Screening of New Antioxidant Molecules Using Flow Cytometry

Serge Ostrovidov,[†] Patricia Franck,[†] Delphine Joseph,[‡] Laurent Martarello,[‡] Gilbert Kirsch,[‡] Francine Belleville,[†] Pierre Nabet,*[,†] and Brigitte Dousset[†]

Laboratory of Medical Biochemistry, School of Medicine, CHU, C.O. Box 34, 54035 Nancy Cedex, France, and Laboratory of Organic Chemistry, Groupe de Synthèse Organique et Hétérocyclique, Faculty of Sciences, University of Metz, 57000 Metz, France

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We present a flow cytometry technique to evaluate the antioxidative properties of molecules on living cells, using a stable murine−murine hybridoma (Mark 3) cell line routinely cultured. Using this technique, intracellular superoxide anions and peroxides were evaluated with dihydrorhodamine (DHR-123) and dichlorofluorescein diacetate (DCFH-DA), respectively. When cells were first incubated for 10 min with either H_2O_2 or the xanthine (X)/xanthine oxidase (XO) system, this flow cytometric technique was capable of evaluating the oxidative stress on cells. Twenty-one new analogues of ellipticine were synthesized and tested for their antioxidative properties compared to vitamin E and Ebselen used as references. A good statistical reflection of the antioxidative activities of these molecules was achieved by analyzing 35 000 cells in each experiment. Among them, the selenated molecule **18** was found to be 10 times more active than Ebselen but 10 000 times less active than vitamin E. Moreover, eight compounds showed glutathione peroxidase-like activities.

Introduction

Reactive oxygen species (ROS) are formed during normal cellular metabolism, but when present in high concentration they become toxic. Lennon et al.[1] and Slater et al.[2] described the relationship between oxidative stress mediated by ROS and cell apoptosis. They reported that exposure of rat insulinoma cells to the oxidative agent 2,4-dimethoxy-1,4-naphthoquinone stimulated proliferation at low concentrations and death by apoptosis and necrosis at high concentrations. Rousseau-Richard et al.[3] observed a positive correlation between the antioxidative activity and the cytotoxic property in a series of hydroxylated ellipticine analogues. We have shown previously[4] that when intracellular antioxidative defenses are overcome by adding H_2O_2 to cultured hybridoma cells, apoptotis is irreversibly induced. Mammalian cells possess intracellular defenses such as superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.16), or glutathione peroxidase (EC 1.11.1.9) in order to protect the cells against excessive levels of free radicals. Also, exogenous addition of compounds such as vitamins (A, E, β-carotene), minerals (selenium, zinc), or proteins (transferrin, ceruleoplasmin, albumin)[5] can provide additional protection. Thus, the search for new nontoxic molecules with antioxidative properties is a very active domain of research.

There are numerous methods to determine the antioxidative properties of a molecule. Some techniques are purely chemical, using fluorescence, chemiluminescence, spectrophotometry, or measurement of O_2 consumption. Moreover, ROS can be evaluated indirectly in cell cultures by enzymatic determination, measurement of malondialdehyde (MDA), or oxidation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Of course, free radicals can be directly determined by NMR, but this is very time-consuming, necessitating large volumes of biological samples, and is not yet widely available.

In the present paper, we describe a useful flow cytometric technique to evaluate the antioxidative properties of various molecules on living cells by using fluorescent probes such as DHR-123 considered to be specific for superoxide anion,[6] as well as DCFH-DA considered to be more specific for peroxides,[7] and other fluorescent probes. With this technique we measured levels of intracellular free radicals at basal state (without oxidative stress) and after oxidative stress induced by H_2O_2 as a main source of peroxides and by the X/XO system as a main source of superoxide anions.[8] Then the effects of putative antioxidant compounds were evaluated by the modifications of these parameters. After validation of our experimental model by the measure of the well-known vitamin E and Ebselen[40] antioxidative activities, we tested a series of 21 new synthetic compounds, analogous to ellipticine, and present the results of this screening. The antioxidative activities of all compounds were considered as efficient when the change in relative fluorescence (Δ) was below the thresholds of relative fluorescence fixed to $\Delta \leq -30\%$ for the fluorescence measured with the probe DHR-123 and $\Delta \leq -50\%$ for the fluorescence measured with the probe DCFH-DA. One of the 21 compounds, the selenated molecule **18**, demonstrated interesting antioxidative properties and was further studied.

Results and Discussion

Screening of Molecules for Their Antioxidative Activity. We screened 21 new analogues of ellipticine for their antioxidative activity, compared to vitamin E and Ebselen (Table 1). The effective molarities cor-

* Corresponding author. Phone: + 33 3 83 85 15 35. Fax: + 33 3 83 85 27 43. E-mail: p.nabet@chu-nancy.fr.
[†] CHU.
[‡] University of Metz.

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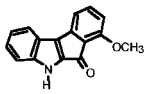
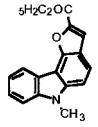
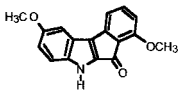
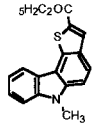
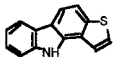
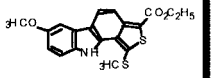
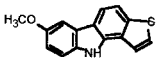
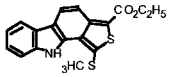
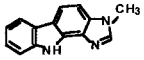
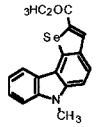
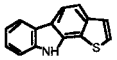
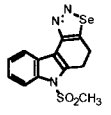
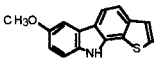
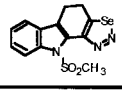
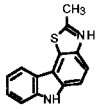
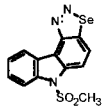
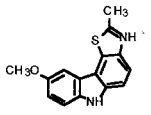
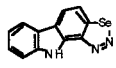
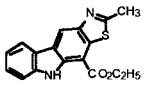
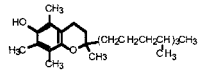
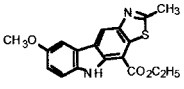
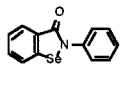
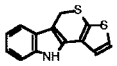
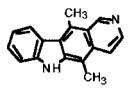
Table 1. Antioxidative Activities of 21 New Analogues of Ellipticine, Vitamin E, and Ebselen

N°	Effective molarities (mol/L)	Chemical structures	N°	Effective molarities (mol/L)	Chemical structures
1	10^{-6}		13	10^{-4}	
2	10^{-5}		14	10^{-4}	
3	10^{-5}		15	fluorescent at 488nm	
4	10^{-6}		16	fluorescent at 488nm	
5	10^{-5}		17	10^{-5}	
6	10^{-5}		18	10^{-6}	
7	10^{-6}		19	10^{-4}	
8	10^{-6}		20	10^{-4}	
9	10^{-6}		21	no effect	
10	10^{-5}		Vit.E	10^{-10}	
11	10^{-5}		Ebse-len	10^{-5}	
12	10^{-5}		-	-	 Ellipticine

respond to the lowest concentration giving a significant antioxidative effect. The values ranged between 10^{-4} and 10^{-6} M for the various synthetic compounds; 10^{-4} M concentrations were often cytotoxic to the Mark 3 cells, with exceptions for molecules **13, 14, 18, 19**.

Molecule **18** was the only selenated molecule that we explored further. Figure 1 shows intracellular ROS levels of Mark 3 cells incubated with increasing concentrations of molecule **18** as determined by flow cytometry using the DHR-123 and DCFH-DA probes. We observed a large antioxidative effect as shown by a decrease in measured fluorescence (compared to cells alone) of −99%, −39%, and −24% for the DHR-123 probe and −91%, −54%, and −43% for the DCFH-DA probe at 10^{-4}, 10^{-6}, and 10^{-8} M, respectively. We checked by in vitro experiment that this decrease in

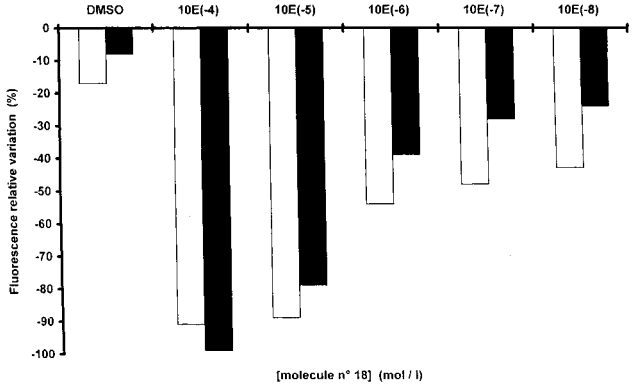


Figure 1. Effects of molecule **18** on intracellular ROS measured by flow cytometry. DMSO being used as solvent, a control (cells incubated for 2 h with 100 μL of DMSO then loaded with the fluorescent probes) was realized. Usually, the free radical scavenging action of DMSO was observed by a decrease in the variation in relative fluorescence ranging from 0% to −10% and −20% for the fluorescence measured by DHR-123 and DCFH-DA, respectively. Mark 3 hybridoma cells were grown in static flasks and incubated for 2 h with different concentrations of molecule **18** solution. ROS were measured with DCFH-DA (□) and DHR-123 (■). The results were compared to results given by cells alone (without any substance). Each compound was tested 3 times, and for each measure, the variation coefficient at the top of the fluorescence peak (FPCV) given by the cytometer was below our fixed threshold (Δ) for the variation in relative fluorescence (with $\Delta \leq -30\%$ and $\Delta \leq -50\%$ for the fluorescence measured by DHR-123 and DCFH-DA, respectively).

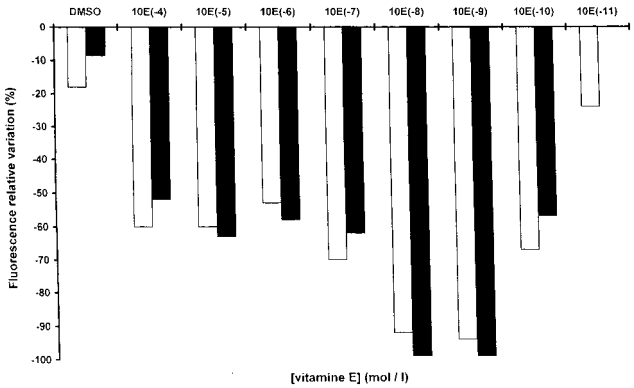


Figure 2. Effects of vitamin E on intracellular ROS measured by flow cytometry. DMSO being used as solvent for vitamin E, a control DMSO (cells incubated for 2 h with 100 μL of DMSO then loaded with the fluorescent probes) was realized. Mark 3 hybridoma cells were grown in static flasks and incubated for 2 h with different concentrations of vitamin E solution. ROS were measured with DCFH-DA (□) and DHR-123 (■). The results were compared to results given by cells alone (without any substance). Each compound was tested 3 times. For each measure, the variation coefficient at the top of the fluorescence peak (FPCV) given by the cytometer was below our fixed threshold (Δ) for the variation in relative fluorescence (with $\Delta \leq -30\%$ and $\Delta \leq -50\%$ for the fluorescence measured by DHR-123 and DCFH-DA, respectively).

fluorescence was not the result of a direct quenching of the tested compounds on the probes (see Experimental Section).

Figure 2 shows the same experiment using a well-known antioxidative molecule, vitamin E. The greatest activity occurred at 10^{-8} and 10^{-9} M for both probes. At 10^{-10} M the antioxidative activity was significantly decreased, with −57% of fluorescence for DHR-123 and −67% of fluorescence for DCFH-DA. At a 10-fold lower

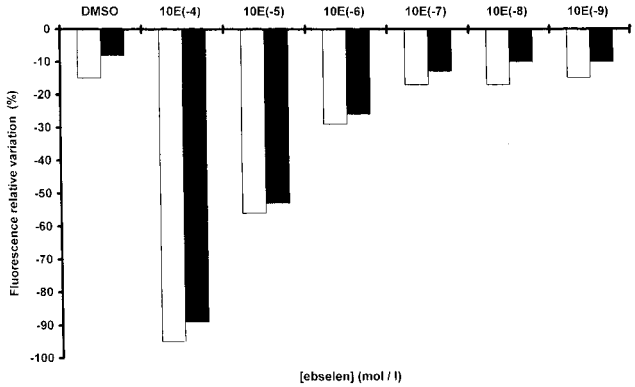


Figure 3. Effects of Ebselen on intracellular ROS measured by flow cytometry. DMSO being used as solvent for Ebselen, a control DMSO was realized. Mark 3 hybridoma cells were grown in static flasks and incubated for 2 h with different concentrations of Ebselen solution. ROS were measured with DCFH-DA (□) and DHR-123 (■). The results were compared to results given by cells alone (without any substance). Each compound was tested 3 times. For each measure, the variation coefficient at the top of the fluorescence peak (FPCV) given by the cytometer was below our fixed threshold (Δ) for the variation in relative fluorescence (with $\Delta \leq -30\%$ and $\Delta \leq -50\%$ for the fluorescence measured by DHR-123 and DCFH-DA, respectively).

concentration (10^{-11} M) antioxidative activity was not significant because the fluorescence decrease was less than the fixed fluorescence thresholds (Δ) and similar to the fluorescence decrease obtained with DMSO alone.

Figure 3 shows the results obtained with Ebselen, another synthetic selenated molecule whose antioxidative activity is well-documented.[40] The results are in the same range as our ellipticine analogue **18** with decreasing fluorescence of −89%, −53%, and −26% for DHR-123 and −95%, −56%, and −29% for DCFH-DA at 10^{-4}, 10^{-5}, and 10^{-6} M, respectively.

Antioxidative Protection against Oxidative Stress and Measurement of Glutathione Peroxidase-like Activity. Figure 4 compares the results obtained with ellipticine analogue **18** to those of vitamin E and Ebselen for their protection against superoxide anions (generated by the xanthine/xanthine oxidase system, X/XO) and peroxides (generated by H_2O_2). This molecule showed a significant antioxidative activity corresponding to 88% of vitamin E activity in the X/XO system and to 70% of vitamin E activity in the H_2O_2 system. However, Ebselen seemed to be ineffective against superoxide anions generated by the X/XO system.

Table 2 shows the glutathione peroxidase-like activity for the different tested compounds at 10^{-4}, 10^{-3}, and 10^{-2} M. From the concentration of 10^{-3} M, Ebselen had glutathione peroxidase-like activity, and at 10^{-2} M the value of this activity was 1048 μmol of oxidized NADP/min/L of Ebselen solution. For the 21 ellipticine analogues tested, we found 8 molecules (**1, 10, 11, 14, 15, 16, 18, 19**) with glutathione peroxidase-like activity at 10^{-2} M. Comparing this activity to that of Ebselen, we observed that four molecules (**14, 15, 16, 18**) were equivalent to 13% of Ebselen activity, while the four other molecules had activities ranging between 21% and 48%. Blanks done without glutathione show that there was not direct oxidation of NADPH by the compounds (Table 3).

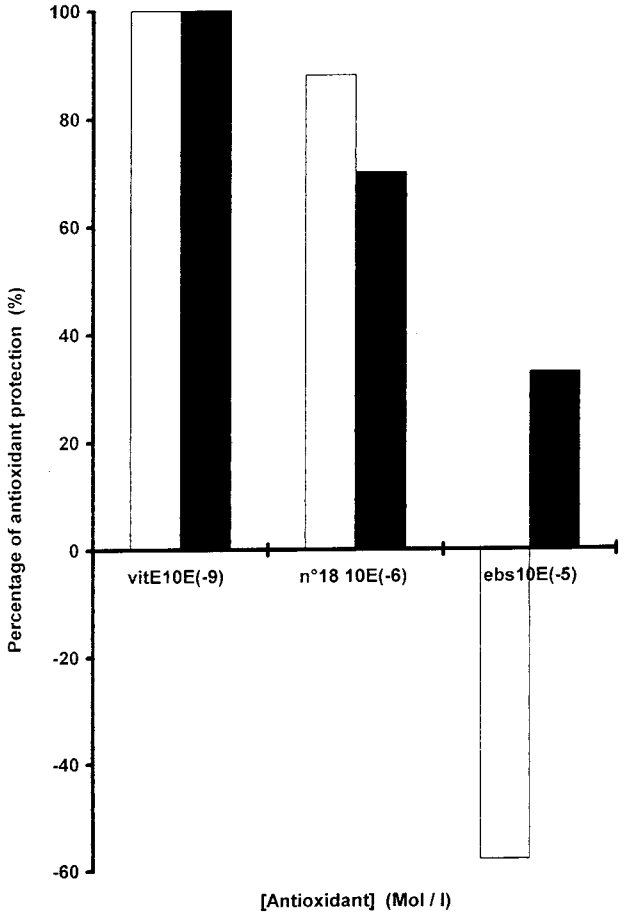


Figure 4. Antioxidative protection of the molecule **18**, vitamin E, and Ebselen for cells incubated with an oxidative stress. Vitamin E showed 100% antioxidative protection. The results for molecule **18** and Ebselen were compared to the vitamin E results. Ebselen seems to increase the rate of superoxide anions: (□) X/XO oxidative stress, fluorescence measured by the probe DHR-123; (■) H_2O_2 oxidative stress, fluorescence measured by the probe DCFH-DA.

Heterocyclic Ellipticine Analogues. Ellipticine is an alkaloid that was isolated by Goodwin[9] from a tropical shrub *Ochrosia elliptica Labill*. In 1967, the antitumor properties of this compound were revealed.[10,11] We present the results of antioxidative properties on 21 new synthetic tetracyclic analogues of ellipticine, in which the pyridine ring was nitrogenated, sulfated, or selenated. This was done using the in vitro flow cytometric technique and the evaluation of internal free radicals of living cells by two commercially available probes.

Our data show that these compounds have antioxidative activity similar to that of Ebselen. Ellipticine analogues **15** and **16** could not be tested in our experimental model since they have a natural fluorescence at 488 nm (which is the excitation wavelength of the flow cytometric technique), whereas all the other compounds do not. As we gated on live cells to measure fluorescence which characterizes the intracellular free radical rates, a sudden decrease in fluorescence was observed for all ellipticine analogues at the highest concentration (10^{-4} M) (with the exception of molecules **13, 14, 18, 19**), due to the cytotoxicity of the compounds themselves. This correlated with low levels of cell viability, as determined by cell size versus granularity (parameters given during the flow cytometric acquisition). Analogue **21** had neither antioxidative nor oxidative activity. Analogue **18,** which is selenated and sulfomethylated, was tested further for its capacity to protect cells against oxidative stress (X/XO and H_2O_2). The results (Figure 4) showed that analogue **18** had important antioxidative activity, which was more efficient against superoxide anions than against peroxides.

Table 2. Measure of the Glutathione Peroxidase-like Activity (μmol of $NADP^+$/L/min) for the Different Molecules

compd	10^{-4} M	10^{-3} M	10^{-2} M	(abs. sample)/(abs. Ebselen) × 100 (%)
1	20	29	369	35
2	11	26	58	6
3	26	25	60	6
4	13	20	47	4
5	29	20	42	4
6	27	20	25	2
7	4	22	60	6
8	20	29	93	9
9	33	35	64	6
10	33	46	506	48
11	40	58	215	21
12	44	71	75	7
13	33	15	53	5
14	26	33	117	11
15	26	9	151	14
16	traces	38	133	13
17	29	31	104	10
18	31	33	140	13
19	18	31	246	23
20	29	33	46	4
21	20	31	51	5
Ebselen	11	111	1048	100
vitamin E	15	33	20	2

It is clear that the aerobic xanthine oxidase reaction produces superoxide anions.[12,13] However, since the 1970s, there has been conflicting literature about the production of singlet oxygen by the same reaction. Some studies reported no production of singlet oxygen by the xanthine oxidase reaction,[14,15] while others observed this production from a reaction between superoxide anions and H_2O_2.[16−19] Spontaneous dismutation of superoxide anions also produces singlet oxygen,[20,21] but it has been reported not to generate detectable levels of singlet oxygen.[22] More recently, Mao et al.[23] showed by ESR that production of singlet oxygen can occur by the reaction between superoxide anions and H_2O_2. In all cases, singlet oxygen production follows that of superoxide anion production,[24] both of them being produced by the X/XO system used in cells culture.[8,25,26] The presence of superoxide anions in the medium can be demontrated by inhibition of the lucigenin fluorescence observed after addition of superoxide dismutase.[27] This superoxide anion detection corresponds to the use of the fluorescent probe DHR-123, considered to be specific for superoxide anion[6] although some authors have shown that it reacts with hydrogen peroxide and peroxynitrite.[28,29] The fluorescent probe DCFH-DA is considered to be more specific for peroxides.[7]

As we measured the antioxidative activity by a fluorescence decrease, one possible interpretation of our results is to postulate that the mean channel shift of fluorescence could be due to a quenching of the fluorescent probes by the added compounds themselves. In vitro experiments show that for all compounds tested this is not the case. Moreover, most of the synthetic compounds responded similarly to vitamin E (Figure 2).

Table 3. Blanks of the Glutathione Peroxidase-like Determinations To Check the Direct Effects of the Compounds Tested on the Oxidation of NADPH

Experiments	Results Glutathione peroxidase activity
Blanks 1 n = 6 0.1 ml water - GSH - H_2O_2 - NADPH Glutathione reductase - PO^4 buffer	13 - 11 - 23 15 - 10 - 10 M = 14
Blanks 2 n = 6 0.1 ml compounds solution - H_2O_2 - NADPH Glutathione reductase (no substrate GSH)	20 - 5 - 40 38 - 33 - 10 M = 24
Assays n = 3 Compound 1 Compound 10 Compound 11 Compound 18 Compound 19 Ebselen } GSH - H_2O_2 - NADPH Glutathione reductase	352 - 343 - 411 M = 369 501 - 525 - 491 M = 506 155 - 197 - 214 M = 215 158 - - - 121 M = 140 182 - 216 - 340 M = 246 1 042 - 1 100 - 1 004 M = 1 048

This response was biphasic with an optimal concentration, whereby the fluorescence decrease is more dominant than at higher concentrations.

Although we tested molecules from the same chemical family, the molecular structures of these compounds are very different. In addition, we do not know the mechanism of antioxidative activity (scavenger, inhibitor of free radical chain reactions, metal chelator, etc.) of these molecules. Consequently, it is impossible to deduce information or make a general rule about structure–activity relationships (SAR) from these screening results (Table 1). However, the following observations can be made:

On some molecules (**4, 7**) the presence of a methoxy group at position 9 increases the antioxidative activity 10-fold versus the same molecule (**3, 6**) without a methoxy group in position 9. It is possible that the methoxy group at this position permits the oxidation of the molecule as for the 9-hydroxyellipticine[3] and produces a very electrophilic quinone imine, which would react with ROS such as superoxide anions or peroxides. The quinones and their reduced form the hydroquinones are very reactive oxidoreductor systems which convert easily and reversibly from the oxidized form (quinone) to the intermediary form (semiquinone) then to the reduced form (hydroquinone). For this sort of system, during the oxidation of the semiquinone to the quinone, superoxide anions and hydrogen peroxide are generated.[30–32] Inversely, the quinone could be reduced by superoxide anions.[33–35] By this fact, the quinone/hydroquinone system is a regulator of the intracellular concentration of certain free radicals. However, this increase of the antioxidative activity by a methoxy group at position 9 does not exist for molecules **2, 9, 11** versus molecules **1, 8, 10**.

Annealing the D-ring seems to influence the antioxidative activity since a relative decrease was observed for catacondensed molecules **10, 11, 19, 21** versus pericondensed molecules **8, 9, 18, 20**.

We expect that the higher the antioxidative activity, the stronger the electronic density is within the molecule. Surprisingly we observed that the aromatization of the C-cycle is an inhibitory factor for antioxidative activity of molecules **20, 21** versus molecules **18, 19**.

The number of heteroatoms carried by the molecule does not seem to increase the antioxidative property (molecules **3, 12, 21**). However, the nature of the heteroatom carried by the D-cycle (molecules **13, 14, 17**) does have some effect on activity, since when the electronic density increases around this atom it becomes more oxidizable and the antioxidative activity increases.

Vitamin E is known as the main membrane antioxidant. It reacts by reducing free radicals.[36–38] The tocopheryl radical produced by this reaction is stable but not very reactive and thus is an inhibitor of radical propagation. There are eight isomers of vitamin E, and in humans, the most abundant and active is α-tocopherol.[39] The results (Figure 2) show that this experimental model efficiently measures vitamin E antioxidative activity against superoxide anions or peroxides. This activity is powerful since maximum effectiveness occurs at concentrations of 10^{-8} and 10^{-9} M.

The glutathione peroxidase-like activity of Ebselen was discovered in 1984.[40,41] Since then, several investigators showed that Ebselen can reduce hydrogen peroxide, organic hydroperoxides, cholesterol hydroperoxides, high-density lipoprotein peroxidation, and phospholipid hydroperoxides[42–45] and can also decrease lipid peroxidation generated by transition metals. Ebselen is active on several substrates such as glutathione, *N*-acetylcysteine, dithiothreitol, or dihydrolipoate.[46–48] However, as it cannot prevent the generation of peroxidation by some radical initiators, like 2,2′-azobis(amidinopropane), and is inefficient against other free

radicals, like phenylpicrylhydrazyl radical,[49,50] Ebselen cannot be taken as a general inhibitor of lipid peroxidation. Moreover, under some circumstances, Ebselen can generate free radicals.[51−53] So, if Ebselen has antioxidative properties,[54] it is a modest antiradical. In fact in vivo, its principal pharmacologic property is to be an antiinflammatory drug inhibiting enzymes implicated in free radical generation at low concentrations.[55] In our experimental model, we observed on Mark 3 cells without oxidative treatment that Ebselen has antioxidative properties against both superoxide anions and peroxides measured by DHR-123 and DCFH-DA, respectively. However, when the cells were treated by oxidative stress, Ebselen inactivated the peroxides generated by H_2O_2 (Figure 4) but increased the rate of superoxide anions generated by X/XO.

Compound **18** showed the most interesting antioxidative properties. Its mechanism of action, however, awaits further clarification. It might act as a scavenger, inhibitor of free radical chain reaction, metal chelator, etc.

Experimental Section

1. Antioxidant Molecules. Compounds **1** and **2** were prepared using a combination of a Japp−Klingemann reaction and a Fischer indole synthesis.[56] Compounds **2−12, 15, 16** were prepared via the Fischer indole synthesis on the corresponding ketone followed by aromatization with DDQ.[57] Compounds **13, 14, 18** were prepared from the 2,3,4,9-tetrahydrocarbazol-1-ones as previously described.[58] Ebselen [2-phenyl-1,2-benzisoselenazol-3(2*H*)-one] is a selenated synthetic molecule having a glutathione peroxidase-like activity[59−61] and was prepared using the procedure of Lesser.[62]

All the new synthetic compounds were characterized by thin NMR (^{1}H, ^{13}C), IR and UV spectra as specified in the cited refs 56, 57, 58; vitamin E (α-tocopherol; Sigma). All compounds were dissolved in DMSO at concentrations ranging from 10^{-4} to 10^{-11} M using 10-fold dilution steps.

2. Flow Cytometry Measurement of Antioxidative Activity. Cell lines: Murine−murine Mark 3 hybridoma cells, secreting IgG1 monoclonal antibodies against rat immunoglobulin α chain, were supplied by Prof. Bazin (Brussels, Belgium). In our laboratory, the cells were routinely cultured in RPMI 1640 medium (Seromed, T121-10) supplemented with 2 mM L-glutamine (Sigma, G1517) and 10% fetal calf serum (FCS) (J. Boys, Reims, France) in an incubator at 37 °C, 5% CO_2, 95% air and humidity. This cell line was chosen because it could be easily cultured in suspension, is very stable, is of lymphoid origin and thus involved with free radicals metabolism, and is of mouse origin.

Culture conditions: All molecules were tested for their antioxidative activities on cells with and without induced oxidative stress. For 10 mL of cell suspension (at the concentration of 5×10^5 cell/mL), seeded in 25-cm^2 static flasks containing the reference medium, 100 μL of the antioxidant solutions was added and the cells were incubated for 2 h at 37 °C, 5% CO_2, 95% air and humidity before analysis by flow cytometry. Controls with 100 μL of DMSO alone were always done.

Cells were oxidatively stressed by either incubation with H_2O_2 or using the X/XO system. First, perhydrol (30% H_2O_2; Merck, Darmstad, Germany) solution at 10^{-3} M concentration was freshly prepared for each experiment. 100 μL (final concentration of 10^{-5} M) were added to the 10 mL of cell suspension in 25-cm^2 static flasks containing the reference medium. Second, xanthine solution (X) (Sigma) 10^{-1} M was prepared in HEPES buffer (Sigma), stored at − 20 °C and used at a final concentration of 8×10^{-4} M. For each experiment, xanthine oxidase solution (XO) (Sigma, x4376) was prepared in HEPES buffer and used at a final concentration of 10^{-2} units/mL. The murine Mark 3 cells were exposed to oxidative stress by incubation for 10 min at 37 °C in the presence of H_2O_2 or X/XO, washed rapidly and then marked with the fluorescent probes for free radical detection by flow cytometry.

Aliquots (1 mL) of the cell suspension (5×10^5 cells/mL) were then incubated at 37 °C with 10 μL of DHR-123 [dihydrorhodamine-123 ($\lambda_{excitation} = 498$ nm, $\lambda_{emission} = 527$ nm), 0.1 mM solution dimethylformamide − stock solution at 10 mM] for 5 min or with 20 μL of DCFH-DA [dichlorofluorescein diacetate ($\lambda_{excitation} = 490$ nm, $\lambda_{emission} = 513$ nm), 0.5 mM solution in dimethylformamide − stock solution at 5 mM] for 20 min. The cells were washed rapidly and stored on ice until flow cytometry was carried out. The probes stock solution was stored at − 20 °C before use.

Flow cytometry: Cells were analyzed using a Coulter Epics C flow cytometer (Coultronic, Margency, France) equipped with a coherent argon laser (Innova 90.5) with an excitation wavelength of 488 nm set at 200 mW power. Green fluorescence was selected using a 525 nm band-pass filter. It was displayed on a 256-channel monoparametric histogram with a logarithmic scale (LGFL). The optical alignment of the apparatus was checked each day and all other settings were unchanged throughout the study. The observed fluorescence reflects the intracellular free radical level. At least 35 000 cells were analyzed for each concentration of antioxidant.

For screening of antioxidative activity on native cells, the fluorescence mean peak channel (MP) was noted and fluorescence expressed by mean fluorescence intensity (MFI) with: $MFI = e^{[\log(1000/256)](MP)}$. Then, the fluorescence relative variation (Δ) was calculated for each sample (S) compared with control (C) (loaded cells without antioxidant treatment and without oxidative stress) with: $\Delta = [(MFI_{(S)} - MFI_{(C)})/MFI_{(C)}] \times 100$.

To consider an efficient antioxidative activity, we determined two thresholds for the variation in relative fluorescence: with $\Delta \leq -30\%$ for the fluorescence measured with the probe DHR-123 and $\Delta \leq -50\%$ for the fluorescence measured with the probe DCFH-DA. For each tested compound, we defined effective molarity as the lowest concentration that produces a variation in relative fluorescence below the fluorescence threshold. A decreased fluorescence indicated a reduction in intracellular free radical level and therefore an antioxidative effect of the tested compound. In contrast, an increased fluorescence shows an augmentation of the intracellular free radical level and therefore an oxidative effect of the tested compound.

For the antioxidant in the presence of an oxidative stress, the antioxidant was used at its effective molarity and the results were expressed as percentage (*P*) of antioxidative protection against an oxidative stress. This percentage was determined as follows: (1) The fluorescence mean peak channel of cells incubated with oxidative molecule (H_2O_2 or X/XO) was defined as 100% of the oxidative stress. (2) The fluorescence mean peak channel of cells incubated with antioxidative then oxidative molecule (H_2O_2 or X/XO) was defined as *x*% of the oxidative stress. (3) And the subtraction 100% − *x*% gives the percentage (*P*) of the antioxidative protection.

3. Verification that Antioxidative Compounds Have No Direct Action on the Probes (quenching or reduction). We have checked, using a spectrofluorometer and mixtures in test tubes that: (a) nonoxidized probes (DHR and DCFH) have no fluorescence by themselves (135−238 units); (b) oxidized probes (rhodamine and fluorescein) by H_2O_2 (200 μM concentration) have a high fluorescence (1191−1532 units); (c) the same oxidized probes in the presence of each antioxidant compound tested (10^{-4} M concentration) have the same fluorescence (1290−1410 with H_2O_2 or 1481−1606 for the naturally oxidized probes).

4. Measurement of Glutathione Peroxidase Activity. We used the Paglia and Valentine method.[65] The principle of this method is the following: First, the sample for which glutathione peroxidase activity must be determined (blood, organic compounds, Ebselen, etc.) is added to reduced glutathione (G-SH) in phosphate buffer, pH 7 (in the presence of H_2O_2). If enzymatic activity is present, glutathione is oxidized (G-S-S-G) in proportion to enzyme activity. Then excesses of

NADPH and glutathione reductase are added and the oxidized glutathione is returned to its reduced form, as NADPH is returned to $NADP^+$. The quantity of $NADP^+$ formed, measured at 340 nm, is proportional to the quantity of G-S-S-G in the sample.

Briefly, 100 μL of the compound solution to be tested (instead of blood) was added to 2.6 mL of phosphate buffer, pH 7, in the presence of H_2O_2 and 0.1 mL of GSH (1.5×10^{-1} M in HCl, 0.01 M). Then 0.1 mL of NADPH (8.4×10^{-3} M phosphate buffer, pH 7) containing glutathione reductase was added. The results are given in μmol of oxidized $NADP^+$/min/L of compound solution.

We have done two blanks: one with 100 μL of water instead of the compound solution to verify absence of glutathione peroxidase-like activity. The other blank contained the compound solution but no added GSH (the substrate), to verify that there was no direct oxidation of NADPH by the organic compounds.

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