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Ex vivo Activities of β-Lapachone and α-Lapachone on Macrophages: A Quantitative Pharmacological Analysis Based on Amperometric Monitoring of Oxidative Bursts by Single Cells

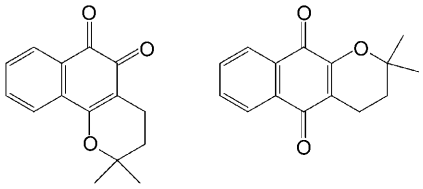
Danielle C. M. Ferreira,[a, b] Issa Tapsoba,[b] Stéphane Arbault,[b] Yann Bouret,[b] Magna Suzana Alexandre Moreira,[c] Antônio Ventura Pinto,[d] Marília O. F. Goulart,*[a] and Christian Amatore*[b]

*β-Lapachone (**1**) has been widely used for its pharmacological activity, particularly against cancer. However, its mechanism of action at the cellular level remains unclear, although a common major hypothesis involves its prooxidant properties. Electrochemical measurements with microelectrodes were taken in order to quantitatively investigate the activity of **1** at different concentrations and several incubation times, on the oxidative bursts released by single macrophages. The exact natures of the electroactive reactive oxygen species (ROS) and reactive nitrogen species (RNS) released by macrophages under the effect of **1** were characterized, and their fluxes were measured quantitatively. This allowed the reconstruction of the primary $O_2^{\bullet-}$ and NO production by the cells. In the first hour, at 10 μM, the decrease in the oxidative burst involved mainly RNS, while the amount of H_2O_2 was found to be higher than in controls. After a longer incubation time—that is, 4 h—at 1 μM, the total amount of ROS and RNS had increased, with significant enhancements of H_2O_2 and NO. In contrast, α-lapachone, the pharmacologically inactive* para*-quinone isomer, was unable to increase the production of RONS by macrophages significantly. Over much longer incubation periods (about one day), however, each quinone induced cell death by apoptosis. All these effects were interpreted by consideration of two different mechanisms involving opposite reactivities of quinones in living cells.*

Introduction

The search for natural products leading to the discovery of promising new drugs has always attracted much attention in pharmacology. The vast biodiversity inherent to regions of tropical rainforests undoubtedly harbours numerous potential compounds that could be used as (or serve as the basis for) inexpensive drugs for the treatment of human or plant diseases, particularly in underdeveloped countries.

β-Lapachone (2,2-dimethyl-3,4-dihydro-2*H*-naphtho[1,2-b]pyran-5,6-dione, **1**) and its position isomer α-lapachone (2,2-dimethyl-3,4-dihydro-2*H*-naphtho[2,3-b]pyran-5,10-dione, **2**) are among such potential candidates. These are naturally occurring products present in the bark of the South American lapacho tree (*Tabebuia avellanedae*), which grows mainly in Brazil. β-Lapachone is also easily synthesized by the chemical transformation of lapachol, which is extracted from the bark of species of the Bignoniaceae family or from lomatiol, a compound isolated from lomatia seeds.[1a,b]



(**1**) β-lapachone (**2**) α-lapachone

Compound **1** presents a large spectrum of pharmacological activities, including antibacterial,[2] antifungal,[2] antimalarial[3,4] and trypanocidal activities.[5–8] β-Lapachone was found in vitro to be the most active compound against bloodstream forms of *Trypanosoma cruzi*, but was completely inactive in vivo in the

[a] *Dr. D. C. M. Ferreira, Prof. Dr. M. O. F. Goulart*
Instituto de Química e Biotecnologia, Universidade Federal de Alagoas
Campus A. C. Simões, Tabuleiro do Martins
Maceió, AL, 57072-970 (Brazil)
Fax: (+55) 82-3214-1389
E-mail: mofg@qui.ufal.br

[b] *Dr. D. C. M. Ferreira, Dr. I. Tapsoba,[+] Dr. S. Arbault, Dr. Y. Bouret, Prof. Dr. C. Amatore*
Laboratoire PASTEUR, Ecole Normale Supérieure
CNRS, UPMC Université de Paris 06, Département de Chimie
24 Rue Lhomond, 75005, Paris (France)
Fax: (+33) 1-4432-3863
E-mail: christian.amatore@ens.fr

[c] *Dr. M. S. Alexandre Moreira*
Laboratório de Farmacologia e Imunidade, ICBS, UFAL
AL, 57020-720 (Brazil)

[d] *Dr. A. Ventura Pinto*
Núcleo de Pesquisa em Produtos Naturais, UFRJ
Rio de Janeiro, 21944-971 (Brazil)

[+] *Present address: UFR SEA Université de Ouagadougou*
03 BP 7021 Ouagadougou 03 (Burkina Faso)

presence of blood.[8] One of the hopes for this compound is its recognized action against cancer cells. Indeed, it is reported to present significant antineoplastic activity against human cancer cell lines originating from leukaemia,[9] prostate,[9,10] malignant glioma,[11] hepatoma,[12] colon,[13,14] breast,[15] ovarian[16] and pancreatic tumours,[17,18] at concentrations in the 1–10 μM range (IC_{50}). Other studies have shown that, in combination with taxol, β-lapachone is an effective agent against human ovarian and prostate xenografts in mice.[16] It is currently in use in phase II clinical trials against pancreatic cancers and was also reported to be effective against nonsmall cell lung cancer (NSCLC).[19] Lee et al. found that β-lapachone in the micromolar concentration range inhibited the viability of human bladder carcinoma T24[20] and human prostate carcinoma DU145[21] cells by inducing apoptosis; this was established by the observation of the generation of apoptotic bodies and DNA fragmentation.

β-Lapachone may be also useful as a potential anti-inflammatory agent to attenuate inflammatory diseases.[22] However, fundamental principles underlying the clinical efficiency of this drug remain to be understood. Moreover, the precise origin of the cytotoxicity of β-lapachone has not yet been clarified. Many possible mechanisms to explain its anticancer[23] and parasiticidal activities have been suggested in the literature; these include possible inhibition of DNA, RNA and protein synthesis, as well as the production of DNA strand breaks in *T. cruzi* parasite[24] or even the poisoning of topoisomerase II.[25,26]

Although β-lapachone has multiple effects, these effects are today known to be cell-type specific. Similarly, the wide variety of active concentration ranges (concentration and duration of exposure) described in previous reports[2–26] suggest that it might involve several mechanisms, depending on its administration protocol and the cell type. Among the numerous proposed mechanisms[23] a central common feature is the rate of formation of reactive oxygen species (ROS)—that is, the amount needed to invoke control of oxidative stress. ROS are known to be toxic to biological systems[27–29] and are therefore probably responsible for tumour cell death during treatment with β-lapachone, as has been reported.[30,31]

Conversely, a decrease in ROS and/or RNS production should decrease oxidative stress damage in redox-imbalanced cells.

ROS and RNS (H_2O_2, ONO_2^-, NO, NO_2^-) femto- or attomolar fluxes released by stimulated single cells may be monitored kinetically and quantitatively by means of platinized carbon fibre ultramicroelectrodes (Figure 1 A).[32–37] Positioning of the active disk surface of the electrode at a micrometric distance from the membrane of a single cell produces a synaptic-like configuration (termed "artificial synapse;" Figure 1 B), in which the electrode surface acts as a quantitative detector of cell secretion. The volume of the sub-picoliter solution lying between the electrode surface and the cell membrane acts as a synaptic cleft in which minute releases of (bio)chemicals provoke high concentration jumps, thereby giving rise to significant current fluxes monitored at the electrode through oxidation of the released species.[33,35] With such a configuration, femtomoles of electroactive species (ROS and RNS in this study) released by an individual cell[35] or by a cell performing within a functional tissue[38–41] can be quantitatively detected and their kinetics of release can be monitored with a precision of about a thousand molecules per millisecond; this means that release can be monitored with a quantitative and kinetic efficiency equivalent to that of a natural synapse.

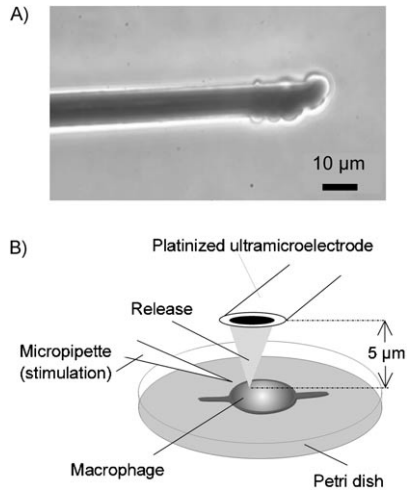


Figure 1. A) Microphotograph of the microelectrode tip that shows the black platinum deposit. B) Scheme summarizing the experimental principle ("artificial synapse" configuration). The mechanical depolarization of a cell membrane is induced by a capillary and used to activate its secretion of electroactive metabolites. These are detected at the surface of a microelectrode.

Much interest has been displayed in the use of amperometry with platinized carbon microelectrodes to analyse and to quantify bursts of reactive oxygen and nitrogen species released by immune cells[37] or skin cells[32,33,36] and their biomedical relevance in the initial oxidative mechanism of carcinogenesis.[32]

This paper reports on an investigation into the changes induced by α- and β-lapachones on oxidative bursts released by single immune cells—macrophages from the cell strain RAW 264.7—by use of the artificial synapse method with amperometry. A specific feature of macrophages is their ability to produce large amounts of ROS and RNS after their activation, which is achievable by multiple biological, chemical or physical agents or means.[42] Macrophages incorporate specific arsenals of calcium-controlled constitutive enzymes, geared towards ready production of large amounts of ROS and RNS in response to different means of activation, including physical membrane stress,[37] because this allows a sudden intake of calcium into the cell cytosol. Model RAW 264.4 macrophages have been incubated for different lengths of time in the presence of various concentrations of β-lapachone and α-lapachone, and their stimulated[37] oxidative bursts have been found to represent the ability of the investigated single cells to produce ROS and RNS.

The exact natures and amounts of released electroactive species (H_2O_2, $ONOO^-$, NO, NO_2^-) were characterized molecularly, and their fluxes were measured and deconvoluted to those of the primary species—$O_2^{\bullet-}$ and NO—that originated all the ROS and RNS detected.

Results

In this paper we use the classical biological terms "antioxidant" and "prooxidant," although their meaning is somewhat unclear. In our understanding, these terms qualify the effect of a compound in reducing or increasing cells' abilities to produce ROS and/or RNS species after stimulation.

The effects of various concentrations of α- and β-lapachones on the production of oxidative bursts by macrophages were investigated by amperometry with platinized carbon microelectrodes. In a first series of experiments, the cell responses were detected at +850 mV versus SSCE, a potential that ensured the simultaneous and quantitative collection of all the electroactive ROS and RNS released during an oxidative burst (H_2O_2, $NO^{\bullet}$, NO_2^- and ONOO).[37] This allowed for the delineation of the global effects (time and concentration dependencies) produced by each quinone on the overall intensity of oxidative bursts. When this had proved informative, a second series of experiments was then conducted at several specific potentials (300, 450, 650, 850 mV vs. SSCE) in order to identify and to characterize each ROS and RNS individually, as well as to quantify their individual fluxes.[37]

Concentration- and time-dependent overall effects of β- and α-lapachones on RAW macrophages

As previously reported,[37,43–45] each oxidative burst produced by a macrophage was characterized as an amperometric spike, which precisely tracked the kinetics of the species released (Figure 2A). Immediately after stimulation of the cell membrane with a sealed glass capillary (Figure 1B), the current resulting from the macrophage response increased sharply, reaching its maximum within about one second. It then gradually subsided until it had decayed to the baseline level after about 50 s (Figure 2A). These general features of amperometric responses, applied to all the results obtained under different conditions, are described below.

Firstly, as illustrated in Figure 2A, a net decrease in the amperometric response was observed after one hour of incubation with β-lapachone (0.1 μM). This indicates that, in the short term, β-lapachone was able to induce a reduction in ROS and RNS release by macrophages. The antioxidant activity of the treatment was more easily characterized by comparing the detected charges (that is, the area of the current/time responses) with or without cell treatment (1 h) with β-lapachone, as illustrated in the inset of Figure 2A. Indeed, according to Faraday's law, the charge (Q) corresponding to the electrolysis of the whole quantity of species reaching the electrode surface represented the total amount of ROS and RNS released under each set of conditions.

The cell responses were measured, as described above, in cells treated for one hour with various concentrations of β-lapachone (0.01, 0.1, 1 or 10 μM). The total amount of ROS and RNS generated by each RAW macrophage was quantified by integrating the current/time responses detected under control conditions or after the β-lapachone incubation conditions (see, for example, Figure 2A, inset). To facilitate the comparison of

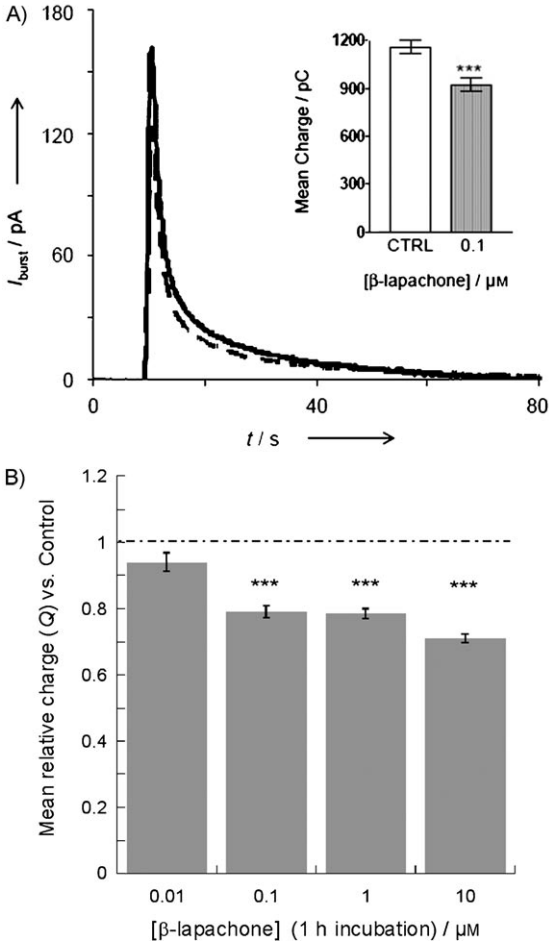


Figure 2. A) Amperometric detection of oxidative bursts after membrane physical depolarization on a murine macrophage (RAW 264.7 cell line) under the control conditions (solid curve) or after 1 hour of incubation with β-lapachone (0.1 μM, dashed curve). Each curve represents the mean of at least 40 different cell responses. Inset: mean coulometric charges (pC) corresponding to the area of the curves shown in A. B) Variations in the mean charges (normalized versus controls), depending on the concentration of β-lapachone; cells were incubated for 1 h at concentrations of 0.01, 0.1, 1 or 10 μM. Each bin represents the relative charge detected after incubation versus that measured for a separate series of controls performed with the same cell preparation. Measurements were conducted with platinized carbon fibre microelectrodes at +850 mV versus SSCE, in phosphate buffer PBS (pH 7.4). The statistical significance (p value) was calculated for each pair of control and β-lapachone experiments and is reported as: * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

effects under each set of conditions depicted in Figure 2B, the mean charge (Q) determined for each set of conditions of incubation was normalized with respect to the mean charge determined for the same cell preparation kept under controlled conditions. In this presentation, therefore, the horizontal line at unity represents the absolute (that is, with no uncertainty attached to it) behaviour of the controls. Values below unity thus denote an antioxidant activity of the treatment, whereas values above unity (see below, Figure 4) indicate a prooxidant one. As indicated in Figure 2B, the normalized charge after incubation with β-lapachone (0.01 μM) did not differ significantly from that of the controls. Increasing the β-lapachone concentration up to 10 μM, while applying the same incubation time

(1 h), caused the charge to decrease slightly. Under these conditions, β-lapachone had a slight but clearly concentration-dependent "antioxidant" effect; that is, increasing its concentration in the incubation solutions resulted in a decrease in the amount of released ROS and RNS relative to the controls (Figure 2B).

The effect of various concentrations of α-lapachone on the production of oxidative bursts by macrophages was also investigated by the same method. No net decrease in the amperometric response was observed in the presence of α-lapachone (1 μM) after one hour of incubation, indicating that, at least in the short term, α-lapachone did not induce any significant alteration in the release of reactive oxygen and reactive nitrogen species by macrophages (Figure 3). However, a close inspection of the shapes of the two curves in Figure 3 revealed that the kinetics of release induced by α-lapachone were different from those observed in the controls, with sharper decays at short times but with longer tails at longer release times. This perhaps indicates some subtle kinetic effects, but this is also at the limit of significance of our measurements. Anyway, this was not comparable to the changes observed for the β-isomer.

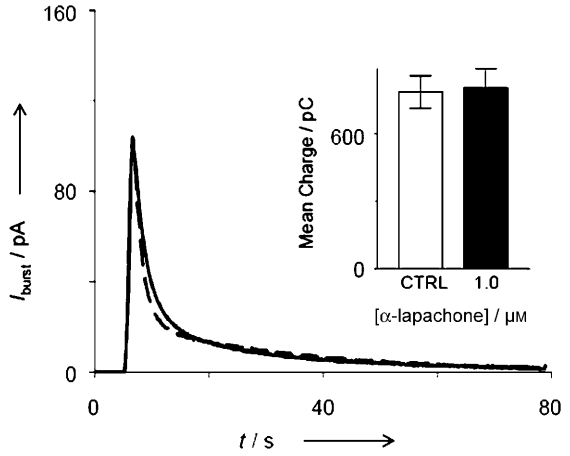


Figure 3. Amperometric measurements of oxidative burst responses from murine macrophages (RAW 264.7 cell line) in the absence of α-lapachone (——) or after 1 h incubation with α-lapachone (1 μM, -----). The inset compares the mean charges resulting from time integration of the mean current responses observed for controls (CTR) or after the treatment with α-lapachone. Measurements were conducted with platinized carbon fibre microelectrodes at +850 mV versus SSCE, in phosphate buffer PBS (pH 7.4), and represent mean values of at least 30 individual measurements in each case.

The responses of macrophages in cells treated for one hour with higher concentrations of α-lapachone—10 and 100 μM—were also analysed. In the incubation using 10 and 100 μM concentrations of α-lapachone and a constant (1 h) incubation time, the charges did not vary significantly relative to the controls (data not shown). The whole set of experiments demonstrated that, under the testing conditions, α-lapachone did not have any essential effect on the amount of released ROS and RNS relative to β-lapachone.

The effect of duration of incubation was investigated by carrying out the same analyses for two series involving either β-lapachone (0.1 μM or 1 μM) or α-lapachone (1 μM or 10 μM).

At 1 and 10 μM concentrations and over a broad range of incubation times, α-lapachone did not produce any statistically significant effect on the oxidative burst responses of macrophages. The only significant change was a decrease observed after 24 h of incubation time at the 10 μM concentration (not shown).

The β-lapachone results differed remarkably, as indicated in Figure 4A and B. In the first hour, slight decreases in the cell responses were observed after their incubation with either 0.1 or 1.0 μM of β-lapachone. In contrast, at longer incubation

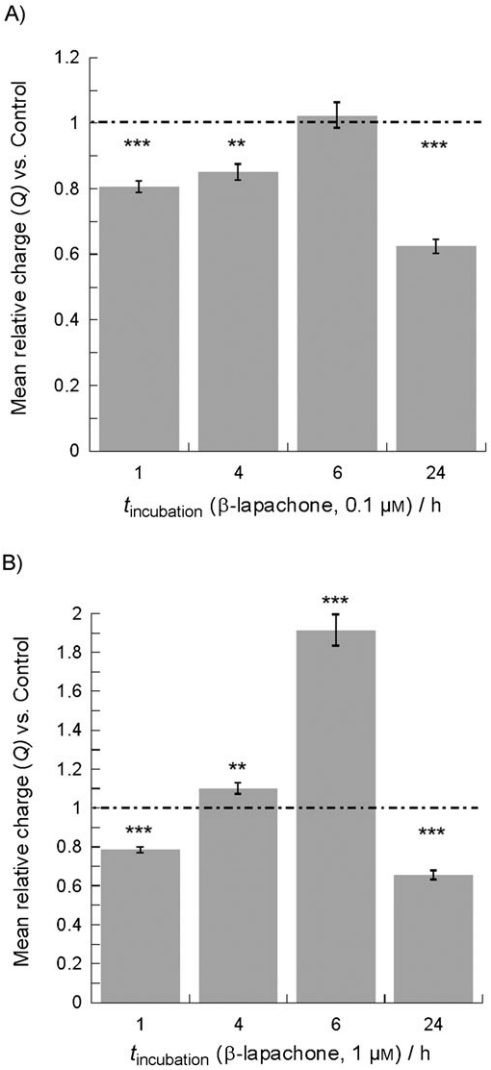


Figure 4. Normalized charges of oxidative bursts detected on macrophages after cell incubation for different periods of time with A) 0.1 μM, or B) 1.0 μM of β-lapachone. Measurements were conducted with platinized carbon fibre microelectrodes by amperometry at +850 mV versus SSCE, in phosphate buffer PBS (pH 7.4). Each bin represents the average value ±standard error of at least 30 different individual cell responses. Each mean result shown is normalized to the mean charge measured for a separate series of controls performed with the same cell preparation. Statistical significance (*p* value) was calculated for each pair of control and β-lapachone experiments and is reported as: * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

times there were evident increases in the entire quantity of ROS and RNS released, whereas at much longer times (24 h) drastic decreases occurred. The time-dependent effect of β-lapachone hence displayed a pronounced peak, which was more evident at higher concentrations (Figure 4B). Because the relative measurement in the absence of treatment is indicated by the horizontal line at unity, this suggests that β-lapachone induced at least three different mechanisms, depending on its concentration and period of action in a cell. At short incubation times the first mechanism was antioxidant, whereas at longer incubation times the second mechanism was prooxidant. Finally, after 24 h of incubation, the prooxidant activity appeared to peak while the antioxidant activity was apparently restored. However, that observation may be an artefact, because the cells simultaneously became less viable. After 4 h of incubation with β-lapachone and at a higher concentration—that is, at least 10 μM—the cells had undergone shrinkage and rounding and showed the characteristic membrane blebbing (bubble formation) seen in cells dying by apoptosis, with loss of most of the cellular organelle structures when compared with control macrophages (Figure 5). This phenomenon was augmented when the incubation time was increased. These morphological observations therefore suggest that the decreases observed after 24 h of incubation (Figure 4A and B) did not reflect a second antioxidant stage but actually an overall decrease in the cell metabolism due to β-lapachone cytotoxicity.

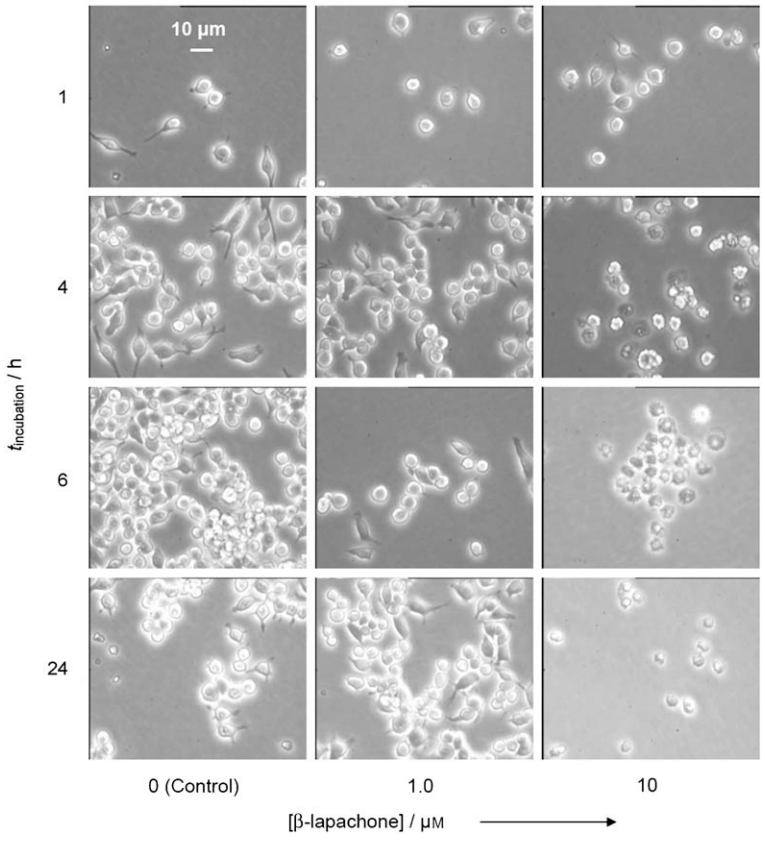


Figure 5. Microscopic observations of controls (left) and of macrophages (RAW 264.7) treated either with 1.0 μM (centre) or with 10 μM (right) of β-lapachone, after 1, 4, 6 or 24 h of treatment, as indicated in each row. The number of cells displayed for each condition is not to be regarded as informative, owing to the variability in cell preparations. However, the changes in cell morphologies are conclusive. The scale bar (10 μm) shown in the top-left photograph (control, 1 h) applies to all the microphotographs shown.

Effect of β-lapachone on the nature of species released during oxidative stress bursts

The results shown in Figures 2, 3, 4 were all obtained from amperometric measurements taken at a single electrode potential (+850 mV versus SSCE) and thus represent the magnitudes of the collective fluxes of all the ROS and RNS released by the tested macrophages under control conditions or after different incubation protocols with β-lapachone.[43] The identification of fluxes of individual species monitored under each set of conditions was based on the use of a specific set of detection potentials.

It has been demonstrated[36,37] that recording of the intensities of oxidative stress responses at four potentials (+300, +450, +650 and +850 mV versus SSCE) allows for the chemical and quantitative characterization of the release kinetics of the four main electroactive ROS and RNS produced by living aerobic cells. In fact, electrochemical currents are additive, but the oxidation of a given species at the platinized carbon surface of the ultramicroelectrode (Figure 1) is only feasible if the electrode potential is sufficiently high. Thus, at +300 mV versus SSCE, only H_2O_2 and $ONOO^-$ can be detected, and the oxidation of each species contributes differently to the resulting current because, although they partially overlap, their electrochemical waves differ significantly. At +300 mV, the oxidation wave for H_2O_2 is almost fully developed, so that its current plateau is reached.[37] At the same potential, the $ONOO^-$ wave is hit on its rising portion, so that only a fraction of its released flux is detected. Conversely, at +450 mV versus SSCE, the two species are quantitatively detected, the electrode potential being sufficiently anodic to hit both wave plateaux, but no other ROS or RNS may be detected at this potential.[37] Each individual flux (ϕ^j) can therefore be extracted from a linear combination of the two current values detected at 300 and 450 mV versus SSCE.[37,46]

$$\phi^{H_2O_2} = \frac{1.88 \times I^{300} - 0.59 \times I^{450}}{2F} \quad (1)$$

$$\phi^{ONO_2^-} = \frac{1.77 \times I^{450} - 2.10 \times I^{300}}{1F} \quad (2)$$

where F is the Faraday. The coefficients in Equations (1) and (2) were established independently, based on the in vitro characterization of each species' electrochemical wave and its coulometry (2e per molecule for H_2O_2 and 1e per molecule for $ONOO^-$).[46]

At +650 mV versus SSCE, NO (1 e per molecule) was detected quantitatively, in addition to H_2O_2 and ONO_2^-. Its individual flux was determined by subtracting the current monitored at +450 mV versus SSCE (due to H_2O_2 and ONO_2^-) from that measured at +650 mV versus SSCE [Eq. (3)]:

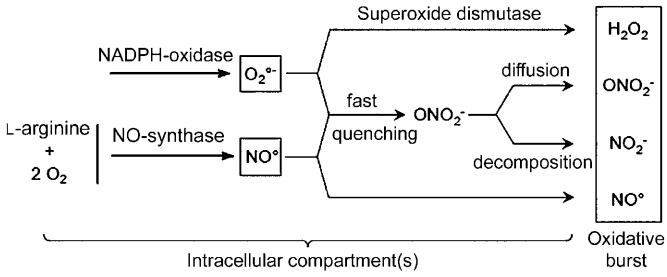
$$\phi^{NO} = \frac{I^{650} - I^{450}}{1F} \quad (3)$$

The current detected at +850 mV versus SSCE represented the sum of the individual currents of all four detectable ROS and RNS, and its increment in relation to that measured at +650 mV versus SSCE afforded the individual flux of nitrite ions (2 e per molecule) [Eq. (4)]:[46]

$$\phi^{NO_2^-} = \frac{I^{850} - I^{650}}{2F} \quad (4)$$

The denominators in Eqs. (1–4) involved different coefficients to account for the different electron stoichiometries of each electrochemical oxidation process ($n=1$ for NO and $ONOO^-$, whereas $n=2$ for NO_2^- and H_2O_2).[46] This shows that the currents measured at +850 mV versus SSCE, as reported in the first section of this study (Figures 2 and 4), were not directly proportional to the sum of the four fluxes, although all the species were oxidized at this potential. Nevertheless, the values shown in Figures 2 and 4 provided direct useful estimates of the magnitude of oxidative stress responses under the conditions examined here.

We had also established previously that the bursts of ROS and RNS observed at single cells follow the initial cell production of only two small reactive molecules that originate all of them: the superoxide radical anion and the nitric oxide radical (see Scheme 1). The H_2O_2 detected at the microelectrode resulted from the rapid, spontaneous or superoxide dismutase-catalysed (SOD-catalysed) disproportionation of $O_2^{\bullet-}$ initially produced by the cell ($2\,O_2^{\bullet-}$ per H_2O_2); similarly, $ONOO^-$ was formed by the extremely rapid, diffusion-limited reaction between $NO^\bullet$ and $O_2^{\bullet-}$ ($1\,O_2^{\bullet-}$ and $1\,NO^\bullet$ per $ONOO^-$). NO_2^- resulted from the spontaneous decomposition of peroxynitrite under our conditions,[37] and hence represented the same proportion of the primary species as in peroxynitrite ($1\,O_2^{\bullet-}$ and $1\,NO^\bullet$ per NO_2^-), whereas excess $NO^\bullet$ freely diffused to the microelectrode surface. Nitric oxide was detected quantitatively, because its reaction with O_2 over the cell–electrode distance (5 μm) was negligible.[47]



Scheme 1. Reaction scheme depicting the origins of the reactive oxygen and nitrogen species detected in the oxidative bursts of RAW 264.7 macrophages.[37]

These stoichiometries enabled the reconstruction of the fluxes of the primary oxidative stress species $O_2^{\bullet-}$ and $NO^\bullet$ on the basis of the fluxes of the individual species (Eqs. 1–4), according to Equations (5) and (6).

$$(\Phi_{O_2^-})^{prod} = 2(\Phi_{H_2O_2})^{mes} + (\Phi_{ONOO^-})^{mes} + (\Phi_{NO_2^-})^{mes} \quad (5)$$

$$(\Phi_{NO})^{prod} = (\Phi_{NO})^{mes} + (\Phi_{ONOO^-})^{mes} + (\Phi_{NO_2^-})^{mes} \quad (6)$$

Although nitrate ions might also be produced in combination with nitrite during the decomposition of peroxynitrite,[48] they could not be considered in evaluation of the initial $O_2^{\bullet-}$ and NO production because they were not detectable at our microelectrodes. However, we wish to emphasize that nitrates (if any were released by the cell) should then contribute equally to the quantification of native quantities of $O_2^{\bullet-}$ and NO, and so should not modify the difference in flux of these species.

Amperometric studies at several potentials are time-consuming, due to the need to take extensive series of measurements to obtain statistically significant data, and so they were only performed for the interesting situations delineated in the series of experiments performed at +850 mV described above. A series of experiments involving the above set of four potentials were conducted after incubation of macrophages with β-lapachone either at 1 μM for 4 h or at 10 μM for 1 h, with use of the same protocols as described above.

The results shown in Figure 6 (10 μM incubation for 1 h) established that, under these conditions, the main effect of β-lapachone was to significantly (compare the reduction of NO production by about 50% of the controls in Figure 6C, right) whereas the production of anion radical superoxide versus the controls remained unaffected (Figure 6C, right). This drastic reduction in NO production was also reflected in the significant decrease in peroxynitrite and a corresponding increase in hydrogen peroxide (see Scheme 1).

The results shown in Figure 7 (1 μM incubation for 4 h) established that after longer incubation periods NO production was restored to the same levels as in the controls, but those of superoxide were significantly increased (Figure 7C, right). Accordingly (Scheme 1), peroxynitrite formation again declined whereas that of hydrogen peroxide increased (Figure 7C, centre).

Discussion

Antioxidant and prooxidant activities of β-lapachone and α-lapachone

The above series of results (Figures 2–7) established unequivocally that β-lapachone presented antioxidant activity on macrophage oxidative bursts over the concentration range of 0.1–10 μM, provided that the incubation times did not exceed one

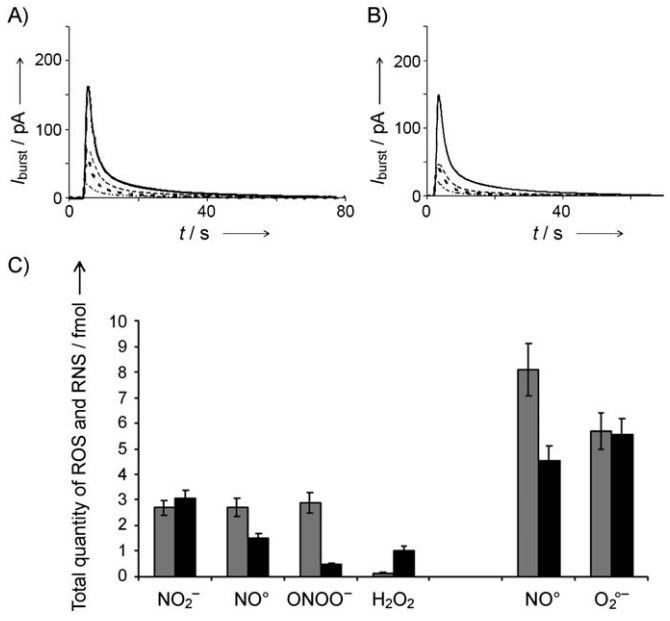


Figure 6. A), B) Comparison of the mean currents (average of at least 30 individual experiments in each case) detected at +850 (solid), +650 (thin dash), +450 (thick dash) and +300 mV (dash-dot) versus SSCE during the amperometric oxidation of ROS and RNS released by RAW 264.7 macrophages, A) under control conditions, or B) after 1 h incubation with β-lapachone (10 μM). C) Left to centre: comparison of the mean quantities (average of at least 30 individual experiments in each case) of each ROS and RNS released by RAW 264.7 macrophages under control conditions (grey bins) or after 1 h incubation with β-lapachone (10 μM, black bins). Right: reconstruction (according to Eqs. 5 and 6) of the primary fluxes of NO and $O_2^{\bullet-}$ released after 1 h incubation (black bins) with β-lapachone (10 μM) or under control conditions (grey bins). Measurements were conducted with platinized carbon fibre microelectrodes in phosphate buffer (PBS, pH 7.4).

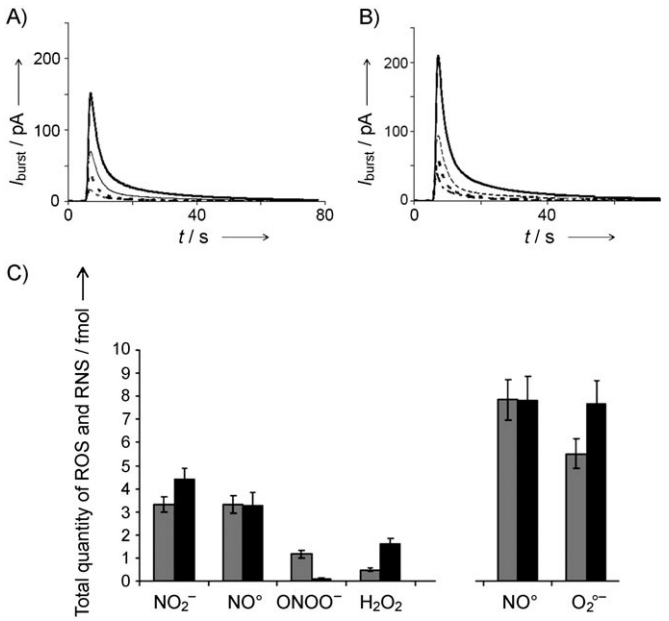


Figure 7. A), B) Comparison of the mean currents (average of at least 30 individual experiments in each case) detected at +850 (solid), +650 (thin dash), +450 (thick dash) and +300 mV (dash-dot) versus SSCE during the amperometric oxidation of ROS and RNS released by RAW 264.7 macrophages, A) under control conditions, or B) after 4 h incubation with β-lapachone (1 μM). C) Left to centre: comparison of the mean quantities (average of at least 30 individual experiments in each case) of each ROS and RNS released by RAW 264.7 macrophages either under control conditions (grey bins) or after 4 h incubation with β-lapachone (1 μM, black bins). Right: reconstruction (according to Eqs. 5 and 6) of the primary fluxes of NO and $O_2^{\bullet-}$ released either after 4 h incubation (black bins) with β-lapachone (1 μM) or under control conditions (grey bins). Measurements were taken with platinized carbon fibre microelectrodes in phosphate buffer PBS (pH 7.4).

hour. Antioxidant activity thus increased with the concentration of β-lapachone. Conversely, when the cells were subjected to the same incubation procedures but for longer incubation times, they increased their mean production of ROS and RNS. This increase proceeded until a new decrease was observed at much longer times (24 h). This later decrease, though, presumably involved severe metabolic changes that ultimately led to apoptosis, culminating at around 4 h (10 μM), as evidenced by the drastic and characteristic morphological changes observed by optical microscopy (Figure 5). For this reason we do not, for the time being, wish to focus on the decrease in oxidative stress illustrated in Figure 4A or B, except to note that it characterizes the consequence of quinone-induced apoptotic behaviour.

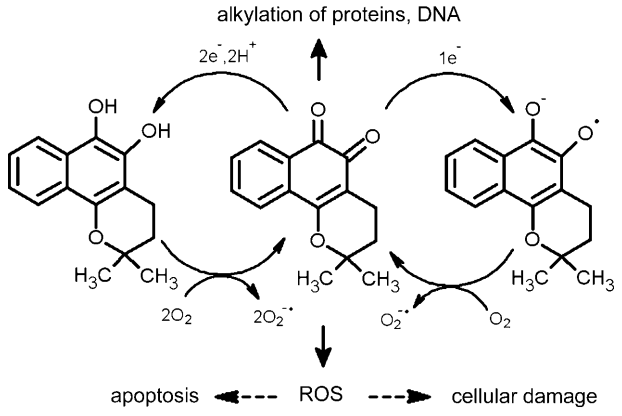
This dichotomy of β-lapachone activity was even more apparent after examination of the production of NO and $O_2^{\bullet-}$ by the cells. Thus, for short incubation times (1 h, Figure 6C, right), the main effect of β-lapachone was to reduce the initial NO production drastically without affecting that of superoxide. Superoxide can be consumed by two main competitive pathways (Scheme 1): its disproportionation (overall second-order kinetics in $[O_2^{\bullet-}]$) or its diffusion-limited reaction with NO (overall first-order kinetics in $[O_2^{\bullet-}]$ and [NO]). A decrease in NO production while constant superoxide production was maintained necessarily favoured an increase in hydrogen peroxide production and a decrease in that of peroxynitrite, as indicated in Figure 6C (left).

After longer incubation times and at an even lower concentration (1 μM, Figure 7C, right), the NO production rate was comparable to that of the controls, whereas superoxide production had increased significantly. The restoration of NO production suggests that the cells may counteract the effect of β-lapachone by increasing their NO synthase activity, or possibly by expressing higher amounts of these enzymes. Indeed, we have recently reported such modulations of NO synthase activity in phagocytes, in which NADPH oxidase activity was genetically down-regulated, with NOS producing both NO and $O_2^{\bullet-}$ in equivalent quantities.[49] In view of that earlier study, it is conceivable that the stress enforced by long incubation with β-lapachone induced overexpression of NOS, so that the initially reduced flux (as observed at short times) of NO and unaltered flux of $O_2^{\bullet-}$ were both increased. Indeed, this would lead to the overall finding that NO production was restored to the same level as in the controls whereas that of superoxide was increased.

In contrast, incubation of macrophages with α-lapachone led to no significant effects on basal levels of ROS and RNS, except for cell death that occurred at excessively high concentrations of α-lapachone. This was an unexpected finding in

view of the great similarity of the two lapachone isomers, but it offered a clue as to the origin of the antioxidant activity found for β-lapachone after short incubation times (Figure 2B). The enzymatic pools responsible for the production of NO are calcium-dependent, whereas *ortho*-quinone derivatives have well established intracellular calcium-chelating properties.[50] The antioxidant effects of β-lapachone observed after short incubation times may be explained by this complexation property, which would decrease the amount of available intracellular calcium ions, a factor that would necessarily reduce the activity of calcium-dependent enzymes such as NO synthases. Conversely, *para*-quinones such as α-lapachone are not able to chelate calcium ions efficiently.[50] They can certainly coordinate them through single bonds, but the steric constraint imposed by the two methyl groups in the quinone ring is undoubtedly too strong to allow for the cooperative clustering of several α-lapachone molecules around one strongly hydrated calcium ion.

The prooxidant activity of β-lapachone observed after long incubation times can be explained on the basis of another well documented property of quinones in living aerobic cells. Quinones are prone to accept electrons from different intracellular redox centres and mediate the formation of superoxide anion radical through redox cycling or "futile cycling" (Scheme 2).



Scheme 2. Redox cycling of β-lapachone; this leads to ROS and RNS production in most cell compartments.

Such increased activity is, in fact, consistent with reports in the literature[9,10] relating to the effects of β-lapachone on tumour cells. Those studies established that this compound exerts prooxidant activities after long incubation times (4 and 6 h) and eventually becomes toxic even for immune cell lines. This was explained in terms of a variety of quinone-induced biological activities, all ultimately based on the fact that they easily accept one and/or two electrons from many cellular electron donors. The reduced quinone derivative may diffuse into many cellular compartments, where it may act as an electron donor to dioxygen (Scheme 2). This would be expected to initiate the intracellular production of reactive oxygen species (ROS), accelerating intracellular oxidative conditions[51] and damaging several cell components,[28,52] including enzymes such as, for example, PTPs (protein tyrosine phosphatases), which catalyse the hydrolysis of phosphoryl groups in tyrosine residues in proteins[53,54] and are emerging as important redox sensors in cells. PTPs each contain a catalytically essential cysteine residue in the signature active site motif, which can be reversibly oxidized by ROS to inactivate the PTP.[55,56] Protein tyrosine phosphorylation is a fundamental mechanism for many signal transduction pathways that control cell growth, differentiation and motility.[54] *Ortho*-quinones have been identified as inhibitors of PTPα, and their mechanism of action has been shown to be mediated by hydrogen peroxide.[57]

Support for this interpretation is again afforded by the difference between the two lapachone isomers. Indeed, the redox cycling and oxygen activation leading to increased levels of ROS is closely related to the quinones' redox potentials.[44,58,59] Consistently with this view, it may be noted that previous experiments with lipoamide dehydrogenase (the enzyme that catalyses redox cycling and superoxide production) established that *ortho*-naphthoquinones catalyse the oxidation of lipoamide by oxygen more efficiently than *para*-naphthoquinones.[60,61] The same trend was previously reported for *Trypanosoma cruzi*,[62] where α-lapachone was found not to induce the release of ROS.

In this respect, it should be noted that because of the absence of a styrene-like structure, as well as the lack of the vicinal electron-withdrawing carbonyl group,[7,63–66a,b] which makes the carbon atoms in these bonds more electron-deficient, the *para*-quinone is reduced at more negative potentials than the *ortho*-isomer [$\Delta E^0 \sim 100$ mV (DMF/TBAP),[63] $\Delta E^0 > 100$ mV (phosphate buffer, pH 7.2)].[65,66] This has also been predicted on the basis of calculated LUMO atomic charges.[67] Such differences in E^0 values may suffice to slow down the rate of redox cycling (Scheme 2), thereby reducing the prooxidant activity of α-lapachone with respect to β-lapachone. According to the Nernst potential law and the Marcus equation, a variation of 100 mV between the two standard potentials implies that for an identical intracellular concentration and over the same duration, β-lapachone would be expected to be about 25 times more active than α-lapachone in the redox cycle (Scheme 2).[68]

Support for this interpretation was provided by this investigation, which found (Figures 6C and 7C) that β-lapachone, within its prooxidant domain of activity, induced strong production of hydrogen peroxide, which is the natural outcome of redox cycling (Scheme 2). α-Lapachone should thus not induce a similar effect before about 24 h, on the basis of their compared redox properties (see above), a result in overall agreement with our observations.

Finally, it should be noted that hydrogen peroxide is normally disproportionated under the influence of catalase. However, this enzyme is deactivated by its substrate when the substrate reaches very high intracellular concentrations. Beyond this threshold, H_2O_2 may accumulate in the cell, in agreement with our findings shown in Figures 7 and 5. Under such conditions, it may diffuse into most of the cellular compartments, where it is prone to evolve by a Fenton reaction[69] into the hydroxyl radical HO•, which is highly reactive to most biological cellular components.

This may explain the cell death process observed after 24 h of incubation. Indeed, sustained production of hydrogen peroxide would be expected to cause increasing damage to most of the cell's machinery through the generation of hydroxyl radicals by the Fenton reaction. Furthermore, in their seminal work, Tagliarino and co-workers[50] observed that the bioactivation of β-lapachone (5 μM) by reductases [for example, NQO1 (diaphorase)] led to futile cycling and consequently to depletion of NADH and NADPH, the electron donors for reductases (NQO1). Exhaustion of reduced enzyme cofactors may therefore be a critical event for the activation of the apoptotic pathway in NQO1-expressing cells after sufficiently long durations of exposure to β-lapachone. This pathway may well explain the cell death that was observed after 24 h of incubation with both lapachone isomers, although the effect of the α-isomer was weaker than that of the β-isomer, again consistently with their distinct reduction potentials. Indeed, higher concentrations and/or prolonged incubation times can initially induce prooxidant activity, but subsequently cause very severe damage to the cell, leading to its death, as we found here at concentrations above 10 μM and incubation times exceeding 6 h.

Conclusions

Electrochemical measurements with microelectrodes are highly interesting for the investigation of biological processes associated with the production of superoxide and/or nitric oxide and their derivatives. The advantages of electrochemical analysis over the traditional biophysical methods include the possible direct detection of the initial onset of oxidative stress conditions, the rapid response time and selectivity of the detection, the possibility of characterizing species that decay rapidly, such as peroxynitrite and NO, and above all, the fact that single cells can be analysed. Taking advantage of the artificial synapse configuration involving a platinized carbon fibre ultramicroelectrode, we were able to investigate the effect of β-lapachone on the nature and strength of cellular oxidative bursts produced by model RAW 264.7 macrophages quantitatively and kinetically. These precise amperometric measurements of minute released quantities performed on single cells confirmed that β-lapachone affects cellular behaviour through two main processes. At short times, the ability of *ortho*-quinones to chelate calcium ions decreases the efficiency of ROS and RNS production by calcium-dependent enzymes, with the overall result that an antioxidant effect is observable. In contrast, after long incubation times, a prooxidant effect becomes apparent, most presumably due to the outcome of redox cycling through which β-lapachone mediates electron transfer to dioxygen, with the result that lethal quantities of ROS and RNS are produced within the cell (Scheme 2). This second mechanism eventually leads to cell death, as reported previously by several authors. In both cases the *ortho* configuration makes β-lapachone more efficient than its *para* isomer. This fact has been ascribed, on the one hand, to a weaker ability to chelate calcium ions, and on the other, to a more negative reduction potential for the *para*-quinone.

Experimental Section

Materials and buffer solutions: Phosphate-buffered saline solution (PBS) was used as buffer throughout the experiments. PBS was prepared from tablets composed of NaCl (137 mM), Na_2HPO_4 (10 mM) and KCl (3 mM, pH 7.4), dissolved in pure water. Water was obtained from a Milli-Q purification system (resistivity = 18 MΩ cm^{-1}, Millipore, Billerica, MA, USA). β-Lapachone (**1**) and α-lapachone (**2**) were synthesized by Prof. Dr. Antonio Ventura Pinto (NPPN, Universidade Federal do Rio de Janeiro, Brazil), and fully characterized by IR, NMR and MS. The analytical data were in full agreement with each molecular structure. Stock solutions in ethanol (5×10^{-3} M) were prepared and stored at 4 °C. All the chemicals were supplied by Sigma (St. Louis, MO, USA) except where indicated otherwise.

Microelectrode fabrication: The procedure for microelectrode fabrication is described elsewhere.[70,71] Briefly, the main steps are as follows: individual carbon fibres (10 μm diameter, Thornel P-55S, Cytec Engineered Materials, West Paterson, New Jersey, USA) were aspirated into glass capillary tubing (1 mm diameter, GC120F-10, Clark Electromedical Instruments, Harvard Apparatus, Edenbridge, UK). Each capillary was then pulled with a microelectrode puller (Model PB-7, Narishige, Tokyo, Japan), and the carbon fibre protruding from the glass tip was subjected to electrochemical deposition of polyoxyphenylene from a precursor solution [allylamine (0.4 M), 2-allylphenol (0.23 M), and 2-butoxyethanol (0.23 M) in water/methanol (1:1, *v/v*)], by a previously described method.[72] Deposition was accomplished by application of a potential of +4 V to a platinum wire counter electrode for 3 min. The polymer deposition was monitored under a microscope. Subsequently, the microelectrodes were washed in distilled water and the polymer was cured for 3 h at 150 °C in order to reticulate it and to form an insulating shield on the carbon fibre surface. The tip of the microelectrode was polished on a diamond particle whetstone microgrinder (Model EG-4, Narishige, Tokyo, Japan) at an angle of 45° for 3 min to expose a clean and regular surface of freshly cut electrochemically active carbon surface. In order to increase the microelectrode sensitivity and selectivity to the reactive oxygen and nitrogen species released by the cells, the polished carbon surface was platinized through reduction of hydrogen hexachloroplatinate (Sigma) in the presence of lead acetate (Sigma) at −60 mV versus SSCE (sodium saturated calomel electrode).[33] The linear increase in the reductive deposition current was recorded, and the process was interrupted when the electrical charge reached the desired value of 30–40 μC. Independent tests showed that this value corresponded to the optimal activity of the electrode surface for these experiments. Under these conditions, detection was quantitative and did not depend on the exact quantity of black platinum deposited.

Cell culture and incubations: The macrophage cell line RAW 264.7 (American Type Culture Collection) was cultured at 37 °C under a CO_2 atmosphere (5%) in Dulbecco's modified Eagle's medium (DMEM) containing D-glucose (1.0 g L^{-1}) and sodium pyruvate (110 mg L^{-1}, Invitrogen, Carlsbad, CA, USA). The medium was supplemented with foetal bovine serum (5%, Invitrogen) and gentamycin (20 μg mL^{-1}, Sigma). The cell lines were maintained at a cell density of $2–5\times10^6$ cells per mL by subculturing every three days following a 1:10 dilution of the cell suspension in fresh medium. Confluent monolayers of RAW 264.7 macrophages were harvested mechanically, resuspended in Petri dishes (35 mm diameter, Nunc, Rochester, NY, USA) and used for electrochemical studies at least 24 h later, a delay that was necessary to allow the cells to recover and start growing. The effects of lapachone derivatives were then assayed by subsequent incubation of cells (1 to 24 h) either with β-lapachone (0.01 μM to 10 μM) or with α-lapachone (1.00 μM to

100 μM), in agreement with conditions applied in former studies of lapachone activities. Solutions were prepared from stock (10 mM) in ethanol and diluted in culture medium before incubation to a final solvent concentration below 1% (*v*/*v*). At this concentration, ethanol affected neither the viability nor the responses of macrophages, as indicated by a comparison of the different control responses before and after incubations with the same solutions but in absence of added quinone of either type.

Single-cell measurements: Amperometric measurements were taken at controlled room temperature ($22\pm1\,^{\circ}C$) on the stage of an inverted microscope (Axiovert 135, Zeiss, Göttingen, Germany) placed inside a Faraday cage. For the experiments, each Petri dish containing macrophages was washed three times with PBS buffer and was then filled with it (4 mL) immediately prior to the measurements.[37] The microelectrode tip was positioned precisely with a micromanipulator (MHW-103, Narishige) at a fixed distance (5 μm) above the surface of an isolated cell. At this point, the tip of a sealed glass microcapillary [1 mm glass rods, GR100–10, Clark Electromedical Instruments, pulled with a PB7 Puller (Narishige) to afford a tip of 1 μm diameter at most] was positioned with a second micromanipulator between the microelectrode surface and that of the cell. The tip was then used to stimulate the cell's response by means of a rapid back and forth movement to trigger a mechanical depolarization of its membrane, returning to its initial position in less than 1 s (Figure 1B). This rapid intrusion of the microcapillary tip into the cell membrane suffices to allow calcium ion to enter the cell cytoplasm and activate the enzymatic pools (NO-synthases and NADPH-oxidases) responsible for generating oxidative stress bursts.[72] The immediate time-dependent burst of ROS and RNS released by the macrophage was detected in real time by amperometry (PRG-DEL amperometric detector, Radiometer Analytical, Copenhagen, Denmark) at a constant potential (*E*) versus a sodium saturated calomel (SSCE) reference electrode. The time-course of the amperometric current was monitored and stored on a PC computer (Dell, Austin, TX, USA) through a D/A converter (Powerlab 4SP, AD Instruments, Colorado Springs, CO, USA) and its software interface (Chart version 5.0). A series of microelectrode potentials (*E*, 850, 650, 450 and 300 mV vs. SSCE) that enabled selective measurements of the time-dependent flux for each species released by the cells was used as determined previously on the basis of in vitro voltammetric studies of the oxidation of independent solutions of H_2O_2, $ONOO^-$, NO and NO_2^- (each at 1 mM in PBS).[37]

Statistics: To account for the cellular variability and to obtain statistically significant information, data were processed on the basis of pairs of control experiments (Q_c) and experimental test conditions (Q_e). At first, a simple analysis of variance was carried out to ascertain that the average values $<Q_c>$ and $<Q_e>$ were significantly different; p values <0.05 were regarded as indicating significant differences. Secondly, it appeared that both $\log(Q_c)$ and $\log(Q_e)$ were distributed as normal distributions (that is, Q_c and Q_e are distributed as log-normal variables). Accordingly, the variable $\log(\rho)=\log(Q_e/Q_c)$ obeyed a normal distribution with a mean $<\rho>$. This allowed us to compute the 90% confidence interval of $\log(<\rho>)$ between $<\rho>-\Delta\rho$ and $<\rho>+\Delta\rho$. The values of ratio $<\rho>$ and its confidence limits, as well as the p value, could thus be determined for each pair of control conditions and experiment conditions and are reported in the figures (* for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$).

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Keywords: amperometry · electrochemistry · electrodes · lapachones · macrophages · oxidative stress

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