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INHIBITION OF OXIDATIVE INSULT IN CULTURED CELLS BY A NOVEL 6-CHROMANOL-CONTAINING ANTIOXIDANT

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Abstract—N18-RE-105 neuronal hybridoma cells were used in a cell culture system to evaluate the protective effects of a novel 6-chromanol-containing antioxidant, U78517F. First, the incorporation of the compound into the cells was evaluated, using a serum albumin carrier. Then the cells were exposed to peroxide-generating compounds, and the cell injury was estimated from the loss of α-aminoisobutyric acid (AIB) transport. We found that U78517F only protected the cells significantly when the degree of oxidative insult was below a certain limit; the measurable protection of cells by U78517F against either cumene hydroperoxide or H_2O_2 was limited to a narrow range of concentrations of the reactive oxygen species generator. Additionally, the protection provided by U78517F was largely localized to the cell membrane and did not extend to protection of mitochondrial function. The action of U78517 was fully consistent with a direct radical scavenging in the cells. The results indicate that the following factors must be taken into account for evaluation of antioxidants in cell culture: (a) the delivery of a compound to cells, especially when the compound is lipophilic; (b) the nature and extent of the oxidative insult used to evaluate protection; and (c) the location of the protective agent in the cells.

Key words: antioxidant; H_2O_2; cumene hydroperoxide; reactive oxygen species; free radicals; radical scavenging; cumulative Gaussian distribution function

In the process of designing and evaluating agents to reduce the damage induced by ROS† in nerve tissues, it is important to develop assays that allow one to characterize the biochemical activity of potential protective agents. A large number of potentially damaging species may be generated at a site of injury, and the introduction of antioxidants into tissue is complicated by a variety of drug delivery problems. Thus, experimental characterization of ROS and protection from ROS in tissues are not trivial issues.

To develop a system that will allow one to characterize the oxidative injury and, ultimately, relate it to the protection by antioxidants, we have been emphasizing the development of cell culture systems where, at the present time, neither the characteristics of ROS relevant to toxicity nor the biochemical mechanism(s) important to the function of anti-oxidants is well documented. Thus, it is important to have novel assays that allow one to measure the insult at the cellular level, as well as the potential protection provided by an antioxidant. In cell cultures, both the amount of ROS introduced into the cell culture and the amount of an antioxidant incorporated into the cells can be controlled and manipulated. Furthermore, knowledge of where and how a given toxic agent acts within a cell will reveal how a protective agent works in modulating ROS-mediated toxicity. Thus, cell culture systems are well suited to the quantitative assessment of the cellular biochemistry of ROS and antioxidants.

To generate toxic effects in cells in culture, a number of ROS generators have been used. During the autooxidation of 6-OHDA in cell culture medium, H_2O_2 is produced, in addition to quinones, superoxide, and other ROS [1–4]. H_2O_2 freely penetrates the cell membrane and affects a number of known biochemical systems within the cell including: activation of the hexomonophosphate shunt with activation of the glutathione redox cycle [5], increased formation of intracellular oxidized sulfhydryls [6], DNA damage [7], loss of intracellular NAD^+ [7], activation of poly-ADP-ribose polymerase [8], a rise in intracellular free calcium [5], and a rapid decrease in ATP [9]. All of these processes occur before loss of plasma membrane integrity, which is determined by using vital stains, by leakage of preloaded ^{51}Cr, or by measuring lipid peroxidation [5, 9]. Thus, the markers described above are used to measure the effects of ROS on intracellular processes. In addition, we have shown that cell surface markers can also be used as markers of ROS action [10]. By virtue of its lipophilicity, cumene hydroperoxide is an example of an ROS generator that remains localized predominantly to the cell membrane and has been shown to initiate lipid peroxidation in cell membranes [11–13].

To quantitate cell toxicity, an assay should relate damage to cell structure and function. The use of a well-defined marker of activity in the membrane allows assessment of the chemistry of radical generation and its relationship to the inactivation of membrane-specific functions. Amino acid transport has been used to assess cell membrane integrity and function [14]. The transport of AIB requires not only the functional integrity of the transport proteins themselves, but also that of the energy metabolism of the cell, since AIB transport is energy dependent [15]. Finally, the membrane must remain intact in order to retain transported amino acids intracel-

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† Abbreviations: AIB, α-aminoisobutyric acid; ROS, reactive oxygen species; 6-OHDA, 6-hydroxydopamine; N18 cells, N18-RE-105 neuronal hybridoma cells; DME, Dulbecco's modified Eagle's medium; EBSS, Earle's balanced salt solution; and MTT, 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

lularly. The many systems that must be preserved in order to sustain AIB transport provide a sensitive means of measuring the several effects of oxidants on cellular biochemistry and the physical integrity of the cell. To protect the AIB transport system against freely diffusible ROS, a compound must protect the transporter protein itself, the energy-generating systems of the cell, and the physical integrity of the membrane. However, if a radical generator that is localized in the membrane is used, as is the case with cumene hydroperoxide, then specific properties of both the damage to the transporter itself and the protective action by a lipophilic potential antioxidant may be measured selectively. Independent measurement of the metabolic function of the cells will allow one to assess damage to the energy-generating systems of cells. For example, staining of mitochondria with a tetrazolium dye can be used as a marker of mitochondrial function [16].

We have shown that the localization and extent of injury induced in culture in a neuronal cell line are dependent on the type of ROS generator used [10]. Furthermore, the uptake of [^{3}H]AIB can be used as a marker for cell surface peroxidative damage induced by cumene hydroperoxide and H_2O_2, since the inactivation is directly proportional to the concentration of free radicals produced. Thus, the application of the appropriate kinetic laws leads to precise quantitative analysis of the inactivation process. On this basis, we proposed a mechanism for damage to the transporter(s) of [^{3}H]AIB [10]. Here, we apply the same mechanism to the analysis of protection by an antioxidant. This analytical process does not require that the details of the mechanism of inactivation or of protection of the transport systems be characterized in every respect. Instead, the analytical approach used here provides a framework that is necessary for the precise assessment of protection, thus enabling us to compare quantitatively the degree of protection provided by different doses of an antioxidant. In the work described below, we evaluate the ability of a novel 6-chromanol-containing compound, designated U78517F, to protect AIB amino acid transporter in the cell membrane from free radicals generated by ROS. We show that protection of these specific biochemical markers in cells depends not only on the concentration of the antioxidant present, but also on the degree of the ROS insult.

MATERIALS AND METHODS

Reagents

H_2O_2 was purchased from the Sigma Chemical Co. (St. Louis, MO), and cumene hydroperoxide was purchased from the Aldrich Chemical Co. (Milwaukee, WI). U78517F was synthesized at The Upjohn Company, and its structure has been given in previous publications [13, 17, 18].

Cells and cell culture

N18 cells are a neuronal hybridoma cell line derived from a mouse neuroblastoma and a Fisher rat embryonic neural retina [19]. N18 cells were plated (2.5×10^4/well) into flat-bottom 96-well plates (Corning Glass Works, Corning, NY) coated with poly-*dl*-lysine (molecular weight 30,000–70,000) (Sigma Chemical Co.). Cells were grown overnight in DME medium supplemented with 44 mM sodium bicarbonate, 16 μM thymidine, 100 μM hypoxanthine, 1 μM aminopterin, and 5% fetal bovine serum and used in experiments the following morning.

Complexing of U78517F with BSA

U78517F was dissolved in 95% ethanol and subsequently evaporated under nitrogen to a volume of approximately 10 μL. Then 1 mL of 3% BSA in PBS, pH 7.4, was added to result in a total drug concentration of 0–5 mM and mixed end-over-end for 1 hr at room temperature. One hundred microliters of the compound in suspension was taken to measure the total amount of compound added, and the remaining sample was centrifuged at 200,000 *g* for 10 min in an ultracentrifuge at room temperature. The supernatant was filtered through a 0.2 μm Acrodisc filter, and the concentration remaining in solution was determined by HPLC using electrochemical detection.

Delivery of U78517F to N18 cells

N18 cells were washed with 200 μL/well of EBSS immediately before use, and then 200 μL/well of DME containing 3% BSA loaded with U78517F at concentrations as noted was added. The cells were incubated at 37° for 1 hr, washed twice with 2 mL EBSS, and then incubated or 2 hr in DME containing 1% ITS$^+$, a nutritional supplement containing insulin, transferrin, and selenous acid (Collaborative Biomedical Products, a division of Becton-Dickinson, Bedford, MA). Cells were removed from 96-well plates by incubating each well with 100 μL of PBS without calcium or magnesium and containing 1 mM EDTA for 10 min at 37°. Cells from a single 96-well plate were washed twice with 10 mL of PBS and then extracted using a Folch extraction [20]. The Folch extraction was done by adding 100 μL of 6 N HCl to 1 mL of washed cells in PBS. Then, 4.5 mL of chloroform:methanol (1:2, v/v) was added to the cells, and the tube was vortexed. An additional 1.5 mL of chloroform and 1.5 mL of 2 M potassium chloride were added followed by vortexing. The extract was centrifuged at approximately 500 *g* for 10 min, and the aqueous phase was discarded. The organic phase was evaporated to dryness under nitrogen, and the extract was brought up in 400 μL with 95% ethanol. Lipid phosphorus was determined on the extracted samples according to the procedure described by Bartlett [21]. Aliquots were taken for quantitation of U74006F or U78517F.

Quantitation of U78517F by HPLC

Weighed standards of U78517F were dissolved in 95% ethanol and quantitated by HPLC. The column used was a Supelcosil (Bellefonte, PA) 5.0 cm × 4.6 mm ID LC-CN column (5 μm). Isocratic elution was used with a mobile phase of 40% (v/v) acetonitrile in 0.02 M disodium phosphate, pH 7.0, containing 0.9% tetrabutyl ammonium perchlorate as an ion pairing agent. Samples eluted from the column at 4–6 min and were detected using a Waters (Milford, MA) model 460 electrochemical detector with a glassy carbon electrode set at 700 mV. Peaks were integrated using a Waters Maxima 820 data analysis system. Samples from BSA solubilization experiments and cell extracts were quantitated based on their peak area compared with known standards of U74006F or U78517F.

Amino acid uptake and mitochondrial reducing activity

N18 cells were washed twice with 100 μL of EBSS and incubated for 1 hr at 37° with EBSS containing 0–1 mM H_2O_2 or cumene hydroperoxide. Cells were then washed twice with 100 μL of EBSS and incubated with 100 μL of EBSS containing 1% ITS$^+$. To evaluate amino acid transport, cells were pulsed for 1 hr at 37° with 100 μL of EBSS containing 1% ITS$^+$ and 0.2 μCi of the nonmetabolizable amino acid analog α-[*methyl*-^{3}H]aminoisobutyric acid ([^{3}H]AIB) (33.5 Ci/mmol, NEN Research Products, Boston, MA). Cells were washed twice with 100 μL of ice-cold PBS and lysed by the addition of 0.2 mL of 0.2 N NaOH. The NaOH was neutralized by adding 0.2 mL of 0.2 N HCl. Neutralized samples were combined with 10 mL of Beckman Ready Safe liquid scintillation fluid in a 20-mL scintillation vial, and their radioactivity was determined in a Packard Tri-Carb scintillation spectrophotometer. The MTT assay used to evaluate mitochondrial function was described previously [16].

Equations and data analysis

The equations describing the assessment of damage generated by ROS in cells, particularly at the cell membrane, have been described previously [10, 13]. The use of the cumulative Gaussian distribution function has been described in detail [10]. Briefly, the square root of the concentration of the ROS generator, e.g. H_2O_2 or cumene hydroperoxide, was used as the independent variable in fitting the cumulative Gaussian distribution function to the data. The use of the square root of the generator concentration is based on the basic kinetics of radical generation, Equation 1, below.

In addition to the multi-site inactivation represented by the cumulative Gaussian function, direct, single-hit inactivation of transporter sites can be delineated by an equation described previously [10]. The following is an extension of the previous derivation for the evaluation of single-hit inactivation of transporters, since it includes incorporation of an additional term to account for radical quenching by an agent added to the cells.

As shown previously [10, 13], the fundamental chemistry of radical formation can be described as:

$$C \xrightarrow{k_i} R^{\cdot} + R^{\cdot} \xrightarrow{k_t} \text{Recombination products} \qquad (1)$$

where C is a radical-generating species, such as cumene hydroperoxide, R$^{\cdot}$ is free radical, k_i is the rate constant of formation of free radicals, and k_t is the rate constant of spontaneous radical recombination.

In the presence of an exogenous radical scavenger, L, the quenching can be described as:

$$R^{\cdot} + L \xrightarrow{k_\ell} X^{\cdot} \qquad (2)$$

where X$^{\cdot}$ is a stable radical, and k_ℓ is the rate constant of reaction between R$^{\cdot}$ and L.

Then, for the early phases of the reaction, the steady state of free-radical generation is given by:

$$\frac{dR^{\cdot}}{dt} = k_i C_0 - k_t R^{\cdot 2} - k_\ell L_0 R^{\cdot} = 0 \qquad (3)$$

where C_0 is the initial concentration of radical generator, and L_0 is the initial concentration of a radical scavenger.

This equation is quadratic in R$^{\cdot}$, with the solution:

$$R^{\cdot} = \frac{-k_\ell L_0 + \sqrt{(k_\ell L_0)^2 + 4k_i C_0 k_t}}{-2k_t}$$

In the absence of a scavenger, the steady-state concentration of radical (R$^{\cdot}$) is directly proportional to the square root of the concentration of the radical generator, C_0. Incorporating this term into the equation for AIB uptake, as was shown previously [10], results in the following equation describing the relationship between uptake (U) of [^{3}H]AIB, the concentration of free-radical initiator (C_0), and the concentration of free-radical scavenger (L_0):

$$U = A_0 e^{-k_d t_p}\left[-\frac{k_\ell L_0}{2k_t} + \sqrt{\left(\frac{k_\ell L_0}{2k_t}\right)^2 + \frac{k_i C_0}{k_t}}\right][1 - e^{-k_0 t_t}]. \qquad (5)$$

The following symbols are used: A_0, uninhibited transport in the absence of free radical-induced damage; k_0, a rate constant for the exit of AIB from cells, which is linear for the conditions used in the experiments described below; t_p, the time of exposure to the radical generating species; and k_d, the rate constant for inactivation of the transporter by free radicals. When t_t, the time of the transport assay, is long so that transport reaches a dynamic equilibrium, as in the experiments described below, the last term, $1 - e^{-k_0 t_t}$, is approximately equal to 1 and can, therefore, be dropped from further consideration.

For the purposes of curve-fitting, the equation then can be expressed in terms of three independent parameters:

$$U = P(1)\, e^{-60[-P(3)+\sqrt{P(3)^2+P(2)C_0}]} \text{ where: } P(1) = A_0 \qquad (6)$$

$$P(2) = \frac{k_\ell k_d^2}{k_t}$$

$$P(3) = \frac{k_\ell L_0}{2k_t} k_d$$

P(1) describes the kinetics of uptake/transport. P(2) is the square of the "radical propagation factor," defined previously [10]. P(3) describes a "protection index" including terms for k_t and for L_0. Fitting this equation to the data measures how well simple radical scavenging by the protective agent can account for protection. The analysis also yields a parameter measuring the protection provided by L_0.

Analysis of data was performed using a nonlinear least squares regression program [22], and theoretical curves were generated using Equation 6 with the best-fit parameters. Statistical significance was assessed by the Akaike information criterion [23] and an F-test, as described by Motulsky and Ransnas [24].

RESULTS

Delivery of U78517F to N18 cells

The quantity of U78517F delivered to cells was linear with the BSA complex and did not appear to be saturable over the range of concentrations tested (Fig. 1). The maximal concentration of U78517F tested, 54 μM, resulted in 7.0 mol% in cells. Note the considerable scatter of the data, indicating that although BSA delivered

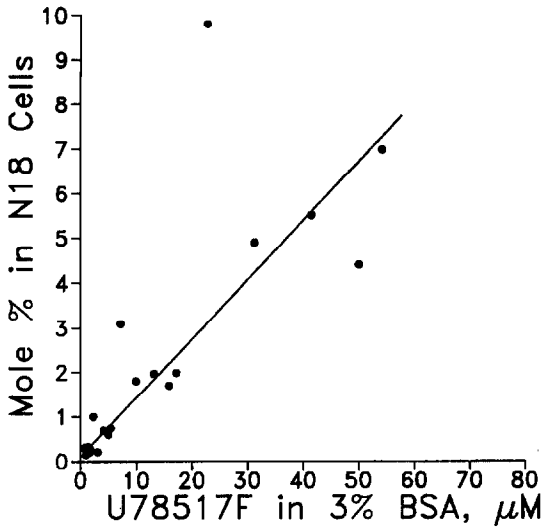

Fig. 1. Association of U78517F with BSA, and association of the complexes with N18 cells. U78517F was mixed with 1 mL of PBS containing 3% BSA and incubated for 1 hr. Association of U78517F with BSA was determined by HPLC from samples following ultracentrifugation at 100,000 *g* of the compound: BSA mixtures. The concentration of U78517F associated with BSA following ultracentrifugation was expressed as a nominal molar concentration. The amount of U78517F delivered to cells at the indicated concentrations was determined by HPLC following Folch extraction from the cells, as described in Materials and Methods. The least squares linear regression fit to the U78517F data is shown as a solid line. The data were pooled from three separate experiments, and each point is the average of duplicate samples.

U78517F to cells in culture, the amount of U78517F ultimately associated with the cells varied significantly even when the concentration of the U78517F–BSA complex was carefully controlled. Therefore, for each experiment described below, we measured directly the amount of compound incorporated in cultures of cells.

Effects of U78517F on AIB transport

Before considering the protective effects of membrane-bound antioxidant, it was necessary to delineate the kinetics of AIB transport under our experimental conditions. For these experiments we used approximately 0.1 μM [^{3}H]AIB in the transport experiments, a concentration well below the K_m of approximately 1 mM reported for AIB transporters [25, 26]. The extracellular concentration of [^{3}H]AIB was not depleted significantly during the time course of the experiment and, thus, pseudo-first-order conditions applied relative to the intracellular concentration of AIB. The concentration of AIB inside the cells ultimately reached dynamic equilibrium with the medium. The presence of U78517F delivered with albumin did not affect AIB transport significantly in N18 cells (data not shown) except at a concentration in the cells higher than 3 mol%, relative to total cellular phospholipid.

Protection of N18 cells from ROS generated from cumene hydroperoxide

Next, we set out to determine if U78517F could protect AIB transport from the effects of cumene hydroperoxide, an oxidative insult more specifically restricted to the plasma membrane than that of H_2O_2. If so, was the protection consistent with simple chemistry of radical scavenging at the plasma membrane? Equation 6 was fitted to the data generated using N18 cells pretreated with U78517F delivered using BSA as the carrier. Both the concentration of U78157–BSA complex added and the amount of U78517F delivered to the cells were measured directly. We calculated the radical propagation factor independently in experiments conducted in the absence of U78517F; then, this constant value was substituted for the radical propagation factor parameter in the equations fitted to the data. Using this approach, the protection index was calculated for different concentrations of U78517F. As shown in Fig. 2, the fit of the equations to data obtained after loading a range of concentrations of U78517F into N18 cells and challenging with a range of concentrations of cumene hydroperoxide was excellent. Similar to the results obtained when H_2O_2 was used, the effect of U78517F in the cells was to shift the concentration-effect curve to the right. Again, more protection was evident at lower concentrations of cumene hydroperoxide, and protection was abrogated at high concentrations of cumene hydroperoxide. In Fig. 3, the protection index is plotted versus the mol%, relative to total cellular phospholipid, of U78517F incorporated into cells, relative to total cellular phospholipid, for two independent experiments. The calculated protection index depended significantly on the concentration of U78517F incorporated into the cells; it was approximately linearly related to the concentration of U78517F below 1 mol% of U78157. Thus, U78517F provided significant protection from cumene hydroperoxide at the plasma membrane, as assessed using AIB uptake. However, at higher concentrations, the protection index leveled off or even decreased. Thus, as delivered by BSA, the protection against cumene hydroperoxide provided by U78157 was most effective over a relatively narrow range of U78517F concentrations.

Protection of N18 cells from ROS generated from 6-OHDA or H_2O_2

Attempts to protect N18 cells from ROS using U78517F were not successful when 6-OHDA was used as the ROS generator (data not shown). This is probably due to the rapid rate of radical generation and the wide variety of radicals and ROS produced by 6-OHDA [10].

As reported previously [10], the concentration-effect curve for H_2O_2 treatment of N18 cells was consistent with a cumulative Gaussian distribution function (Fig. 4), corresponding to action at multiple sites in the cells by H_2O_2 and its products. Since no significant decrease in uptake of AIB was evident at low concentrations of H_2O_2, i.e. 3 μM H_2O_2 or less, the effects of U78517 cannot be assessed at these low concentrations of H_2O_2. At higher concentrations of H_2O_2, addition of U78517F resulted in modest protection against the action of H_2O_2. For example, at 1.1 mol% U78517F, the concentration–effect curve of H_2O_2 was shifted significantly to the right (Fig. 4). U78517F displayed the most protection against 40 μM H_2O_2 or less. No protection was detectable in cells treated with 50 μM H_2O_2 or more.

A range of U78517F concentrations was used to explore the protective effects at various concentrations of H_2O_2. The cumulative Gaussian distribution function

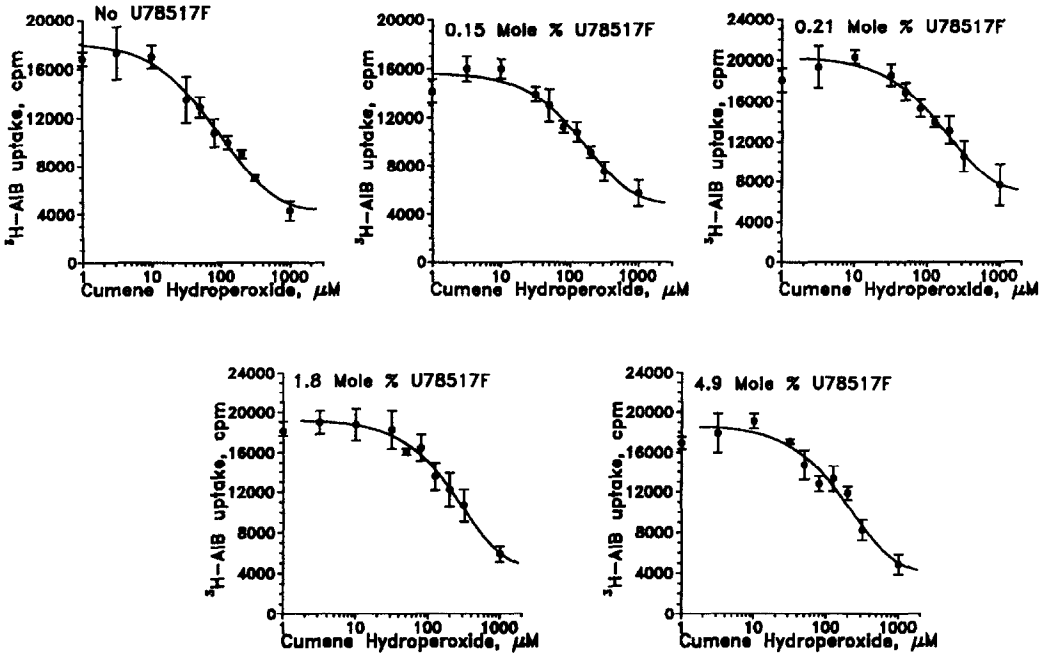

Fig. 2. Protection of AIB transport in N18 cells by U78517F. N18 cells were pretreated with U78517F:BSA complex for 60 min before washing and adding the concentrations of cumene hydroperoxide indicated for an additional 60 min. The cells were washed again before [^{3}H]AIB was added, and uptake was evaluated after 60 more min. The values shown are the means ± SD for 4 replicate measurements from one representative experiment of two experiments carried out. The amount of U78517F associated with the cells was determined by HPLC analysis of Folch extracts of cells incubated in parallel cultures for each concentration of U78517F used. Incorporation is expressed as the mol% of U78517F relative to cell phospholipids and is indicated above the relevant panel in the figure. Equation 5 was fitted to the data using nonlinear least squares regression, and the lines indicate the agreement between the data and the model proposed for the chemistry of inactivation and protection.

was fitted to the data to calculate the mean value for inactivation of AIB transport by H_2O_2 at each concentration of U78517F delivered to the cells. Note that the mean value of the Gaussian distribution corresponds to inactivation of 50% of the uptake of [^{3}H]AIB. By equating damage linearly with loss of AIB uptake, one can define the mean toxic concentration as an IC_{50}. The results are summarized in Table 1. As little as 0.3 mol% of U78517F provided significant protection against H_2O_2, and protection increased modestly to a maximum of approximately a 2-fold rightward shift in the mean concentration of H_2O_2 required to inactivate AIB transport in the presence of 3 mol% or more of U78517F.

We also observed the effects of a range of concentrations of H_2O_2 on the ability of U78517F to protect mitochondrial function, as assessed by the MTT assay. The mean concentration of H_2O_2 required to inactivate mitochondrial function, i.e. the concentration of H_2O_2 resulting in loss of 50% of the MTT staining, was 30 ± 3 μM H_2O_2. This indicates that it takes 1.5-fold more H_2O_2 to inactivate the mitochondrial function than to inactivate the AIB transport under our experimental conditions. No change in the H_2O_2-induced mitochondrial toxicity was detected even at the highest concentration of U78517F used. That is, the mean concentration required to inactivate the mitochondria was 30 μM H_2O_2 in the presence or absence of U78517F. The results indicate that the protection afforded by U78517F is largely restricted to the plasma membrane of the cell.

DISCUSSION

Our results highlight several processes that must be controlled in order to evaluate the effects of oxidative insult on cells and to assess the efficacy of anti-oxidative compounds in cultured cells. First, it is necessary to define the properties of the oxidative insult and to analyze quantitatively the damage to the cells. Characterization of the insult includes its dependence upon the rate of generation of ROS and the shape of concentration–effect curves. Additionally, the cellular location of damage resulting from a particular radical-generating species needs to be determined. Finally, since it is likely that the action of any antioxidant can be overwhelmed in the face of a very large ROS insult, it is necessary to quantitate the action of a potential antioxidant over a range of ROS concentrations.

We restricted our studies to the situation where the protective agent was not present in the solution external to the cells during the oxidative insult. Instead, we loaded the cells with the antioxidant in order to evaluate the efficacy of U78517F in cells without the complication of reactions taking place between the compound and ROS external to the cells. Under these conditions, U78517F was able to protect the N18 cells against the effects of H_2O_2 and cumene hydroperoxide. However, protection was observed only over a relatively narrow range of concentrations of U78517F incorporated in the cells. Also, at high concentrations of H_2O_2 or cumene

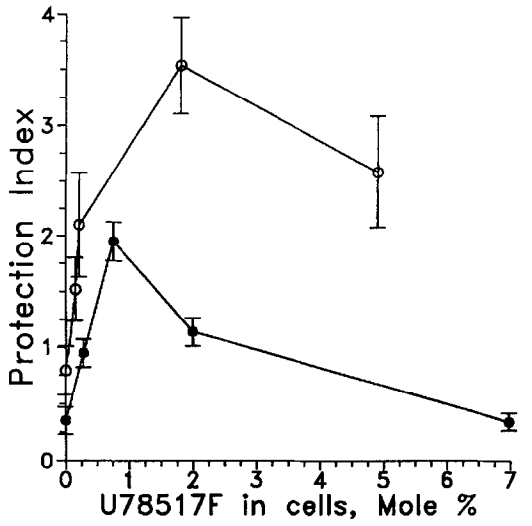

Fig. 3. Relationship between the incorporation of U78517F in N18 cells and protection from cumene hydroperoxide. The protection indices were calculated at each concentration of incorporated U78517F by fitting the data to Equation 5 using nonlinear least squares regression analysis. The protection index indicates the degree of protection provided by a given concentration of U78517 and was calculated for each of the concentrations of U78517F shown in Fig. 2 (○) and for a second, independent experiment (●). The protection index is a summary term that includes the rate constants for free radical generation and spontaneous recombination, as well as the concentration of U78517 and its rate constant for free radical scavenging. The values shown are means ± SD for the protection index calculated from a complete concentration–effect curve of cumene hydroperoxide concentration versus [^{3}H]AIB uptake at a single concentration of U78517F. For example, each panel of Fig. 2 is used to generate a single mean and standard deviation for one protection index shown in this figure.

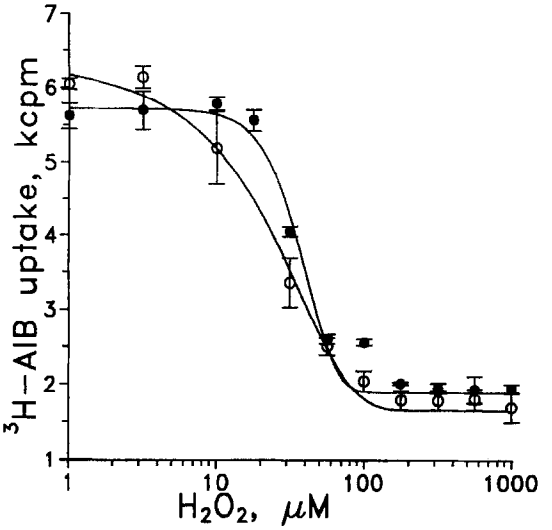

Fig. 4. Effect of H_2O_2 on AIB transport in N18 cells and protection by U78517F. N18 cells were pretreated for 1 hr with culture medium (○) or 2.3 μM U78517F associated with BSA (●); then the cells were washed and exposed to H_2O_2 at the concentrations indicated for an additional 60 min. The cells were washed again, and the amount of [^{3}H]AIB taken up by the cells in 60 min was determined. The treatment resulted in the incorporation of 1.1 mol% U78517F, relative to total cellular phospholipid. The equation for a cumulative Gaussian distribution was fitted to the data using nonlinear least squares regression, and the results are shown as solid lines superimposed on the data. The values shown are means ± SD for quadruplicate determinations at each H_2O_2 concentration. The curve for the cells treated with U78517 was shifted significantly to the right ($P < 0.01$, as determined by an F-test of the fit of a Gaussian curve with a mean H_2O_2 concentration of 22 μM compared with a mean H_2O_2 concentration of 37 μM). The mean concentrations of H_2O_2 resulting in inactivation of AIB transport are presented in Table 1 along with the mean concentrations from a range of concentrations of incorporated U78517F.

hydroperoxide, U78517F was not able to protect the cells. The results indicate that both the type and concentration of the ROS generator and the concentration of the potentially protective agent in cells must be considered in order to design a system where protection of cells is to be measured. No agent is likely to protect cells from a massive and rapid onslaught of radicals; both intracellular and exogenously added protective species can surely be overwhelmed by an insult of sufficient magnitude. Thus, if we are to design experiments to assess the efficacy of potentially protective compounds, we must control very closely the rate of radical production. Additionally, from a practical standpoint, the rate of radical generation at a site of injury in tissue must be low enough to be compensated for by endogenous cellular systems; otherwise, death of the organism would follow post haste. Intervention can probably only be beneficial therapeutically in cases where supplementation of the endogenous antioxidants can provide an edge for survival of some cells without disrupting the metabolic functions of the cells, which depend intimately on chemical oxidation and reduction reactions.

In addition to controlling and understanding the generation of ROS, a cell culture system also allows study of the delivery of potentially protective compounds, as well as evaluation of their relative efficacy once delivered. Especially for compounds that are lipophilic, the use of a carrier is required in order to allow maximal transfer of the compound of interest into the cellular target compartment [27]. Due to the highly lipophilic nature of U78517F, its concentration in aqueous culture medium at physiological pH is negligible. This presents a significant problem when trying to deliver the compounds to cells in culture. If added from a high concentration stock in organic solvent, U78517F molecules will precipitate on dilution into aqueous medium. Therefore, it is necessary to carry the compounds to the cells by some means. We did not want to use detergents, since these can result in cellular damage. We chose albumin since this is an abundant serum protein present *in vivo* with sites specific for association with some hydrophobic compounds [28]. For compounds that are soluble in aqueous solutions at physiological pH, the amount of compound delivered is usually proportional to the concentration of the compound in the extracellular medium. However, for two-phase delivery systems, or those dependent on a carrier molecule like BSA, delivery is determined not only by the nominal concentration of the compound, but also by the concentration of the carrier and the partitioning from the carrier either into the cytoplasm or into the cell membrane. Thus, in the case of a two-phase system, the amount of a compound delivered to cells is not necessarily directly proportional to the amount of compound added extracellularly. If bind-

Table 1. Effect of H_2O_2 on AIB transport in N18 cells

[U78517:BSA] complex in medium (μM)	U78517 in cells (mol%)*	Mean toxic concentration of H_2O_2† (μM)
0	0	22 ± 3
0.7	0.3	30 ± 1
2.3	1.1	37 ± 2
7.2	3.1	40 ± 3
23	9.8	39 ± 3

* Mol% is the ratio of the moles of U78517F incorporated in the cells and moles of the lipid phosphate × 100.

† The mean toxic concentration was determined by fitting the equation for a Gaussian distribution to the data generated after treating a series of microtiter wells containing N18 cells with the concentration of U78517F:BSA complex indicated. For each concentration U78517:BSA, N18 cells were treated with H_2O_2 at each of 10 concentrations ranging from 1 to 1000 μM, as shown in Fig. 4. Four replicate wells were treated at each concentration of H_2O_2. [^{3}H]AIB transport was measured as described in Materials and Methods. The mean toxic concentration corresponds to the concentration of H_2O_2 that results in inactivation of 50% of the total [^{3}H]AIB uptake. The values shown are means ± SD.

ing of the compound to the carrier is not simple and stoichiometric, delivery may be complex.

In conclusion, we set out to explore the biochemical and cellular details of ROS and began to explore how a drug can work to protect cells from an oxidative insult. The toxicity of ROS conforms to relatively simple kinetics and, thus, a cell culture system can be used for exploring cellular and biochemical details of the toxic events. The type of ROS used to generate the radicals, the rate of radical production, and the location of both the toxic species and the potential protective agents must be taken into account in order to begin to understand the biochemistry of the system. Especially in the case of lipophilic compounds proposed for use in protecting against lipid peroxidation, the means of delivering the compounds to cells must also be considered. From these foundations, we will be able to explore the biochemistry and cellular biology of additional antioxidant compounds.

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