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Exposure of Cells to Hydrogen Peroxide Can Increase the Intracellular Accumulation of Drugs

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Abstract: One of the fastest growing areas of scientific research involves aspects of oxidative stress, either causes of or results from. Despite the enormous quantity of literature on the topic, surprisingly, the effects of oxidative stress on the pharmacokinetics of drugs have not been previously investigated. This is an extremely important concern, considering that the degree of oxidative stress that the human body experiences is known to be widely variable. Oxidative stress may be transiently increased, as is the case with some inflammatory episodes, or it may be chronically elevated, as is the case in some disease states, in aging, or with smokers. This report examines the influence of oxidative stress on the pharmacokinetics of model drugs utilizing cells in culture. Specifically, the effect of subtoxic, short-term exposure to hydrogen peroxide was investigated. Low micromolar, single doses of hydrogen peroxide were shown to cause dramatic increases in the apparent intracellular accumulation of model compounds with different physicochemical properties in different cell types. To examine the mechanistic basis for this, we evaluated possible hydrogen peroxide induced changes in cells including (1) intracellular pH, (2) membrane integrity, and (3) membrane fluidity (i.e., lateral membrane diffusion). We found no significant changes in pH or membrane integrity, but results were consistent with changes in hydrogen peroxide mediated reductions in lateral membrane diffusion, which we postulate facilitated the accumulation of the test substrates. Although studies presented here were all done in cell culture systems, we believe the findings could have substantial therapeutic relevance and warrant further investigations, which may provide reasons why drugs often have anomalous pharmacokinetic behavior and disproportionate dose−response relationships in certain patient populations.

Keywords: Hydrogen peroxide; drug permeability; membrane fluidity; daunorubicin; Oregon Green

Introduction

Understanding the reasons for pharmacokinetic variability of drugs represents an important area of research in which much has been accomplished. Research has provided mechanistic insight into reasons for variability that results from age, gender, diet, disease state, and genetics to name a few.[1−4] Despite the progress that has been made, there is still much work to do in order to achieve the ultimate goal of individualized dosing of drugs to obtain an appropriate pharmacodynamic response. Episodes in which patients

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receive hypo- or hypertherapeutic doses are still quite commonplace, some of which are known to result from alterations in drug metabolizing enzyme expression and/or activity,[5] altered drug absorption,[6] and changes in drug distribution,[7] to name a few. However, the effects of hydrogen peroxide (H_2O_2), the physiological levels of which can vary considerably, on the distribution of drugs has not been previously examined to our knowledge.

H_2O_2 is not a very reactive chemical species on its own and does not oxidize most biological membranes. The molecule's oxidative capacity typically arises from its conversion to reactive hydroxyl radicals, which can occur following exposure to UV light or by interaction with transition metal ions. Levels of H_2O_2 below 50 μM are suggested to have limited toxic effects on most cell types. Interestingly, at these low concentrations the molecule is believed to function as an inter- and intracellular signaling molecule, which may provide a physiological basis for fluctuations in its concentrations.[8] Moreover, H_2O_2 is known to fluctuate under various disease states and environmental conditions; therefore this prompted us to examine if such fluctuations could influence the accumulation of drugs in cells. Examples of factors leading to alterations in H_2O_2 levels and oxidative stress include diseases such as diabetes mellitus, arthritis, hypertension, ischemia−reperfusion, ethnicity, age, gender, and body mass index.[9−13] A number of these factors have long been known to influence the pharmacokinetics of drugs and therefore the pharmacodynamic response of drugs but for reasons not related to H_2O_2; therefore this research has the potential to allow us to obtain a more complete mechanistic insight into the observed variability.

In this work we examined the effects of physiologically relevant levels of H_2O_2, which was introduced to cells grown in culture. The influence of the cellular uptake of two model compounds was subsequently evaluated with or without H_2O_2 pretreatment. Dramatic increases in the intracellular accumulation of drugs were observed following H_2O_2 pretreatment. To investigate the mechanism, we evaluated three likely H_2O_2-induced changes to cells that could foster the observed increases in drug accumulation. These were (1) alterations in intracellular pH, (2) diminished plasma membrane integrity, and (3) changes in plasma membrane fluidity. Only the latter of the three was found to change to a significant extent. Collectively, the data suggest that the accumulation of drugs in cells may increase as a consequence of H_2O_2-mediated alterations in plasma membrane dynamics.

Experimental Section

Cell lines and Reagents. Daunorubicin was purchased from Oakwood Products Inc. (West Columbia, SC). Lucifer Yellow, 2′,7′-difluorescein (Oregon Green 488), 1,1′-dioctadecyl-3,3,3′,3′-tetramethyllindocarbocyanine percholate ($DiIC_{18}$), and (5- and 6-) carboxyl SNARF1 AM acetate ester were purchased from Molecular Probes (Eugene, OR). WST-1 was obtained from BioVision (Mountain View, CA). Hydrogen peroxide 30% in water was purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals and reagents were obtained from Sigma (St. Louis, MO).

HL-60 cells were kindly provided by Dr. Yueshang Zhang (Arizona Cancer Center, University of Arizona). Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM Hepes, 1 mM sodium pyruvate, 0.1% penicillin, 0.1% streptomycin and were maintained at a density of 1×10^5 to 1×10^6 cells/mL. CCD-1064SK human foreskin fibroblasts were purchased from ATCC (Manassas, VA) and were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin. All cells were grown at 37 °C in a humidified 5% CO_2 atmosphere. All experiments with cells were conducted between passage numbers four and six.

H_2O_2 Cytotoxicity Evaluations. Cell sensitivity to H_2O_2 was determined using WST-1 according to the manufacturer's protocol. Briefly, HL-60 cells were seeded in triplicate in 96-well culture plates at 5×10^4 cells/well and exposed to a range of hydrogen peroxide concentrations for a period of 2 days. At this time 10 μL of WST-1 reagent was added to each well and the plates were returned to the incubator for an additional 2 h. The absorbance of each well was measured at 450 nm in a Thermo Electron microplate reader (Waltham, MA). IC_{50} value experiments with the fibroblasts were identical to HL-60 cells except that the seeding density was 1×10^4 cells/well and the cells were allowed to adhere

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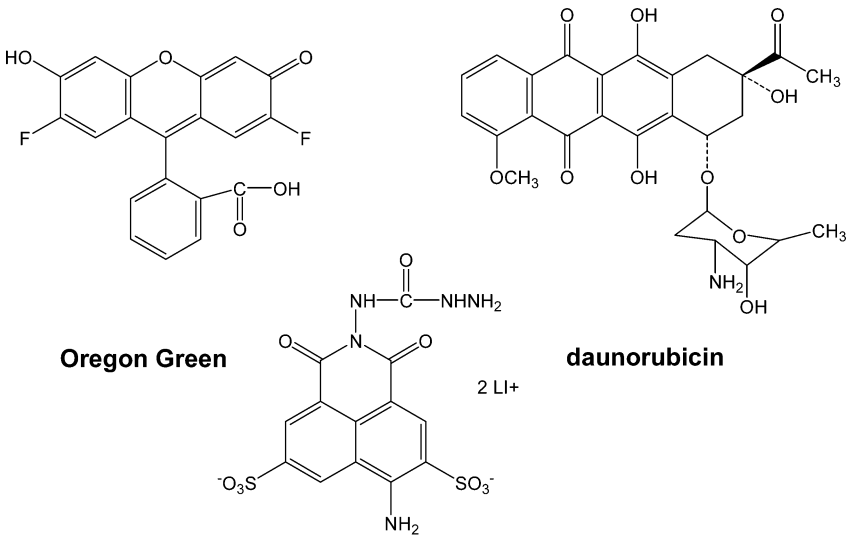

Figure 1. Structures of fluorescent molecules used to evaluate the influence of H_2O_2 on plasma membrane permeability. Both Oregon Green and daunorubicin are relatively hydrophobic and membrane permeable. Oregon Green is a weakly acidic molecule. Daunorubicin is weakly basic. Lucifer Yellow is a polar molecule known to be membrane impermeable.

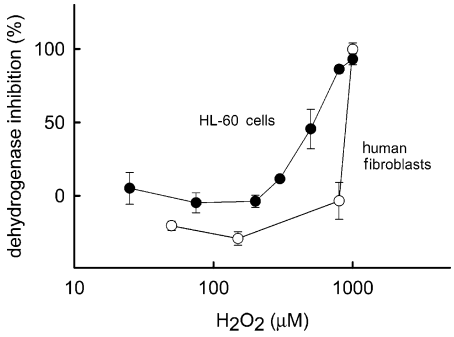

Figure 2. Cytotoxicity evaluations for H_2O_2 on the human leukemic cell line HL-60 (●) and on a human skin fibroblast (○). Concentrations of H_2O_2 used in this work were 50 μM since it is close to being physiologically relevant and did not display any significant toxicity to cells. Reported values are means ± SD for three independent determinations.

overnight prior to hydrogen peroxide additions. IC_{50} was defined as the concentration of drug causing 50% dehydrogenase inhibition as compared to control cells.

Drug Accumulation Studies. Using results from H_2O_2 IC_{50} evaluations we chose to incubate cells with 50 μM H_2O_2, which is a concentration that has minimal antiproliferative effects on either cell line. Fibroblasts were seeded at a density of 1×10^5 cells/mL and allowed to adhere overnight. HL-60 cells were seeded at a density of 5×10^5 cells/mL. Medium containing 50 μM H_2O_2 was added to the cells and placed in the cell culture incubator for 2 days, after which time cells were washed three times with warm PBS. Cells were subsequently exposed to Oregon Green 488 (OG, 1 μM for HL-60 cells and 2 μM for fibroblasts), daunorubicin (DNR, 50 nM, both cells), or Lucifer Yellow (LY, 2.2 mM, both cells). The cells were incubated with OG and DNR for 3 h and LY for 30 min. Following drug exposure cells were washed twice with warm PBS to remove unincorporated drug and were viewed using a Nikon Eclipse 80í microscope equipped with epifluorescence. The FITC filter set was used to view the LY and OG. The Texas Red filter set was used to view the DNR. Images were captured using an Orca ER camera (Hamamatsu Corp., Japan) and analyzed by MetaMorph version 6.2 (Universal Imaging Corp.). With microscope settings kept constant H_2O_2 treated cells were compared to untreated control cells to observe differences in drug accumulation.

Cytosolic pH Measurements. H_2O_2 treated fibroblasts were compared to control fibroblasts to determine if the reagent caused any differences in cytosolic pH. Using (5- and 6-) carboxyl SNARF1 AM acetate ester, a pH-sensitive probe, cytosolic pH was determined using a protocol outlined in our earlier work.[14] Briefly, following H_2O_2 treatment, as previously described, cells (10^7) were incubated with 4 μM (5-and 6-) carboxyl SNARF1 AM acetate ester for 1 h. Cells were then washed twice with warm PBS, resuspended in PBS (pH 7.4), and placed in a cuvette. Using a PTI spectrofluorometer, emission intensities were measured at 580 and 640 nm while using an excitation of 514 nm. The ratios of the emission intensities were used to determine the cytosolic pH as referenced.[15]

Fluorescence Recovery After Photobleaching (FRAP). Fibroblasts with or without H_2O_2 treatment were incubated

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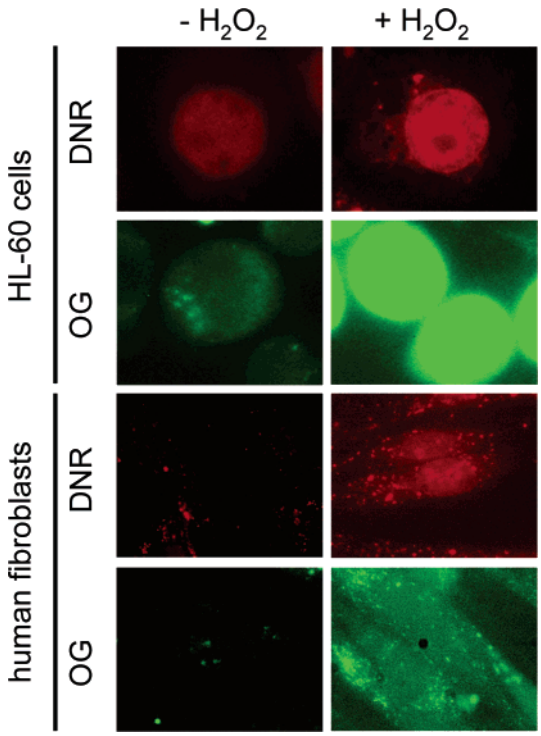

Figure 3. H_2O_2 pretreatment increases the cellular accumulation of daunorubicin (DNR) and Oregon Green (OG) in both HL-60 cells (top micrographs) and human skin fibroblasts (bottom micrographs). Designated cells ($+H_2O_2$) were incubated in culture media supplemented with 50 μM H_2O_2 for 2 days, washed, and subsequently incubated for 3 h with Oregon Green (1 μM for HL-60 cells and 2 μM for fibroblasts) or daunorubicin (50 nM, both cells) in cell culture media at 37 °C. Cells were subsequently washed and visualized using a fluorescence microscope. Identical microscope settings were employed to allow for meaningful comparisons. Micrographs are representative of at least 5 separate trials.

with 100 μM $DiIC_{18}$ in Hanks' Balanced Salt Solution (HBSS) for 10 min to allow the lipophilic dye to incorporate into the plasma membrane. Cells were then washed twice with warm HBSS and viewed with a LSM 510 Zeiss confocal microscope at room temperature. A defined rectangular region of interest (ROI) was set and kept constant in size throughout the experiment. A designated area of the cell plasma membrane was photobleached using a 488 nm argon laser exciting at full power for 3 s. Images were collected at 565 nm before and after the photobleaching at 100 ms intervals for a total of 11 s. Images were monitored for diffusion of unbleached dye from areas adjacent to the bleached region using ImageJ (NIH software). The fluorescence intensity of the photobleached region was quantified as a function of time using cells with and without H_2O_2 pretreatment.

Results and Discussion

Drug Selection and Evaluation of H_2O_2 Toxicity. To examine the effects of H_2O_2 on drug accumulation, two cell lines were chosen for investigation. The first was the human leukemic cell line HL-60, and the second was a normal human skin fibroblast. For each of the cell lines it was important that concentrations of H_2O_2 presented to cells were reasonably physiologic and were not significantly toxic. Others have suggested that concentrations of H_2O_2 below 50 μM were nontoxic to most cells.[8] To investigate the toxicity of H_2O_2 in the cells used in this study we evaluated H_2O_2 IC_{50} values. As is shown in Figure 2, the IC_{50} values for the HL-60 cells and for the human fibroblasts were well above the 50 μM concentration used in our evaluations. Moreover, using a Trypan Blue exclusion method we show that there is negligible growth inhibition at this concentration (See Supplemental Figure 1 in the Supporting Information).

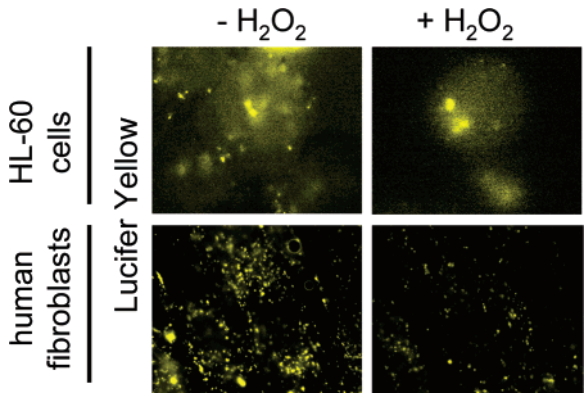

Figure 4. The influence of H_2O_2 on the plasma membrane permeability of a membrane impermeant probe Lucifer Yellow in HL-60 cells (top panels) and in human fibroblasts (bottom panels). See Figure 3 for experimental details. Lucifer Yellow was incubated with all cells at a concentration of 2.2 mM for 30 min in cells with ($+H_2O_2$) or without ($-H_2O_2$) pretreatment. Intracellular punctate staining observed in the micrographs is typical of endocytic uptake of polar molecules such as Lucifer Yellow.

We chose two model compounds for this investigation, namely, the fluorescent weakly basic anticancer agent daunorubicin and the fluorescent weakly acidic dye Oregon Green (see Figure 1 for structures). Evaluating the fluorescence of cells with and without H_2O_2 treatment allowed us to visually document changes in drug accumulation and intracellular distribution. All settings on the fluorescence microscope were kept constant to allow for meaningful comparisons. In Figure 3, one can observe significant increases in accumulation for both compounds following H_2O_2 pretreatment. Drug accumulation was also investigated in cells experiencing hydrogen peroxide pretreatment at variable hydrogen peroxide concentrations and exposure times (Supplemental Figure 2 in the Supporting Information). There was found to be no correlation of drug accumulation with either hydrogen peroxide exposure time or hydrogen peroxide concentration.

Investigating the Mechanism for H_2O_2-Induced Drug Accumulation. One reason for the observed increase in accumulation seen with daunorubicin could be rationalized if the cytosolic pH decreased after H_2O_2 pretreatment. This was an important consideration since hydrogen peroxide at

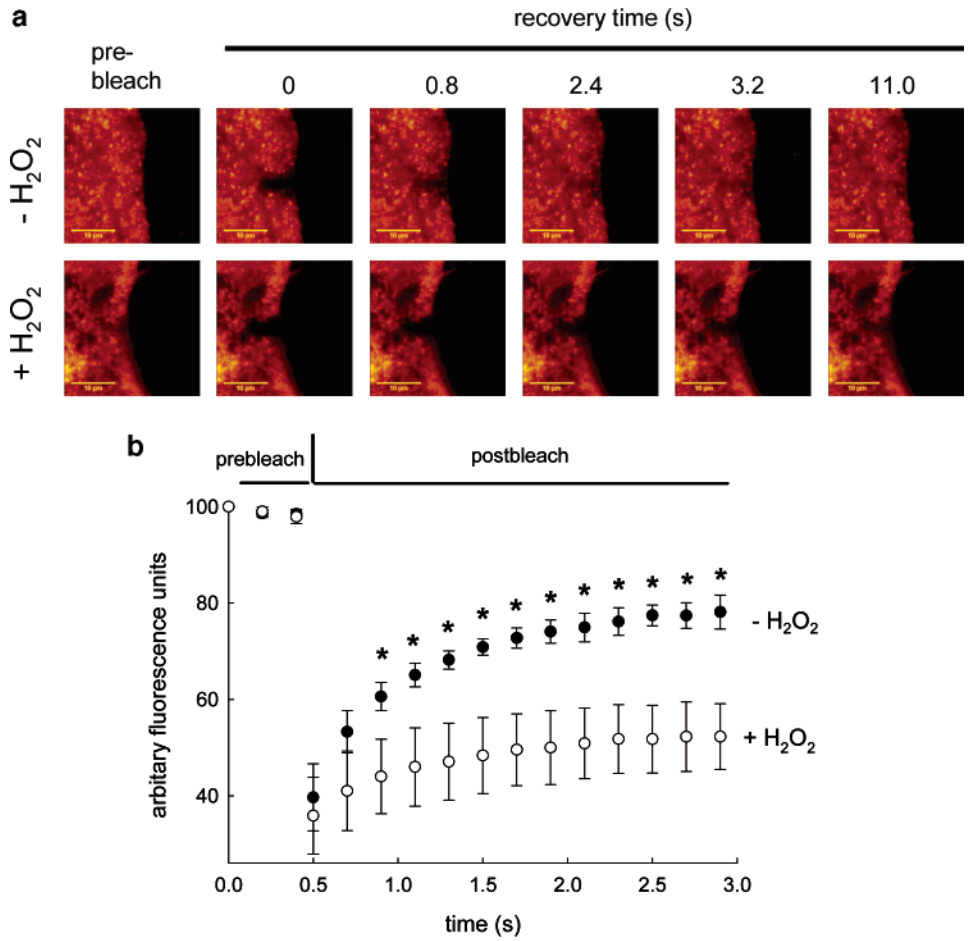

Figure 5. Analysis of membrane fluidity in fibroblasts with ($+H_2O_2$) or without ($-H_2O_2$) pretreatment by using a fluorescence recovery after photobleaching method (FRAP, see Experimental Section). Cells were treated with or without H_2O_2 as indicated on the legend. Cells were subsequently labeled with 100 μM $DiIC_{18}$ for 10 min, which specifically labels the plasma membrane. (a) Micrographs of plasma membrane containing $DiIC_{18}$ before photobleaching (left micrographs) and micrographs of the bleached area as a function of time. The presented image is representative of at least three trials. (b) Quantitative analysis of the fluorescence intensity of the photobleached area of the plasma membrane relative to prebleached levels as a function of time are plotted. Recovery of fluorescence with control (●) and H_2O_2 pretreated cells (○) over a 3 s time period are presented. The recovery time is significantly faster in cells without H_2O_2 pretreatment ($-H_2O_2$) than in cells pretreated with the agent ($+H_2O_2$) suggesting that H_2O_2 pretreatment causes a decrease in membrane fluidity. Data are the mean of three independent experiments ± SD. Data points for treated and control cells at H_2O_2 concentrations indicated with "*" are statistically different ($p < 0.05$).

slightly higher levels (100 μM) had been previously shown to decrease the cytosolic pH significantly.[16] If this were the case, the lower pH could make pH partitioning into the cytosol more favorable for a weakly basic drug like daunorubicin because of the increase in ionization (and a reduction in membrane permeability) when the drug enters the cytosol. One reason for selecting Oregon Green for evaluation in this study was that it was fluorescent and weakly acidic. Correspondingly, if cytosolic pH were to decrease, this could actually lead to decreases in cytosolic concentrations for this compound after H_2O_2 pretreatment, as would be in agreement with pH-partitioning theory. As is shown in Figure 3, the levels of Oregon Green also were significantly elevated in cells treated with H_2O_2, which is consistent with little change in cytosolic pH caused by H_2O_2. In order to further confirm this, cytosolic pH in HL-60 cells with or without H_2O_2 pretreatment was experimentally determined and the pH was shown not to change to significant enough extent to alter drug accumulation (i.e., observed pH values were 7.57 ± 0.02 and 7.50 ± 0.03, for cells with and without H_2O_2 treatment, respectively, $n = 3$, P-value < 0.04). Together, these findings strongly reduce the possibility that changes in intracellular pH could account for the observed changes in drug accumulation illustrated in Figure 3.

A second possibility for increased accumulation of these agents following H_2O_2 pretreatment could occur if the physical integrity of the plasma membrane was compromised as a result of the treatment. In other words, it might be

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possible that the H_2O_2 treatment could increase the "leakiness" of the plasma membrane which could facilitate the diffusion of drugs into the cytosol. To investigate this possibility we incubated cells with or without H_2O_2 pretreatment with Lucifer Yellow. This fluorescent dye is well-known to be membrane impermeable due to its polar nature (see Figure 1), and if the plasma membrane integrity was compromised we would expect to see increases in cytosolic accumulation of this molecule following H_2O_2 pretreatment. As is shown in Figure 4 there were no significant increases in cytosolic levels following the H_2O_2 treatment suggesting that the plasma membrane remained intact. Lucifer Yellow, as with any cell membrane impermeable molecule, typically enters cells only by fluid phase endocytosis. This is visually observed as punctate staining occurring in both cells with or without treatment, which can be observed in Figure 4.

A third possible explanation for enhanced drug accumulation following H_2O_2 pretreatment could be expected to occur if the agent caused changes in the fluidity of the plasma membrane. To evaluate this we utilized an increasingly popular technique for this purpose referred to as fluorescence recovery after photobleaching (FRAP).[17] Briefly, a fluorescent molecule which incorporates specifically into the plasma membrane is incubated with cells. Then a strong fluorescent beam is focused on a small defined area of the plasma membrane to bleach the molecules in the beam's path. The rate at which unbleached molecules from surrounding portions of the plasma membrane diffuse to the bleached area is an indicator of plasma membrane fluidity. When comparing the recovery rate following bleaching we routinely observed that untreated cells were able to recover faster than cells treated with 50 μM H_2O_2 (Figure 5). This result suggests that upon exposure to H_2O_2 the plasma membrane becomes more rigid. This is consistent with the work of others who have shown that oxidative stress typically leads to decreases in membrane fluidity.[18,19] Intuitively, it would seem logical that increases in membrane fluidity should favor passive permeability; however, this is not the case. This phenomenon is actually consistent with the recent work of Sharma and colleagues who have evaluated a number of different intestinal absorption enhancers and found a correlation between membrane rigidity levels and increased passive permeability of a number of drugs.[20]

It is important to point out that we do not believe this effect is caused by a H_2O_2 mediated reduction in the activity of drug transporter proteins that are often present on the plasma membrane of multidrug-resistant cancer cell lines. Although we have not evaluated the fibroblast for expression of transporters, we have previously evaluated the HL-60 cell line and did not identify any transporters associated with this cell line using commercially available antibodies to common transporter proteins.[14]

The demonstrated correlation between H_2O_2 exposure and the concomitant increase in drug accumulation represents a substantial finding. Interestingly, additional studies (see Supplementary Figure 2) investigating the time and concentration dependence suggest that higher concentrations of H_2O_2 do not lead to more pronounced accumulation as may be expected. The results may suggest that patients experiencing oxidative stress (or an increase in H_2O_2 levels) may have an increased response to a given dosage of a drug relative to a patient with decreased oxidative stress, or those patients chronically taking antioxidants with their medications. This could be a very important factor in our continued efforts to provide more individualized dosing of drugs. Moreover, localized areas of the body receiving enhanced oxidative stress, such as the case with ischemia and reperfusion, may receive higher amounts of drugs relative to other areas of the body.

Conclusion

We believe that in the future it may be warranted to consider a patient's level of oxidative stress and/or H_2O_2 levels prior to making appropriate dosage recommendations for drugs with very narrow therapeutic indexes. Moreover, this work should significantly aid in the long-term goal of gaining a complete understanding, on a mechanistic level, of the myriad reasons responsible for variations in dose−response relationships among patients.

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Supporting Information Available: Figures depicting the influence of H_2O_2 exposure on the viability of the human leukemic cell line HL-60 and human skin fibroblasts using the Trypan Blue exclusion assay (Supplemental Figure 1) and results of investigation of drug accumulation in cells experiencing hydrogen peroxide pretreatment at variable hydrogen peroxide concentrations and exposure times (Supplemental Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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