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Abstract

Apatone™, a combination of menadione (2-methyl-1,4-naphthoquinone, VK_3) and ascorbic acid (vitamin C, VC) is a new strategy for cancer treatment. Part of its effect on tumor cells is related to the cellular pro-oxidative imbalance provoked by the generation of hydrogen peroxide (H_2O_2) through naphthoquinone redox cycling. In this study, we attempted to find new naphthoquinone derivatives that would increase the efficiency of H_2O_2 production, thereby potentially increasing its efficacy for cancer treatment. The presence of an electron-withdrawing group in the naphthoquinone moiety had a direct effect on the efficiency of H_2O_2 production. The compound 2-bromo-1,4-naphthoquinone (BrQ), in which the bromine atom substituted the methyl group in VK_3, was approximately 10- and 19-fold more efficient than VK_3 in terms of oxygen consumption and H_2O_2 production, respectively. The ratio $[H_2O_2]_{produced}$ / $[naphthoquinone]_{consumed}$ was 68 ± 11 and 5.8 ± 0.2 (µM/µM) for BrQ and VK_3, respectively, indicating a higher efficacy of BrQ as a catalyst for the autoxidation of ascorbic acid. Both VK_3 and BrQ reacted with glutathione (GSH), but BrQ was the more effective substrate. Part of GSH was incorporated into the naphthoquinone, producing a nucleophilic substitution product (Q-SG). The depletion of BrQ by GSH did not prevent its redox capacity since Q-SG was also able to catalyze the production of reactive oxygen species. VK_3/VC has already been submitted to clinical trials for the treatment of prostate cancer and has demonstrated promising results. However, replacement of VK_3 with BrQ will open new lines of investigation regarding this approach to cancer treatment.

Key words: Cancer; Apatone™; Ascorbic acid; Hydrogen peroxide; 2-Bromo-1,4-naphthoquinone

Introduction

The combination of ascorbic acid (vitamin C, VC) and menadione (2-methyl-1,4-naphthoquinone, vitamin K_3, VK_3) has been widely explored in several studies *in vitro* (1-5) and *in vivo* (5-8) as a new therapy against cancer in which the use of both vitamins demonstrated a synergistic action compared to the administration of either vitamin alone (1,9). The United States Patent and Trademark Office (USPTO) approved a patent and assigned the trademark name Apatone™ (Indian Creek Medical Technologies) with serial number 78475364 to this compound. Apatone™ is characterized by the administration of a combination of VC and VK_3 to target and kill cancer cells. Apatone™ was used in end-stage prostate cancer patients who were not responding to conventional therapy. The treatment was considered safe and effective since, of the 15 patients who continued Apatone™ for more than 12 weeks, only 1 died after 14 months of treatment (10).

The antitumor activity of the VC/VK_3 combination has been associated with the generation of reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2) (3). This reaction occurs when VK_3 is non-enzymatically reduced by ascorbate to form the VK_3 equivalent semiquinone free radical and dehydroascorbate. The transient semiquinone free radical is reoxidized to its quinone form by molecular oxygen, thus generating ROS such as superoxide radical anion ($O_2^{-\bullet}$), H_2O_2 and hydroxyl radicals ($^\bullet OH$) (3). One characteristic of tumor cells seems to be directly related to their susceptibility to VC/VK_3 therapy. This includes the reduced level of ROS detoxifying enzymes such as catalase, superoxide dismutase (SOD) and glutathione (GSH) peroxidase (11,12), which elicits a redox imbalance. In this regard, the VC/VK_3 combination provokes an additional oxidative

stress characterized by decreased intracellular thiol levels, increased intracellular Ca^{2+} levels and lipid peroxidation in cells already deficient in their intrinsic antioxidant protection (13). The VC/VK$_3$ combination also inhibits the glycolytic pathway (14), which is the primary mechanism responsible for respiration in cancer cells, known as "The Warburg Effect" (15). Additionally, VC accumulates in tumor cells through isoforms of the glucose transporter (GLUT) family, i.e, GLUT1, 3 and 4 (16,17). An important aspect of the signaling pathway involved in the cytotoxicity of VC/VK$_3$ is that, besides necrosis and apoptosis, the antitumor activity of this vitamin combination has been attributed to a new type of cell death first observed in 1993 (18) and named autoschizis in 1998 (19). In contrast to apoptosis, the activation of caspase 3 is not verified in autoschizis (20). Autoschizis is also characterized by the excision of cytoplasmic fragments (7,9,21).

Since a pro-oxidative imbalance in tumor cells provoked by incubation with VC/VK$_3$ seems to underlie the pharmacological mechanism for cancer treatment with this drug combination, we studied and compared the efficiency of different VK$_3$-related compounds, searching for higher efficacy in the production of H_2O_2 when these molecules catalyze the autoxidation of ascorbic acid.

Material and Methods

Chemicals

Ascorbic acid, VK$_3$, 2-bromo-1,4-naphthoquinone (BrQ), 2-methoxy-1,4-naphthoquinone (MQ), SOD, catalase, GSH, oxidized glutathione (GSSG), *N*-ethylmaleimide (NEM), cytochrome C, ortho-phthalaldehyde (OPA), and reduced nicotinamide adenine dinucleotide (NADH) were purchased from Sigma-Aldrich Chemical Co. (USA). H_2O_2 was prepared by diluting a 30% stock solution and calculating the concentration from the absorption of the solution at 240 nm (λ = 43.6 M^{-1} cm^{-1}). All reagents used for solutions, buffers, and mobile phases were of analytical grade.

Measurement of oxygen consumption

The consumption of dissolved molecular oxygen during the redox cycling reactions was monitored using a YSI 5300A Oxygen Monitor (USA). Unless otherwise stated, the reaction mixture contained 500 µM ascorbic acid and 10 µM naphthoquinones (BrQ, VK$_3$, or MQ) in 10 mM phosphate-buffered saline (PBS), pH 7.4, at 37°C. Before the beginning of the reaction, the opened reaction cuvette containing PBS and naphtoquinones was equilibrated to reach air saturation at 37°C. Then, the electrode was inserted and the reaction triggered by adding ascorbic acid.

Measurement of H_2O_2 production

The production of H_2O_2 during the redox cycling reac-

tion was monitored using an amperometric detector and a biosensor specific for H_2O_2 (TRB 4100, World Precision Instruments, USA). Initially, the instrument was calibrated using H_2O_2 standards ranging from 10 to 200 µM and an analytical curve was constructed to convert ΔpA to the H_2O_2 concentration. Unless otherwise stated, the reaction mixture contained 500 µM ascorbic acid and 10 µM naphthoquinones (BrQ, VK$_3$ or MQ) in 10 mM PBS, pH 7.4, at 37°C.

Measurement of naphthoquinone consumption

For the measurement of naphthoquinone consumption during the redox cycling, the reaction mixtures consisting of 10 µM naphthoquinones (BrQ or VK$_3$) and 500 µM ascorbic acid in 10 mM PBS, pH 7.4, at 37°C were incubated for 15 min and the remaining concentration was measured by HPLC coupled to a diode array detector set at 265 nm (Jasco, USA). The analyses were conducted isocratically on a Luna C18 reversed-phase column (250 x 4.6 mm, 5 µm) with a mobile phase consisting of 0.1% formic acid:methanol (40:60, v/v). The flow rate was 1.0 mL/min.

Measurement of GSH consumption and GSSG production

The effect of GSH on the redox cycling reaction was studied in terms of the production of ROS, depletion of GSH and production of GSSG using OPA as a fluorescence derivatization reagent and HPLC analysis, as previously described (22) and as explained below.

GSH assay. After incubation of the reaction mixture consisting of naphthoquinones (BrQ or VK$_3$) and GSH in the presence or absence of ascorbic acid, a 50-µL aliquot was removed and added to 500 µL 0.1% EDTA in 0.1 M Na$_2$HPO$_4$, pH 8.0. A 300-µL amount of 0.1% EDTA in 0.1 M Na$_2$HPO$_4$ and 20 µL 0.1% OPA in methanol were added to a 20-µL aliquot of this mixture. This final mixture was incubated at 25°C for 15 min in the absence of light and injected (20 µL) into the HPLC system.

GSSG assay. Next, 200 µL of the same reaction mixture as used for analysis of GSH was incubated at 25°C with 200 µL NEM for 25 min in the absence of light to interact with the remaining GSH present in the sample. A 750-µL amount of 0.1 M NaOH was added to this mixture and an aliquot of 20 µL was removed and added to 300 µL 0.1 M NaOH and 20 µL 0.1% OPA in methanol. This final mixture was incubated at 25°C for 15 min in the absence of light and injected (20 µL) into the HPLC system.

HPLC method. The analyses were carried out on a Luna C18 reverse-phase column (250 x 4.6 mm, 5 µm) using an HPLC-fluorescence detection system (Jasco). The mobile phase consisted of 15% methanol in 25 mM NaH$_2$PO$_4$ (v/v), pH 6.0. The flow rate was maintained at 1.0 mL/min. The detection of the fluorescent derivative was observed at 350 nm excitation and 420 nm emission. Standard curves

were constructed to calculate the consumption of GSH and formation of GSSG (data not shown).

Identification of the GSH-conjugated product

The identification of the product obtained when BrQ was incubated with GSH (100 µM) was performed by direct injection of the previously HPLC-purified product into the mass spectrometer operating in negative ion mode (LCQ Fleet Ion Trap Mass spectrometer, Thermo Scientific, USA). The negative molecular ion peak [M - 1] = 462.05 corresponded to the product that was generated by the substitution of the bromine atom at position 2 of the naphthoquinone moiety with GSH, leading to Q-SG (MW 463.09). An additional confirmation of this product was the fragment observed at 306.16, which corresponded to GS$^-$. The reaction conditions were 10 µM BrQ and 100 µM GSH for 3 min in PBS at 37°C.

Analysis of superoxide production

The generation of a superoxide anion during the redox cycling was measured by adding 100 µM cytochrome c to the reaction mixture containing 10 µM naphthoquinones and 50 µM VC in PBS, pH 7.4. The blank for absorbance measurements consisted of cytochrome c and BrQ in PBS. An HP8452 diode array spectrophotometer (Agilent, USA) was used to measure absorbance. The reaction was initiated by adding VC and then scanned at 30-s intervals. A sharp peak at 550 nm is characteristic of reduced cytochrome c generated by a superoxide anion.

Measurement of the effect of NADH on ascorbic acid consumption

The effect of NADH on the redox cycling reaction was studied by measuring the rate of consumption of ascorbic acid in the presence or absence of this reduced coenzyme. The reaction mixture contained 100 µM NADH, 10 µM naphthoquinones (BrQ or VK$_3$) and 50 µM VC in PBS, pH 7.4. The blank for absorbance measurements contained PBS and naphthoquinone. An HP8452 diode array spectrophotometer (Agilent) was used to measure the variation in absorbance. The reaction was initiated by adding VC and then scanned at 30-s intervals. The band centered at 270 nm was chosen to monitor ascorbic acid consumption and the band centered at 340 nm was followed to monitor the oxidation of NADH.

Results

We studied the capacity of naphthoquinone derivatives in terms of their efficacy as generators of H$_2$O$_2$ when combined with ascorbic acid. The naphthoquinone moiety is an important pharmacophoric element for cytotoxic activity (23) and the reduction potential of the aromatic compounds is sensitive to the presence of substituent groups in the ring (24). Thus, we compared the redox properties of menadione with BrQ and MQ (Figure 1). These compounds were chosen because bromine is an electron-withdrawing atom and, conversely, methoxy is an electron-donating group stronger than the methyl group present in the parent molecule (24). Hence, we were able to analyze both electronic effects on the redox cycling of the naphthoquinone moiety driven by ascorbic acid. The concentration of ascorbic acid (500 µM) and naphthoquinones (10 µM) were chosen to confirm the catalytic effect of the latter compound and are of the same proportions of compounds used in Apatone™ (10). Figure 2A shows that the presence of an electron-withdrawing group in the naphthoquinone moiety provoked a significant increase in the rate of oxygen consumption compared to menadione or to the methoxy derivative. As observed, approximately 75% of the dissolved molecular oxygen was depleted within 1 min of reaction using BrQ, compared to only 5% using VK$_3$ or 7% MQ. In these experiments, the reaction medium was previously equilibrated at 37°C. Hence, the relative concentration of 100% is equivalent to approximately 200 µM of dissolved oxygen in PBS. Therefore, considering that only 10 µM BrQ was added to the reaction mixture, this is evidence of its efficient recycling during the reaction course. Figure 2B depicts the comparison the relative initial rate of oxygen depletion measured in the linear phase of the reaction. Considering that the higher efficiency of BrQ as a redox cycling agent could offer a therapeutic advantage since it could be applied in a lower-dose regimen, we tried to find the lowest concentration of BrQ that was as efficient
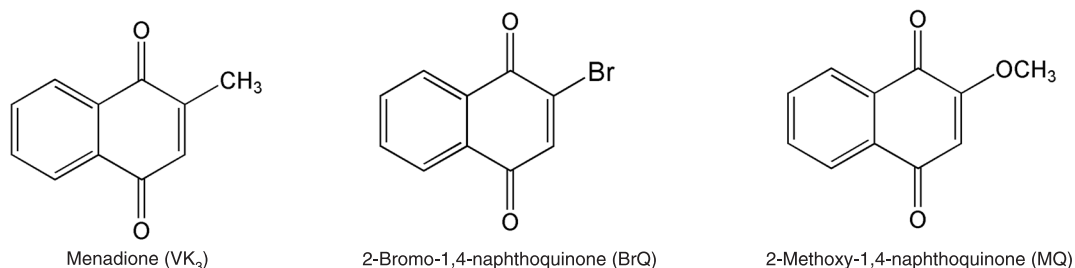
Menadione (VK$_3$)   2-Bromo-1,4-naphthoquinone (BrQ)   2-Methoxy-1,4-naphthoquinone (MQ)

Figure 1. Molecular structures of the naphthoquinones.

as VK$_3$. Figure 2C shows that using about one-hundredth the concentration of BrQ, the rate of oxygen depletion was still comparable to that obtained with VK$_3$.

The superoxide anion generated by the redox cycling of the naphthoquinone was transient, being promptly dismuted to H$_2$O$_2$. The evidence of a peak at 550 nm when cytochrome c was added to the reaction medium was a confirmation of the production of superoxide anion (Figure 3A and B). Here we followed the production of H$_2$O$_2$ using a specific electrode and an amperometric detector, which was previously calibrated with standard solutions of H$_2$O$_2$. The results displayed in Figure 3C and D confirmed the higher catalytic effect of BrQ compared to VK$_3$ and MQ. The addition of catalase during the course of the reaction confirmed the formation of H$_2$O$_2$ (Figure 3E).

We used the rate of H$_2$O$_2$ production as an experimental approach to study the effect of the reactants in the redox cycling reaction. As depicted in Figure 3F, the rate of H$_2$O$_2$ production was dependent on the concentration of ascorbic acid. On the other hand, the addition of SOD, which increased the rate of H$_2$O$_2$ production by catalyzing the dismutation of the superoxide anion, did not provoke any alteration (Figure 3G and H). Taken together, these experiments demonstrated that the rate-determining step in the redox cycling reaction is the interaction between ascorbic acid and BrQ.

From another point of view, the combination of ascorbic acid and naphthoquinone is a way to exacerbate the autoxidation of ascorbic acid. In other words, naphthoquinone acts as a catalyst for these reactions (3). However, part of the naphthoquinone can be transformed to other products, which, consequently, decreases its capacity for ROS production. For this reason, we measured the consumption of naphthoquinones during the reaction course. We noted that VK$_3$ was consumed less than BrQ (Figure 4). However, considering the production of H$_2$O$_2$ within the same time interval, the ratio [H$_2$O$_2$] μM / min$_{produced}$ / [naphthoquinone]. μM / min$_{consumed}$ was 68 ± 11 and 5.8 ± 0.2 for BrQ and VK$_3$, respectively, which revealed the higher efficacy of BrQ as a catalyst for the autoxidation of ascorbic acid.

In addition to acting as a catalyst for the generation of H$_2$O$_2$, naphthoquinone may have other effects in the cellular medium. First, as an electrophilic molecule, naphthoquinone is able to react with the intracellular antioxidant and nucleophilic GSH. The consequence of this reaction is the depletion of the intracellular antioxidant reserve (25). Here, we found that both VK$_3$ and BrQ reacted with GSH, but BrQ was the more effective substrate. Part of the GSH was incorporated into the naphthoquinone, producing a nucleophilic substitution product (Q-SG; Figure 5). Another part of the GSH was oxidized to GSSG (Figure 6).

The depletion of GSH took place in the absence and, without a significant difference, in the presence of ascorbic acid, but it did not avoid the redox cycling reaction. Considering that the consumption of BrQ by GSH could hamper
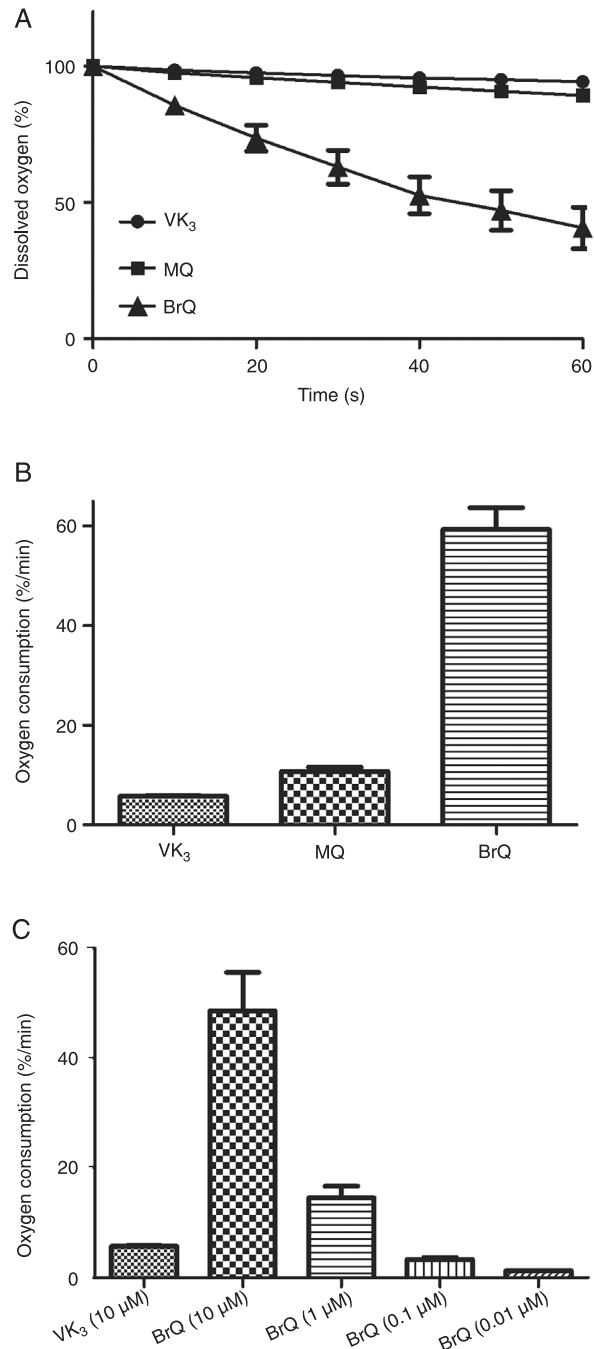

Figure 2. Consumption of dissolved molecular oxygen. *A*, Kinetic profiles of oxygen depletion. *B*, Rate of depletion (% oxygen/min). The reaction mixtures consisted of naphthoquinones (10 μM VK$_3$, MQ or BrQ) and vitamin C (500 μM) in PBS, pH 7.4, at 37°C. *C*, Effect of the concentration of BrQ on oxygen depletion. The results are representative of at least three different experiments. VK$_3$ = 2-methyl-1,4-naphthoquinone; MQ = 2-methoxy-1,4-naphthoquinone; BrQ = 2-bromo-1,4-naphthoquinone.
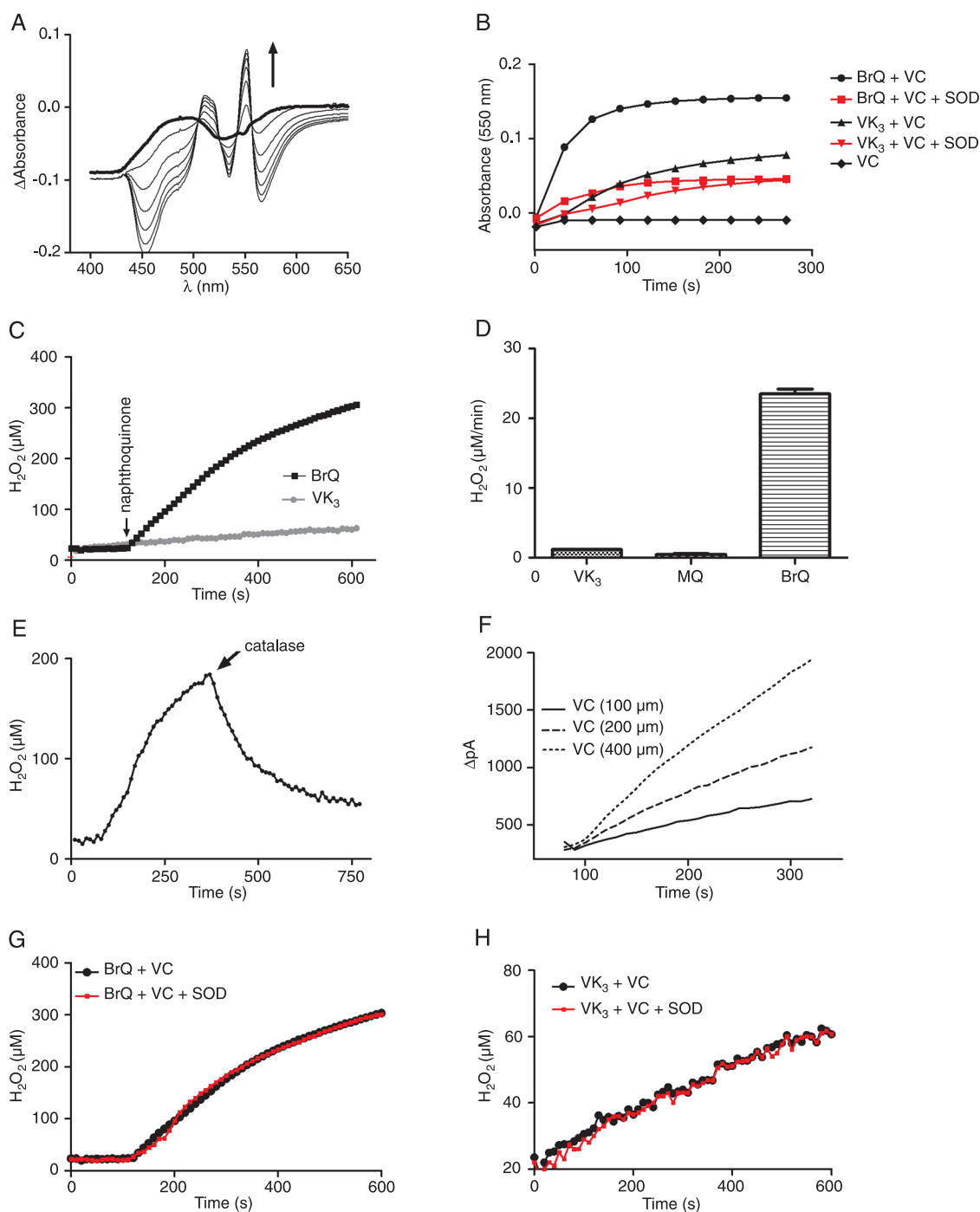
Figure 3. *A*, Detection of the formation of transient superoxide anion during the redox cycling reaction (VC/BrQ) by the reduction of cytochrome c (100 µM). Arrow shows the direction of absorbance increase as the reaction proceeds. *B*, Effect of SOD (3 mg/mL) on the production of transient superoxide anion. *C*, Kinetic profile of H_2O_2 production during the redox cycling reactions. *D*, Rate of H_2O_2 production. *E*, Evidence of the formation of H_2O_2 (VC/BrQ, catalase 3 mg/mL). *F*, Effect of ascorbic acid on the rate of H_2O_2 production in the reaction with BrQ. *G*, Effect of SOD (3 mg/mL) on the H_2O_2 production (VC/BrQ). *H*, Effect of SOD on H_2O_2 production (VC/VK$_3$). The reaction mixtures consisted of naphthoquinones (10 µM) and VC (500 µM) in PBS, pH 7.4, at 37°C. The results are representative of at least three different experiments. VC = vitamin C; BrQ = bromoquinone; SOD = superoxide dismutase; VK$_3$ = 2-methyl-1,4-naphthoquinone; MQ = 2-methoxy-1,4-naphthoquinone.

its efficacy in the generation of ROS, we tested the capacity of the substitution product Q-SG as a catalyst for the production of ROS in the presence of ascorbic acid. In this case, BrQ and GSH were incubated for 25 min to allow time for the total conversion to Q-SG. Ascorbic acid was then added and oxygen was measured. The results depicted in Figure 7 show that the redox cycling reaction was also effective using Q-SG.

We also monitored the consumption of ascorbic acid and the effect of the addition of the coenzyme NADH. As depicted in Figure 8, the addition of NADH to the reaction mixture consisting of BrQ and VC promoted its oxidation and inhibited the depletion of ascorbic acid. Again, BrQ was significantly more effective than VK_3, which had a small effect on ascorbic acid depletion and no effect on NADH oxidation.
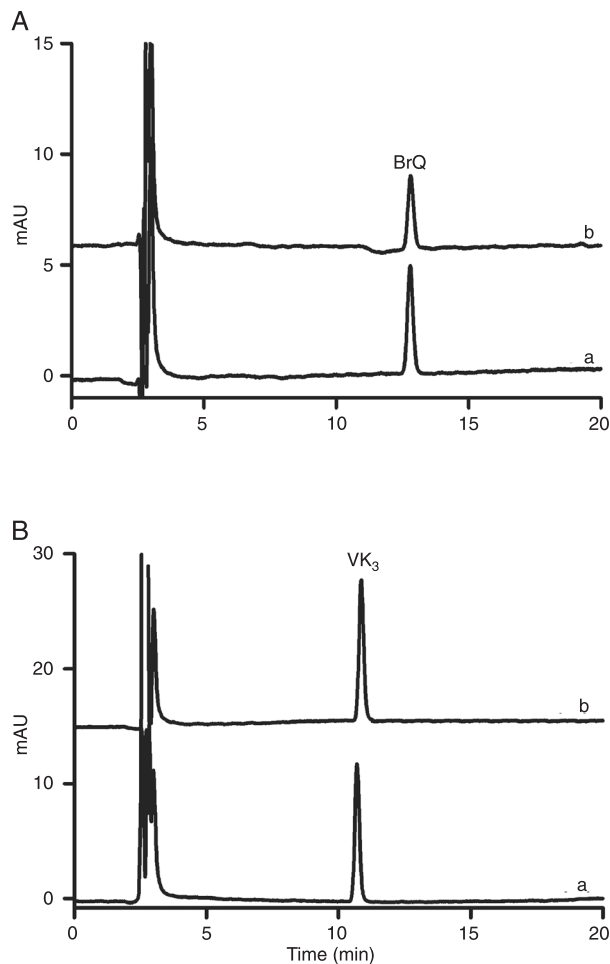

Discussion

ROS, particularly H_2O_2, have dose-dependent, but somehow opposite effects on cancer cells. For instance, H_2O_2 at low concentration acts as a second messenger in the pathways that lead to increased cell proliferation through activation of important receptor tyrosine kinases, cytokines and transcription factors such as activator protein 1, hypoxia-inducible factor-1, nuclear factor-κB (26-28), and p53 (29). Other events related to H_2O_2 signaling involve the induction of the expression of proto-oncogenes such as c-*fos*, c-*jun* and c-*myc*, thus contributing to the expression of genes related to proteins of the cell cycle that promote persistent cell proliferation, apoptosis inhibition and metastases (26). On the other hand, the increased concentration of ROS at the cellular level is considered to be an effective way to promote the death of cancer cells (30,31). For instance, a high concentration of H_2O_2 induces cell cycle arrest and DNA alterations, including DNA damage, mutations

Figure 4. Consumption of the naphthoquinones during the redox cycling reaction. *Aa*, Pure BrQ (10 µM); *Ab*, VC/BrQ reaction. *Ba*, Pure VK_3 (10 µM); *Bb*, VK_3/VC reaction. The reaction mixtures consisted of naphthoquinones (10 µM) and VC (500 µM) in PBS at 37°C and the remaining concentration of naphthoquinones measured after 15 min. BrQ = bromoquinone; VC = vitamin C; VK_3 = 2-methyl-1,4-naphthoquinone.
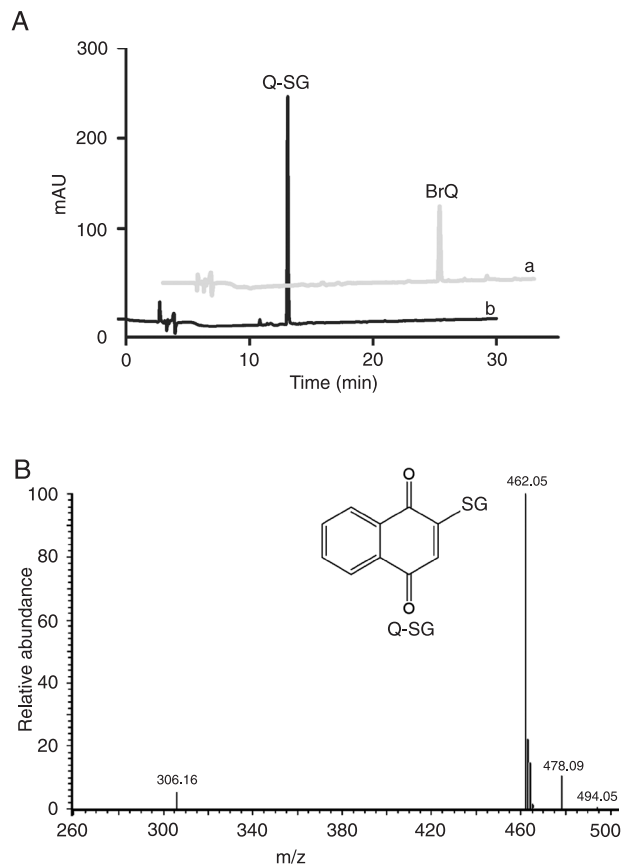
Figure 5. Reaction between BrQ and GSH. *Aa*, Pure BrQ (10 µM); *Ab*, consumption of BrQ and production of Q-SG. *B*, Mass spectrum of Q-SG. The reaction conditions were BrQ (10 µM) and GSH (100 µM) for 3 min in PBS at 37°C. BrQ = bromoquinone; GSH = glutathione; Q-SG = nucleophilic substitution product.

and genetic instability, which trigger alternative signaling pathways and changes in cellular metabolism leading to
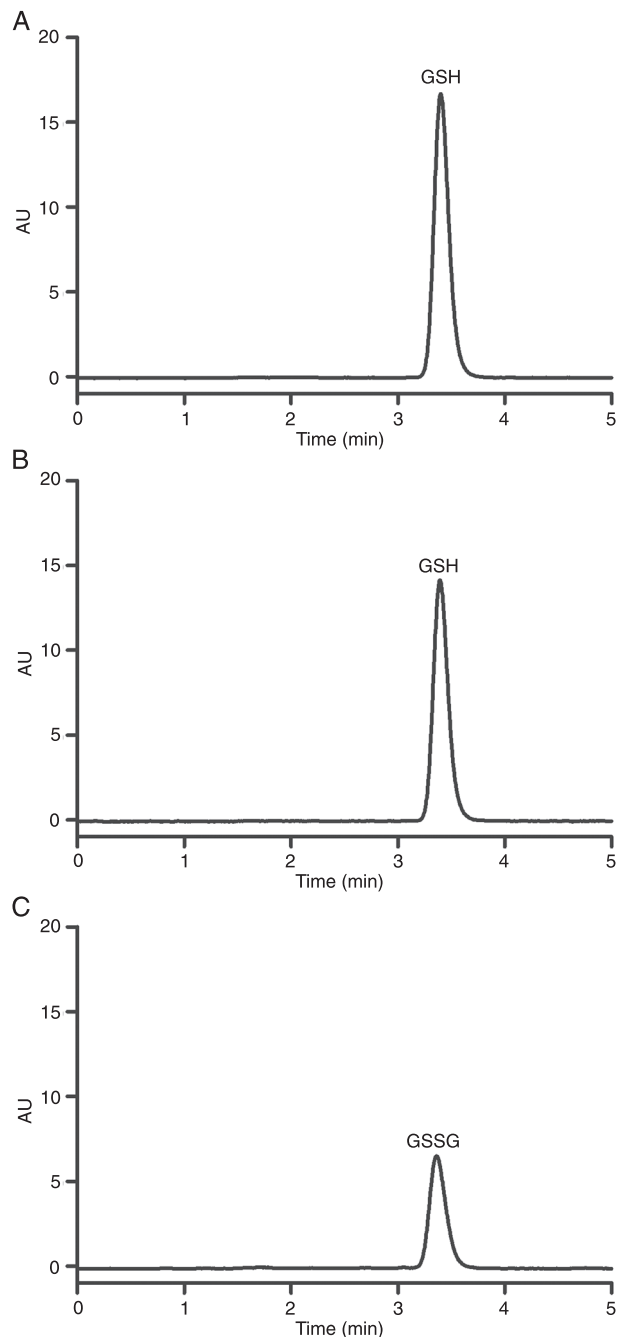
cell death (31). Considering this last characteristic, several therapeutic approaches to cancer are based on or linked to the exacerbation of the generation of ROS in tumors. Obviously, this is the case with the use of VK_3/VC in several *in vitro* (1-5,9,14,18-21,32) and *in vivo* (6-8,10,14) models. It is important to note that, when used alone, both vitamins present cytotoxic effects through mechanisms that include the generation of H_2O_2 (2), but the combined administration of VC/VK_3 shows synergistic and selective cytotoxic activity at doses as much as 50 times lower than either vitamin alone (1). It is well established that, in the VC/VK_3 combination, the latter vitamin works as a catalyst for the autoxidation of ascorbate, increasing the intracellular concentration of H_2O_2, inducing the G1/S and G2/M cell cycle, blocking the cell cycle, and decreasing DNA synthesis, among other cellular events, where the exacerbated production of H_2O_2 is of pivotal importance (2,32).

Considering these findings, the present study was performed to search for new and more efficient drug combinations that could enhance the production of ROS. The VC/BrQ pair presented here was more effective than the well-established VC/VK_3 combination in terms of the production of ROS, particularly H_2O_2. This augmented potency is related to the electron-withdrawing feature of the bromine atom compared to the methyl group present in VK_3. Indeed, the presence of electron-withdrawing groups at aromatic rings increase their redox potential and, consequently, their pro-oxidant capacity (33). This property was observed here, since BrQ was more effective in the oxidation of ascorbic acid and GSH and the production of a superoxide anion and H_2O_2. These results are consistent with the increased redox cycling of quinones of higher redox potential (4).

Figure 6. Consumption of GSH and production of GSSG. *A*, Pure GSH (100 µM); *B*, remaining concentration of GSH after the redox cycling reaction; *C*, production of GSSG after the redox cycling reaction. The reaction conditions were bromoquinone (10 µM) and GSH (100 µM) in PBS, pH 7.4, at 37°C. After 15 min, aliquots of the reaction mixtures were removed and submitted to derivatization and analysis. GSH = glutathione; GSSG = oxidized glutathione.
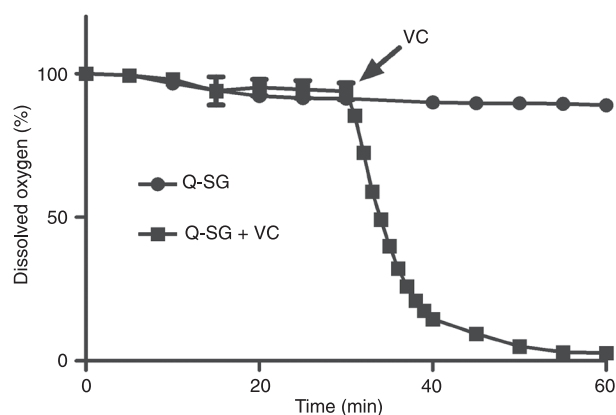

Figure 7. Consumption of dissolved molecular oxygen by Q-SG. BrQ and GSH were previously incubated for 30 min to guarantee the total conversion to Q-SG. The reaction conditions were BrQ (10 µM) and GSH (100 µM) in PBS, pH 7.4, at 37°C. Ascorbic acid (500 µM; VC) was added at the indicated time. Q-SG = nucleophilic substitution product; BrQ = bromoquinone; GSH = glutathione.

The success of the VC/VK$_3$ combination is also related to the fact that tumor cells have an increased capacity to accumulate VC compared to normal cells (16). Indeed, a characteristic of tumor cells is the uptake of dehydroascorbate through one or more glucose transporter isoforms, including GLUT1, GLUT3 or GLUT4 (34). This oxidized form of ascorbate is produced in the pro-oxidant microenvironments around tumors. Once inside these cells, dehydroascorbate is reduced back to ascorbate by NADH-dependent semidehydroxyascorbate reductase or glutathione-dependent dehydroxyascorbate reductase (35), which prevents its reverse transport and, consequently, results in the accumulation of up to 100-fold higher concentrations of VC compared to the extracellular milieu (17,34). We propose that this property of the VC/VK$_3$ combination can also be improved by using BrQ instead of VK$_3$, since the oxidation of ascorbate to dehydroascorbate was more effective using BrQ.

Another important difference between normal and tumor cells is the increased use of the glycolytic pathway by the latter (15). For example, the use of Apatone™ provokes an 80% decrease in the respiration of tumor cells due to a 30% inhibition in the activity of glyceraldehyde-3-phosphate dehydrogenase

and 100% depletion of cellular NAD$^+$ (36). The high level of dehydroascorbate has also been proposed to be involved in the inhibition of other enzymes in this metabolic pathway, including hexokinase and glucose-6-phosphate dehydroge-
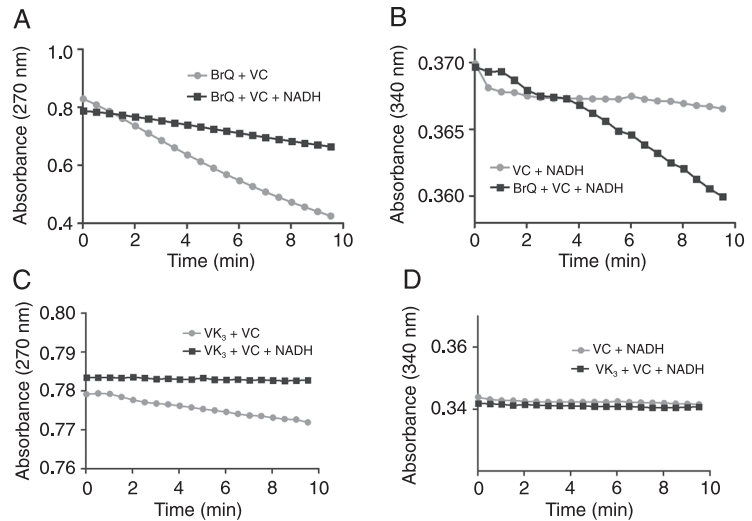
Figure 8. Oxidation of NADH during the redox cycling reaction. *A,C*, Oxidation of ascorbic acid monitored by its absorbance at 270 nm. *B,D*, Oxidation of NADH monitored by its absorbance at 340 nm. The reaction mixtures consisted of BrQ or VK$_3$ (10 µM) and VC (50 µM) in PBS at 37°C in the presence or absence of NADH (100 µM). NADH = nicotinamide adenine dinucleotide; BrQ = bromoquinone; VK$_3$ = 2-methyl-1,4-naphthoquinone; VC = vitamin C.
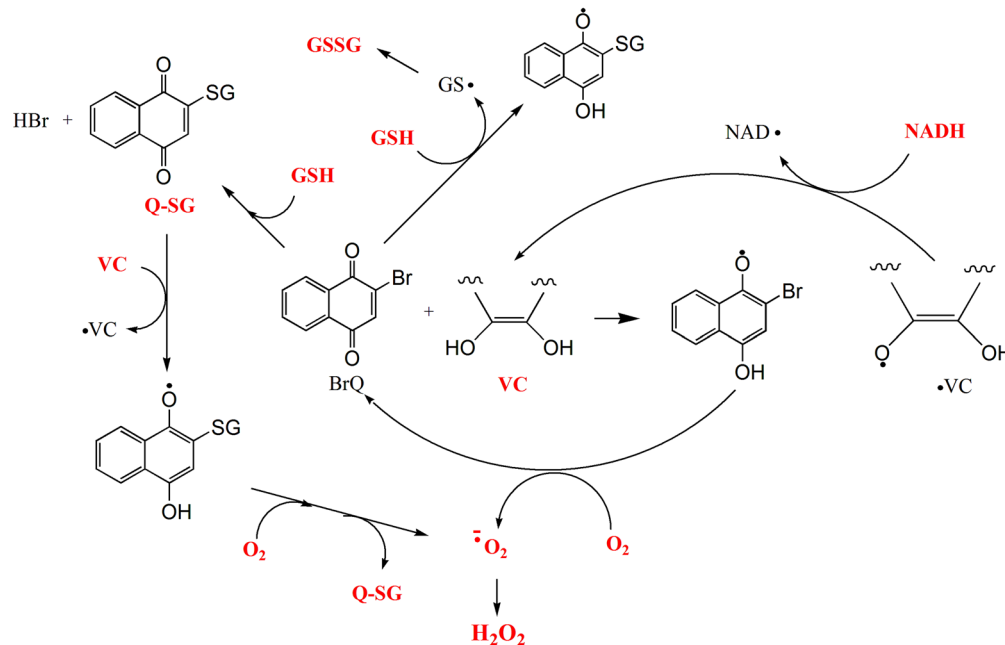
Figure 9. Pathways proposed for the BrQ/VC redox cycling reaction in the presence or absence of GSH or NADH. The compounds that were measured by its consumption or production are marked in red. BrQ = bromoquinone; VC = vitamin C (simplified structure of ascorbic acid); GSH = glutathione; NADH = nicotinamide adenine dinucleotide; Q-SG = substitution product of the reaction between BrQ and GSH.

nase (37). In addition to the high levels of ROS produced by BrQ/VC, we demonstrated that this combination, but not VK_3/VC, can chemically deplete the coenzyme NADH, which could have direct implications for the glycolytic pathway.

The VC/VK_3 combination also decreases the intracellular GSH level and initiates membrane lipid peroxidation. The oxidation of GSH has been correlated with the release of mitochondrial Ca^{2+}, thus activating endonucleases, lipases and proteases leading to signaling pathways related to cell death (13). This is another feature that could be improved using BrQ/VC. First, this drug combination was more effective in the depletion of GSH, leading to the production of its oxidized form, GSSG. Second, BrQ also reacted directly with GSH, leading to a compound in which the bromine was replaced with GSH. This molecule, which we called Q-SG, did not lose its catalytic effect on ascorbic acid autoxidation, which enhanced its efficacy, since, in addition to the depletion of GSH, the products of the reaction were still able to generate ROS.

In conclusion, we performed a complete chemical study of the redox cycling reaction using BrQ/VC (Figure 9). Our results show that the substitution of VK_3 by BrQ significantly increases the generation of a pro-oxidative state compared to VK_3. As we have demonstrated, the concentration of BrQ could be reduced by a factor of 100 and still maintain an effect similar to that of VK_3. This property could lead to a lower-dose regimen for cancer treatment. We propose that this combination should be applied in *in vitro* and *in vivo* studies that use Apatone™.

Acknowledgments

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