 20% in 1 min, and this level was maintained until minute 10. All
optimized MRM transitions and retention times are shown in Table 1.

Fragmentation patterns of the probes and O$_2^-$-specific products were recorded by a Q Exactive HF (Thermo Fisher Scientific, Waltham, MA) mass spectrometer with a high resolution of 60,000. The fragment mass identity was verified using ACD Spectrus Processor 2017.2.1 software. Assigned fragment masses differed by $<0.001$ Da from theoretically calculated masses, and assignments are shown in Figure 2.

Sample preparation for fluorogenic probe stability tests and metabolite extraction

The stability of all three fluorogenic probes was tested in Hank’s solution (Thermo Fisher Scientific, Waltham, MA, 14025) to evaluate autoxidation rates under cell-free conditions. Each probe (2 $\mu$L) was incubated in 50-$\mu$L Hank’s solution for 0, 10, 30, 60, and 150 min under the following three conditions in triplicate: 25$^\circ$C with light, 25$^\circ$C without light, and 37$^\circ$C without light. Two hundred microliters of acetonitrile and 20 $\mu$L of 1 mM internal standard (chloramphenicol) were added, vortexed (800 rpm) for 5 min at room temperature, and centrifuged at 8000 $g$ for 5 min at 4$^\circ$C to extract the probes. Twenty microliters of each supernatant was analyzed by LC-MS/MS to evaluate the autoxidation rates.

Metabolite extraction and determination of ROS status in a cell line

For proof of principle, ROS signatures were induced or scavenged by chemical treatments to detect compartment-specific signals for HE, mito-HE, and HPr$^+$ using the established LC-MS/MS method. HepG2 cells were treated with two chemical ROS stimuli, H$_2$O$_2$ (100 $\mu$M) and rotenone (1 $\mu$M) for 2 h, as well as with the ROS scavenger NAC (1 mM) for 20 h. HepG2 cells were cultivated in Dulbecco’s modified Eagle medium (Life Technologies, New York, NY) containing 4.5 g/L glucose, supplemented with 10% fetal bovine serum (Siliantes, Munich, Germany), 1% penicillin–streptomycin–neomycin (Invitrogen, Carlsbad, CA), at 37$^\circ$C in a humidified atmosphere of 5% CO$_2$. Cells were split into six-well cell culture plates (9 cm$^2$) and grown to full confluence. After treatment, cells were incubated with either 2 $\mu$M HE, mito-HE, or HPr$^+$ for 30 min at 37$^\circ$C in biological triplicates.

Cells were washed with ice-cold phosphate-buffered saline twice, and immediately lysed in 200 $\mu$L ice-cold phosphate-buffered saline containing 0.1% Triton X-100. The cell suspensions were transferred into 1.5-mL black tubes to avoid light exposure and placed on ice for further disruption by shear forces induced by 10 repeated injections through a 26-G cannula. The homogenates were centrifuged for 5 min at 600 $g$. The resulting supernatant was used for LC-MS/MS analysis.

LC-MS/MS instrument settings

Supernatant (20 $\mu$L) was injected, and compounds were separated on a LC instrument (1290 series UHPLC; Agilent, Santa Clara, CA), coupled online to a triple quadrupole hybrid ion trap mass spectrometer QTrap 6500 (Sciex, Foster City, CA). All transitions and compound-specific settings are shown in Table 1. Data acquisition was performed at an ion spray voltage of 5.5 kV in a positive mode for the electro spray ionization source, N$_2$ as the collision gas was set to medium, the curtain gas was at 30 psi, the ion source gases 1 and 2 were at 50 and 70 psi, respectively, and the interface heater temperature was set to 350$^\circ$C. MS data have been deposited into the publicly accessible repository PeptideAtlas under the identifier PASS01157.

Data evaluation and statistics

Metabolite identification was based on three levels: (i) the correct retention time, (ii) three transitions, (iii) and matching MRM ion ratios of tuned pure metabolites as described previously (2). Peak integration was performed using MultiQuant$^\text{TM}$ software v.2.1.1 (Sciex). All peaks were reviewed manually and adjusted if necessary. The peak area of the first transition per metabolite was used for subsequent calculations. An internal standard was used to normalize all LC-MS/MS runs for instrumental variations. HE, mito-HE, and HPr$^+$ were used to normalize the respective oxidized derivatives for the total input in cell culture experiments. Two-tailed unpaired t-tests were performed.

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Authors’ Contributions

Y.X. performed the experiments, analyzed the data, and prepared the figures. D.M. conceived the study and wrote the article.

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| Abbreviations Used |
|---------------------|
| 2-OH-E⁺ = 2-hydroxyethidium |
| 2-OH-mito-E²⁺ = 2-hydroxy-mito-ethidium |
| 2-OH-Pr²⁺ = 2-hydroxypropidium |
| di-E⁺ = diethidium |
| di-mito-E²⁺ = di-mito-ethidium |
| di-Pr²⁺ = dipropidium |
| E⁺ = ethidium |
| H₂O₂ = hydrogen peroxide |
| HE = hydroethidine |
| HepG2 = human liver cancer cell line |
| HPLC = high-performance liquid chromatography |
| HPr⁺ = hydropropidine |
| LC-MS/MS = liquid chromatography–tandem mass spectrometry |
| mito-E²⁺ = mito-ethidium |
| mito-HE or mitoSOX red = mito-hydroethidine |
| MRM = multiple reaction monitoring |
| MS = mass spectrometry |
| NAC = N-acetyl-cysteine |
| NDS = nitrosodisulfonate |
| O²⁻ = superoxide |
| Pr²⁺ = propidium |
| ROS = reactive oxygen species |