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The spermostatic and microbicidal actions of quinones and maleimides: towards a dual purpose contraceptive agent

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Running title: Spermicide-microbicide development

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ABBREVIATIONS: cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; AKAP, A kinase anchoring protein; MOMP, major outer membrane protein; HIV, immunodeficiency virus; AIDS, acquired immune deficiency syndrome; STD, sexually transmitted disease; N9, nonoxynol 9; BWW, Biggers, Whitten and Whittingham medium; IgG, immunoglobulin G; FITC, flourescein isothiocyanate; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DHE, dihydroethidium; SyG, Sytox green; DTT, dithiothreitol; *p*CMBS, *p*-chloromercuribenzene sulfonic acid; Ptx, pentoxifylline; dbcAMP, dibutyryl cyclic adenosine monophosphate; PBS, phosphate buffered saline; BSA, bovine serum albumin; MALDI-TOF. matrix-assisted-laser desorption-ionization-time-of-flight mass spectrometer; MBP, maltose binding protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TI, therapeutic index; EDTA, ethylenediaminetetraacetic acid; ROS, reactive oxygen species; HPLC, high pressure liquid chromatography; Et, ethidium; 2OHEt, 2-hydroxyethidium; LUMO, lowest unoccupied molecular orbital; sAC, soluble adenylyl cyclase, *p*-BQ, *p*-benzoquinone; BM, bismaleimide; 2,6DCBQ, 2,6 dichloro-*p*-benzoquinone; *p*-NQ, *p*-naphthoquinone; 2,6-DMBQ, 2,6 dimethoxy-*p*-benzoquinone; Mena, menadione; Spz, human spermatozoa

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ABSTRACT

There is an urgent need to develop safe, effective dual-purpose contraceptive agents that combine the prevention of pregnancy with protection against sexually transmitted disease. Here we report the identification of a group of compounds that on contact with human spermatozoa induce a state of 'spermostasis', characterized by the extremely rapid inhibition of sperm movement without compromising cell viability. These spermostatic agents were more active and significantly less toxic than the reagent in current clinical use, nonoxynol 9, giving therapeutic indices (ratio of spermostatic to cytotoxic activity) that were orders of magnitude greater than this traditional spermicide. Although certain compounds could trigger reactive oxygen species generation by spermatozoa, this activity was not correlated with spermostasis. Rather, the latter was associated with alkylation of two major sperm tail proteins that were identified as A Kinase Anchoring Proteins (AKAP3 and AKAP4) by mass spectrometry. As a consequence of disrupted AKAP function, the abilities of cAMP to drive PKA-dependent activities in the sperm tail such as the activation of SRC and the consequent stimulation of tyrosine phosphorylation, were suppressed. Furthermore, analysis of microbicidal activity using *Chlamydia*, revealed powerful inhibitory effects at the same low micromolar doses that suppressed sperm movement. In this case, the microbicidal action was associated with alkylation of MOMP, a major Chlamydial membrane protein. Taken together, these results have identified for the first time a novel set of cellular targets and chemical principles capable of providing simultaneous defense against both fertility and the spread of sexually transmitted disease.

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Introduction

The World Health Organization has highlighted the need to develop novel, safe, effective dual-purpose contraceptive agents that combine prevention of pregnancy with protection against sexually transmitted disease (World Health Organization, 2002). Currently, over 40 million people are living with human immunodeficiency virus (HIV) and 18,000 new infections are notified every day (UNAIDS/WHO, 2006). In 2006 alone, the acquired immune deficiency syndrome (AIDS) claimed around 3 million lives. The pandemic in sexually transmitted disease (STD) is not confined to HIV. Infection rates for a wide variety of other pathogenic bacteria (syphilis, Chlamydia and gonorrhea) and protozoa (trichomoniasis) are also increasing, leading to around 340 million new infections a year (World Health Organization, 2006). At the same time, the world's population continues to grow exponentially, rising from 5 billion in 1988 to 6 billion in just 12 years. The urgent need for new contraceptive methods is illustrated by the fact that the family planning needs of over 120 million couples go unmet every year, resulting in an annual worldwide abortion rate of 46 million (World Health Organization, 2006). Many of these terminations take place under unsafe and insanitary conditions causing the deaths of 68,000 women a year, with a further 5 million being temporarily or permanently disabled (World Health Organization, 2006). In light of these data, the development of dual action microbicides/spermicides that will empower women to protect themselves against pregnancy and STDs is of critical importance.

One of the first candidates for such a product was nonoxynol 9 (N9). This detergent is an approved spermicide that is currently used in a wide variety of contraceptive barriers and creams. Twenty years ago, N9 was found to inactivate HIV *in vitro* (Malkovsky et al., 1988) suggesting a promising role for this compound in the search for a dual action spermicide/microbicide. However, subsequent trials demonstrated that

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while N9 might provide some protection against STDs such as gonorrhoea and Chlamydia, it tended to irritate the vaginal mucosa facilitating, rather than preventing, the transmission of HIV (Van Damme et al., 2002).

Given the inadequacy of current technology, it is clearly important that new leads are identified for spermicide development that specifically target the spermatozoa. In this context, surface-active agents such as N9 or benzylkonium chloride, while effective cytotoxic agents, lack any element of specificity in their mechanism-of-action (Hughes et al., 2007). The other approaches to spermicide development, including acid buffers, gramicidin, chlorosugars and gossypol, similarly lack effector mechanisms that are specific to the male germ line (Hughes et al., 2007). In this article, we report the development of a novel class of spermostatic agent that does not kill spermatozoa but instantly arrests their movement by targeting key proteins in the sperm tail. The same molecules also possess anti-microbial activity as exemplified by their suppressive effect on Chlamydia infectivity. This group of compounds therefore represent a major advance in our capacity to engineer safe, effective dual-purpose spermicide/microbicides for clinical use.

Materials and Methods

Compounds. All chemicals were purchased from commercial suppliers and were of the highest purity available. Chemical structures and formulae for all compounds are given in Supplementary Figure 1 and Supplementary Table 1. Antiphosphotyrosine monoclonal antibody (clone 4G10) and anti-mouse immunoglobulin G (IgG) horseradish peroxidase conjugate were from Upstate Biotechnology (Lake Placid NY) while mouse anti-phosphorylated SRC (pY416) monoclonal was from Calbiochem (La Jolla, CA, USA). Flourescein isothiocyanate (FITC)-labeled anti-mouse IgG was obtained from Sigma (St Louis, MO, USA).

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Sperm preparation. Human semen samples were obtained from a panel of donors assembled for the Reproductive Science Group of the University of Newcastle under Institutional and State Government ethical approval. All procedures were carried out in accordance with the Declaration of Helsinki. After a 48 h abstinence, samples were produced by masturbation into sterile specimen containers and delivered to the University within 1 h of production. Following liquefaction, the semen samples were processed by discontinuous Percoll gradient centrifugation to generate low- (50% Percoll) and high- (100% Percoll, isotonic) density Percoll fractions as described previously (Aitken et al., 1996). Cells collected from the high density Percoll fractions were suspended at a final concentration of 2.0×10^7 cells/ml in Biggers, Whitten and Whittingham medium (BWW; Biggers et al., 1971). For certain experiments, seminal plasma was collected from the top of the Percoll gradient.

Spermicidal activity. The effect of quinones on sperm motility in BWW was assessed via the Sander-Cramer assay (Sander and Cramer, 1941). 5 µl of the compound to be tested was added to 95 µl of sperm suspension prepared in BWW. Percentage sperm motility was assessed after 20 sec incubation at room temperature (RT). Sperm were classed as motile if any tail movement was observed. Each concentration of compound was tested in triplicate for both motility and vitality. Vitality was measured using eosin exclusion staining (World Health Organization, 1992).

Quinone stock solutions were prepared at a concentration of 50 mM in DMSO and 5 µl of stock solution was added to BWW to prepare working solutions to determine the ED_{50} value (the concentration at which 50% of the spermatozoa were immobilized). For time course experiments, in which spermatozoa were given a transient exposure to a given compound and then their motility evaluated over time, sperm were incubated with 20 or 50 µM quinone for 20 sec, centrifuged and resuspended in fresh medium. Sperm

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motility and vitality were then counted as described above at regular intervals throughout the observation period. Sperm motility assays in oxygen depleted BWW, seminal plasma and in the presence of antioxidants are described in the Supplementary file on Methodology.

Motility of demembranated-reactivated sperm. Demembranation and reactivation of sperm was performed as described by Yeung *et al*. (1988). A no-ATP control was included to confirm that the cells had been successfully demembranated and were immotile. The Sander-Cramer motility assay was ultimately performed as described above.

Cytotoxicity and Therapeutic index. Cytotoxicity of quinones in McCoy cells (a mouse fibroblast cell line) was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described in detail in the Supplementary file on Methodology. Therapeutic indices (TI) giving a relative measure of spermostatic activity to cytotoxicity, were calculated as the inverse log of ED_{50} minus the inverse log of LD_{50}:

1. $$TI = \log(1/ED_{50}) - \log(1/LD_{50})$$

DHE assay using flow cytometry. Superoxide formation in response to quinone exposure was measured with the fluorescent probe dihydroethidium (DHE) while cell viability was simultaneously monitored using Sytox Green (SyG) (De Iuliis et al., 2006). The assay is described in detail in the Supplementary file on Methodology.

Cyclic voltammetry and associated electrochemical parameters. Cyclic voltammetry was performed at RT on a VMP Potentiostat (Perkin Elmer, Wokingham, UK) using a Ag/AgCl reference electrode and Pt counter and working electrodes. The procedure is described in detail in the Supplementary file on Methodology.

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Protein thiol expression. Following incubation with 50 µM of a given compound for 20 min, the spermatozoa were centrifuged and washed twice with BWW. The cells were incubated with 1 mM DTT at 37°C for 10 min. The sperm suspensions were then centrifuged, washed twice with BWW and resuspended. Protein thiols were labeled with a fluorescent probe BODIPY TMR Cadaverine IA (Invitrogen, Mount Waverley, VIC, Australia) at a final concentration of 0.1 mM for 1 h in the dark. Sperm proteins were then extracted with sodium dodecyl sulphate (SDS) buffer (Ecroyd et al., 2004) and quantified using the bicinchoninic acid assay (Pierce, Rockford, IL, USA) in accordance with the Lowry method. SDS PAGE was conducted using 7.5% polyacrylamide gels at 20 mA constant current.

Tyrosine phosphorylation. Sperm were suspended in BWW at a density of 2.0 x 10^7/ml and treated with 100 µM quinone (compounds 11 and 15), diamide (compound 50), *p*-chloromercuribenzene sulfonic acid (*p*CMBS) or DMSO vehicle control for 15 min at RT. The sperm suspensions were then centrifuged, resuspended at 5 x 10^6 cells/ml and divided into two Falcon tubes. One half was centrifuged, the supernatant removed and proteins extracted with SDS extraction buffer. The other half of the sperm suspension was incubated with 3 mM dibutryl cyclic AMP (dbcAMP) and 1 mM of a phosphodiesterase inhibitor (pentoxifylline; Ptx) for 90 min, after which the sperm suspension was centrifuged and proteins extracted with SDS as described above. The percentages of motile and vital spermatozoa were recorded after 15 and 90 min. SDS PAGE was conducted with 2 µg solubilized sperm protein using 7.5% polyacrylamide gels at 20 mA constant current. Immunodetection of phosphotyrosine residues was performed at RT as described (Ecroyd et al., 2004), using an anti-phosphotyrosine monoclonal antibody (clone 4G10; Upstate). Phosphorylated proteins were detected using an enhanced chemiluminescence kit according to the manufacturer's instructions

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(Amersham, Buckinghamshire, UK).

Immunolocalization of phosphorylated SRC. Capacitated cells were prepared in BWW without $CaCl_2$ (BWW-Ca^{2+}) but supplemented with 3 mM Ptx, and 5 mM dbcAMP. Following incubation, the spermatozoa were fixed in 4% paraformaldehyde, washed 3 × with phosphate-buffered saline (PBS), aliquoted onto poly-L-lysine coated glass slides and air-dried. All subsequent incubations were performed in a humid chamber at 37°C. The cells were permeabilized with 0.2% Triton X-100 for 15 min, rinsed with PBS and blocked with 10% serum/ 3% BSA for 1 h. Slides were washed 3 × with PBS for 5 min and incubated in a 1:50 dilution of primary antibody (pY416) at 4°C overnight. Slides were then subjected to 3 × 5 min washes with PBS and incubated in a 1:100 dilution of FITC-conjugated secondary antibody for 2 h at 37°C. Slides were again washed and mounted in 10% mowiol 4-88 (Calbiochem) with 30% glycerol in 0.2 M Tris (pH 8.5) with 2.5% 1,4-diazobicyclo-[2.2.2]-octane. Cells were finally examined using either a Zeiss Axioplan 2 fluorescence microscope or an LSM laser scanning confocal microscope equipped with argon and helium/neon lasers. Control incubations in which spermatozoa were incubated in either an irrelevant primary antibody (anti-His Tag) or secondary antibody only were routinely included in all analyses.

Protein identification. Sequencing of gel plugs using the University of Newcastle matrix-assisted laser desorption ionisation-time-of-flight mass spectrometer (MALDI-TOF) (Amersham, Buckinghamshire, UK) was performed as described by Baker *et al*. (2005). MALDI-TOF mass spectrometry protein identifications were also confirmed at the Australian Proteome Analysis Facility (APAF) using MALDI-TOF MS/MS. Details of the plug preparation and spectrometry methods are included in the Supplementary file on Methodology.

Chlamydia infectivity–MOMP. The transformed *Escherichia coli* (DH5 {pMMM3})

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expressing the pMAL-c2 vector encoding recombinant maltose binding protein (MBP)-MOMP fusion protein was a generous gift from Harlan Caldwell (Rocky Mountain Labs, Hamilton, Mont.). The MOMP was produced and purified as described previously (Berry et al., 2004). Recombinant maltose binding protein-MOMP (MBP-MOMP) was treated with 50 µM *p*-benzoquinone (11), 2,6-dimethoxy-*p*-benzoquinone (3) and bismaleimide (35) for 20 min at RT. MBP-MOMP was incubated with 1 mM of the fluorescent thiol probe monobromobimane (mBBr) for 10 min prior to SDS extraction. SDS PAGE was conducted using 15% polyacrylamide gels at 100 V for approximately 2 h.

Microbicidal Activity. *C. muridarum* (ATCC VR-123, Virginia, USA), formally the mouse pneumonitis biovar of *C. trachomatis* (MoPn), was grown by inoculation of McCoy cell monolayers in Dulbecco's minimal essential medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 µg/mL Streptomycin sulfate, 2 µg/ml Gentamycin and 20 mM HEPES. Elementary bodies were purified using a discontinuous Renografin gradient. McCoy cells were plated at 5 x 10^4 cells/well the day before use in 48 well plates. The compounds were diluted to the concentrations that reflected their spermostatic activity: *p*-naphthoquinone (compound 19, 5 µM), 2,6-dichloro-*p*-benzoquinone (compound 12, 5 µm), bismaleimide (compound 35, 4 µm), menadione (compound 25, 150 µm) and *p*-benzoquinone (compound 11, 21 µM). Each treatment was made up to a volume of 100 µl with the compound and/or spermatozoa and had 2000 IFU's *C. muridarum* added. Treatments were incubated for 1 h at 37ºC, 5% CO_2. They were then added to the wells containing McCoy cell monolayers and diluted 1:1. Plates were incubated for 4 h, after which the seed was removed and replaced with fresh media containing 1 µg/ml cycloheximide. They were then incubated for 24-30 h at 37ºC:5% CO_2. Cells were fixed using 100% methanol, stained using ABC/DAB staining system and counted as described previously (Berry et al., 2004). For control treatments,

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100 µl 3% H_2O_2 and 100 µl media containing 1 µg/ml penicillin/streptomycin were used and treated as above.

Statistical analysis. All experiments were repeated at least 3 times on independent samples and the results analyzed by ANOVA using the SuperANOVA program (Abacus Concepts Inc, CA, USA) on a MacIntosh G5 computer; *post hoc* comparison of group means was by Fisher's PLSD test. Differences with a *P* value of <0.05% were regarded as significant.

Supplemental data. The supplemental data comprises a list of the chemical structures (Supplementary Figure 1) and formulae (Supplementary Table 1) of the cohort of compounds used in this study; when individual compounds are first mentioned in the text the compound number in Supplementary Table 1 is given in parentheses, thereafter the compound is referred to by its chemical name alone. Supplemental Table 2 gives a detailed description of the spermostatic activities and TI values for all of the compounds assessed in this study while Supplementary Table 3 lists the DHE responses and cell viabilities. Supplementary Table 4 gives details of the data used for the scattergrams reported in Figs. 2C and D. The Supplementary Methodology file gives details of sperm motility assays, the cytotoxicity assay, the DHE assay for superoxide, cyclic voltammetry determinations and protein identification protocols. Supplemental data can be found with this article online at http://molpharm.aspetjournals.org.

Results

Certain quinoid structures possess spermicidal activity log orders of magnitude greater than nonoxynol 9. This study originated in a proteomic analysis of human spermatozoa which revealed the presence of quinone oxidoreductase (IPI

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00000792.1) in the cytoplasm of these cells (Baker et al., 2007). In light of this finding we hypothesized that exposure of human spermatozoa to appropriate quinoid structures would trigger a one-electron reduction of these molecules to form an unstable semiquinone that would then redox cycle with the generation of reactive oxygen species. Since human spermatozoa are known to be particularly susceptible to oxidative stress (Aitken, 1989, 1999) and the latter are known to have a detrimental effect on sperm motility (Alvarez et al., 1987; de Lamirande and Gagnon, 1992; Aitken et al., 1993; Aitken and Baker, 2006), it was anticipated that quinones might constitute a new lead in the search for spermicidal compounds to replace N9. The spermicidal activities of a wide range of quinones, hydroquinones and Michael acceptors (Supplementary Figure 1; Supplementary Table 1) was therefore examined. Of the 77 compounds examined, the anthraquinone, (compound 29) is not strictly a quinone because it has no isolated double bond conjugated to a carbonyl group. Nevertheless, it was included as a structurally similar compound, not expected to be easily reduced to a semiquinone or to act as an alkylating agent. The list also included compounds that should be poor alkylating agents, such as duroquinone (compound 15), and others with electron withdrawing substituents such as *p*-chloranil, which should be a good alkylating agent. In addition, known redox cycling quinones such as menadione (Criddle et al., 2006) were included along with other compounds such as the maleimides (compounds 34-36), cinnamates (compounds 40, 42-45) and vinyl sulphones (compounds 51-52) that are Michael acceptors incapable of such redox related activity.

The assay employed for the spermicidal assessments was based on the industry standard, the Sander-Cramer assay, and examined the ability of a given compound to immobilize suspensions containing millions of human spermatozoa within 20 sec. For the purpose of this assay, we recovered highly motile spermatozoa from the base of the high density region of a 2 step discontinuous Percoll gradient and suspended these cells

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in a modified Tyrode's medium at a standard concentration of 20×10^6 /ml. Table 1 illustrates the results obtained for the 31 most active compounds analysed in this study, arranged in order of their spermicidal activity; Supplemental Table 2 contains the complete data set. For reference, the current industrial standard, N9, was included and exhibited an ED_{50} value of 130 μM. The most active quinones were 400 times more active than N9. Table 1 also reveals that, in general, halogenated quinones were more spermicidal than their parent quinones, with the exception of *o*-chloranil (compound 16) and 2-bromo-*p*-naphthoquinone (compound 23). The most active benzoquinones were dimeric and/or halogenated; moreover, the 2,5-substituted bromo-, chloro- and iodo-benzoquinones (compounds 6, 55, 56) were equally active (ED_{50} of approximately 3 μM). Methyl and phenyl groups reduced activity unless the quinone also possessed a halogen. For instance, 2-chloro-5-phenyl-*p*-benzoquinone (compound 58) was more active than 2-chloro-*p*-benzoquinone (compound 10), but 2-phenyl-*p*-benzoquinone (compound 7) was less active than *p*-benzoquinone (compound 11). Likewise, 2,6-dibromo-3,5-dimethyl-*p*-benzoquinone (compound 54) was more active than 2,5-dibromo-*p*-benzoquinone (compound 56). Electron donating OH, methoxy and long alkyl groups rendered the benzoquinones inactive as seen in 2,6-dimethoxy-*p*-benzoquinone (compound 3), vitamin K_1 (compound 32) and 2,3-dimethoxy-5-methyl-*p*-benzoquinone (compound 62).

Quinones can alkylate nucleophiles such as protein thiols if one of the carbons on the electrophilic double bond of the quinoid ring is unsubstituted or bears a good leaving group. All spermicidal quinones possessed a carbon which could be attacked by nucleophiles. However, the number of unsubstituted positions on the quinoid ring did not correlate with activity. There were six non-spermicidal benzoquinones (compounds 1, 3, 5, 14, 18, and 62) that exhibited an unsubstituted position capable of undergoing

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nucleophilic attack. Furthermore, the benzoquinone, thymoquinone (compound 5), was not spermicidal despite the presence of two unsubstituted carbons (C_3 and C_6).

Naphthohydroquinone (71) was the only spermicidal hydroquinone in this series. This compound is known to autoxidise rapidly in solution compared to other hydroquinones, so its activity was probably due to naphthoquinone formation (Munday, 1997). Benzo-hydroquinones (compounds 46, 72-76) were not spermicidal in the Sander Cramer assay. This observation is in keeping with previous studies indicating that addition of 2,5-ditertbutylhydroquinone (100 μM), a Ca^{2+} ATPase inhibitor, to human spermatozoa had no effect on motility for up to 6 hours (Perry et al., 1997).

Analysis of cytotoxicity reveals a high therapeutic index for many candidate compounds. Table 1 and Supplemental Table 2 also present the therapeutic index (TI) for each of the compounds tested. The TI is a measure of non-specific cytotoxicity secured using McCoy cells in conjunction with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). In this assay, the tetrazolium ring of MTT is cleaved by the mitochondrial dehydrogenases of viable cells forming purple formazan crystals which are soluble in acidified isopropanol. The TI represents the ratio of spermicidal activity to non–specific cytotoxicity calculated as the inverse log of the spermicidal ED_{50} value, minus the inverse log of LD_{50} [$TI = \log(1/ED_{50}) – \log(1/LD_{50})$]. In this assay, the compound in current clinical use, N9, yielded a TI value of 0.4. While some quinones were more cytotoxic than N9 (juglone, 2-bromo-*p*-naphoquinone and *p*-naphthoquinone), the 12 most spermicidal compounds listed in Table 1 gave TI values of ≥1.6. Since the TI score is a log value, this indicates that the values recorded for these compounds were at least 10 times higher than that recorded for N9, and in the case of the most active compounds,100 times greater. The highest therapeutic index was given

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by 2,3,7-trichloro-5-hydroxy-*p*-naphthoquinone (compound 22), which gave a TI value of 2.4. Parallel assessments of cytotoxicity with Hela cells gave excellent TI values for the most promising compounds examined including 2,2'-bis(*p*-naphthoquinone) (TI = 2.2), *p*-benzoquinone (TI = 1.1), 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (TI = 1.3), 2,3,7-trichloro-5-hydroxy-1,4-naphthoquinone (TI = 1.7) that were also orders of magnitude greater than N9 (TI = -0.3).

Analysis of sperm viability reveals that most active quinones are spermostatic rather than spermicidal. Parallel assessments of sperm vitality using eosin exclusion revealed that, unlike N9, the ability of the compounds listed in Table 1 to suppress sperm movement was not related to a loss of viability, which remained at control levels (80-95%) for all compounds tested. In other words, these compounds were not strictly spermicidal, they were spermostatic; rapidly and specifically disrupting sperm movement without having any significant impact on cell survival. The specificity of this action explains why many of these compounds compounds exhibited such excellent TI values.

The spermostatic action of bioactive quinones was irreversible. This was established in an experiment in which sperm movement was suppressed with a 20 second exposure to 20 or 50 µM *p*-benzoquinone or *p*-naphthoquinone. The cells were then pelleted by centrifugation (500 $\times$ *g* for 5 min), resuspended in fresh medium BWW and examined for motility over the ensuing 28.5 hours. No resuscitation of motility was observed.

We also confirmed that this permanent spermostatic action was exhibited when vigorously motile spermatozoa from the high density region of Percoll gradients were resuspended in seminal plasma at a concentration of 20×10^6/ml. As indicated in Table 2, a range of benzoquinones, naphthoquinones and maleimides were assessed in the

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presence of seminal plasma at doses of 50 and 100 µM. Under these conditions, the *p*-naphthoquinones retained their activity (compounds 19, 22, 24, 27 and 71), as did the bismaleimide (compound 35). However the benzoquinones (compounds 6 and11) either lost activity or their effectiveness became extremely variable. Even after 5 min exposure, benzoquinones such as *p*-benzoquinone or 2,5-dichloro-*p*-benzoquinone were inactive. This could not have involved the selective 2 electron reduction of these compounds in seminal plasma because hydroquinones (compounds 38 and 46) were more active in seminal plasma than in a balanced salt solution. Thus hydroquinone was inactive in medium BWW at >500 µM (Supplemental Table 2) whereas the same compound was active at 50 µM in the presence of seminal plasma (Table 2). An alternative explanation is that this loss of spermostatic activity when benzoquinones are exposed to seminal plasma involved the one electron reduction of these compounds to redox-cycling semi-quinone intermediates (see below).

Mechanism of action studies reveal the alkylation of key targets in the sperm tail to be more important than redox cycling activity. In order to confirm that the spermostatic effect of quinones was a direct action on the motility apparatus of the sperm tail, and not an indirect effect mediated by changes in the plasma membrane or cytoplasm, a Triton X demembranation-ATP reactivation model was used (Yeung et al., 1988). With this model system, spermatozoa are demembranated with 0.08% Triton X-100 in the presence of DTT (1 mM) and EDTA (0.5mM) and reactivated by the addition of 1 mM ATP and 50 µM cAMP. Under these conditions motility is reactivated in around 70% of spermatozoa and remains at a high level for at least 20 min. Both *p*-benzoquinone and *p*-naphthoquinone instantly immobilized such reactivated spermatozoa in a dose dependent fashion (Fig. 1A,B). The ED_{50} values were higher than those recorded in intact cells because quinone bioavailability would have been

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compromised by the presence of DTT, the presence of which was essential for the operation of the reactivation model. Nevertheless, such results suggest a direct effect of quinones on the cytoskeletal elements of the sperm tail, rather than an indirect effect dependent on the integrity of the sperm plasma membrane, the status of the cytosol or the intracellular availability of ATP/cAMP.

In principle, quinones could target key elements of the flagellum as a consequence of their ability to redox cycle and generate free radicals or as a result of their inherent alkylating activity. The fact that quinones were still spermostatic in demembranated-ATP reactivation models, where redox cycling would not be expected to occur due to the loss of oxido-reductases, suggested that this activity was not involved in the suppression of motility. This conclusion was reinforced by the observation that both *p*-benzoquinone and *p*-naphthoquinone were extremely effective spermostatic agents in oxygen-depleted media (Fig. 1C,D). Indeed *p*-naphthoquinone was more active in oxygen-depleted medium (ED_{50} = 3.8 µM) than in normal medium BWW equilibrated with air (ED_{50} = 20.7 µM), suggesting that redox cycling actually counteracted the spermostatic activity of such compounds. In keeping with this conclusion, neither superoxide dismutase (SOD; 300 U) nor catalase (3000 U) had any effect on the spermostatic activity of 100 µM *p*-naphthoquinone (Fig. 2A). Similarly, addition of a membrane permeant SOD mimetic [Mn(III)tetrakis(1-methy-4-pyridy)porphyrins pentachloride] at a dose of 25 mM, did not influence the spermostatic activity of 100 µM *p*-naphthoquinone (Fig. 2B).

Further evidence that redox cycling was not involved in the mechanisms by which quinones exhibited spermostatic behaviour, came from an analysis of ROS generation using a flow cytometry assay that has been validated for spermatozoa (De Iuliis et al., 2006). This assay deployed dihydroethidium (DHE) as the probe and revealed no discernable relationship between the spermostatic activity of a given compound and its redox cycling activity (Table 3; Supplemental Table 3 for full data set). Fig. 2C illustrates

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this point by plotting the spermostatic activity of the compounds listed in Table 3 against their redox activity measured with DHE. This analysis revealed that compounds that were only moderately effective as spermostatic agents, such as *o*-chloranil (compound 16) or *o*-naphthoquinone (compound 31) were extremely redox active. Conversely, compounds that completely suppressed sperm movement in a matter of seconds, such as bismaleimide, did not trigger a ROS response in a majority of cells; the complete data set for this scattergram is contained in Supplementary Table 4.

HPLC analysis of the DNA sensitive fluorochromes generated from DHE in the presence of spermatozoa also revealed no relationship between the suppression of sperm movement and the ratio of 2OH-ethidium (2OHEt; a unique reaction product generated by the reaction of DHE with superoxide anion) to ethidium (Et; the non-specific 2–electron oxidation product of DHE) (Fig 2D,E; Supplementary Table 4). Menadione generated the highest levels of 2OHEt and yet was not spermostatic while 2,3-dichloro-*p*-naphthoquinone, *p*-benzoquinone and 2,5-dichloro-*p*-benzoquinone were extremely spermostatic and yet generated low levels of this product (Tables 1 and 3; Fig. 2E). An unexpected general trend was observed such that as the one electron reduction potentials of the compounds became more negative, the ratio of 2OHEt to Et increased (r = -0.58; P<0.05). This suggested that the one electron reduction of the quinone to a semiquinone radical was being enzymatically driven by quinone oxidoreductase.

If redox cycling and ROS generation were not causally involved in the suppression of sperm movement then the only alternative mechanism was the alkylation of key thiols. In order to examine this hypothesis, spermatozoa were exposed to a range of quinones or maleimides followed by DTT to reduce any remaining disulphide bonds. Thiol expression was then detected with BODIPY TMR Cadaverine 1A (Fig. 3A). This analysis revealed a close association between the ability of a given compound to suppress free thiol expression and its spermostatic activity (Fig. 3A). Thus highly

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spermostatic quinones such as *p*-benzoquinone (Fig 3A), *p*-chloranil and 2,2-bis-*p*-naphthoquinone suppressed thiol expression by human spermatozoa, while inactive quinones such as lapachol or menadione were ineffectual in this regard. Similarly within the maleimides tested, the only compound to exhibit spermostatic activity (bismaleimide) suppressed thiol expression, while related alkylating agents (*N*-ethylmaleimide and iodocetamide) displaying no spermostatic activity, were ineffective. Thus, complexation of sperm thiols was not a general property of alkylating agents; the ability of certain reagents to covalently bind the major thiols expressed by human spermatozoa was a specific attribute of these particular molecules. Similarly, the interaction between these molecules and sperm thiols did not extend to other cell types. McCoy cells (a mouse fibroblast cell line) expressed a large number of potentially susceptible thiols and yet no loss of thiol expression was observed when these cells were targeted with spermostatic agents such as *p*-benzoquinone or *p*-naphthoquinone (Fig. 3B).

The major targets for thiol alkylation by spermostatic agents are AKAP3 and AKAP4. The two major proteins alkylated by spermostatic quinones exhibited molecular masses of 82 and 110 kDa (Fig, 3A). These proteins were excised from the gels and identified by mass spectrometry as AKAP3 and AKAP4 (A Kinase Anchoring Proteins 3 and 4). These molecules are major constituents of the sperm flagellum and act as scaffolds for the assembly of PKA and associated molecules, such as ropporin, involved in the orchestration of flagellar movement. PKA is, in turn, responsible for phosphorylating a variety of downstream targets that are essential for the control of sperm movement. One of the key proteins targeted by PKA in this way is the promiscuous tyrosine kinase pp60c-SRC (Baker et al., 2006). The activation of this kinase results in a global upregulation in phosphotyrosine expression in proteins that are largely located on the fibrous sheath and mitochondria of the sperm tail. If the

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spermostatic agents identified in this study interfere with the ability of AKAPs to orchestrate cAMP signalling in the flagellum, then we should see an immediate downregulation in the activation of SRC and consequent suppression of phosphotyrosine expression in the sperm tail. As illustrated in Fig. 3 (C, D and E), addition of the spermostatic quinone, *p*-benzoquinone, led to a dramatic cessation of phospho-SRC expression in the presence of dbcAMP. Similarly, addition of this quinone to human spermatozoa completely suppressed both the basal and the cAMP-induced stimulation of tyrosine phosphorylation in human spermatozoa (Fig. 4A,B; lane 2). The non-specific thiol oxidant, diamide, also suppressed both motility and tyrosine phosphorylation (Fig. 4A,B, lane 4). Although this compound was not strictly spermostatic (in that it could not immobilize spermatozoa with 20 sec) it was capable of suppressing sperm movement over the more prolonged time course (15 min) associated with these tyrosine phosphorylation experiments. In contrast, quinones (duroquinone; lane 3) or alkylating agents (*p*CMBS, lane 5) that lacked spermostatic activity had no effect on tyrosine phosphorylation and none of these reagents interfered with cell viability (Fig. 4B).

The specificity of this effect on sperm movement was unrelated to the hydrophobicity parameter, LogP, the pKa of the corresponding hydroquinone or the LUMO coefficient (data not shown). However for a collection of 17 benzoquinones, a parabolic relationship was evident between spermostatic activity and both the one electron reduction potential ($E^1_{1/2}$) and E_{LUMO} (Fig. 4C,D) suggesting that there is an optimal electron reduction potential range for the expression of spermostatic activity.

Spermostatic quinones possess antimicrobial activity. Antimicrobial activity was assessed using the infection of McCoy cells with *Chlamydia muridarum* elementary

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bodies as a model system (Fig. 5). An infection dose of 1000 IFU was selected because Eckert *et al*. (2000) found that 625 IFU is the highest concentration transmitted by Caucasian females. Both antibiotics and 3% H_2O_2 were found to significantly suppress Chlamydial infectivity in this model system ($P < 0.001$; Fig. 5A). Furthermore, all of the quinones tested (*p*- naphthoquinone, *p*- benzoquinone, 2,6 –dichloro-*p*-benzoquinone, bismaleimide and menadione) also significantly reduced Chlamydial infection rates in this model. In subsequent experiments 2,2-bis-*p*-naphthoquinone was also shown to significantly suppress Chlamydia infectivity at doses as low as 1 µM ($P < 0.001$). The concomitant presence of spermatozoa had no impact on the microbicidal activity of these compounds (Fig 5A). Analysis of ROS generation in McCoy cells using DHE as the probe revealed a highly significant increase with a majority of the quinones tested (data not shown). However, bismaleimide exhibited no capacity to generate ROS even though this compound was extremely effective against Chlamydia. Thus, while the H_2O_2 result illustrated in Fig. 5A, clearly indicated that ROS alone could inhibit Chlamydial infectivity, it is not the only microbicidal mechanism, alkylation is another possibility. In keeping with this suggestion, spermostatic quinones such as *p*-benzoquinone and bismaleimide were found to suppress thiol expression by recombinant Chlamydial Major Outer Membrane Protein (MOMP) (Fig. 5C). A quinone that was not spermostatic (2,6-dimethoxy-*p*-benzoquinone) had minimal effect on thiol expression by MOMP. Thus an important parallel exists in the mechanisms by which this group of quinones and maleimides can suppress sperm movement and disrupt the infectivity of Chlamydia involving the selective ability of these compounds to complex key thiols

Discussion

The results secured in this study represent a new lead in our attempts to develop dual-purpose formulations capable of providing women with simultaneous protection

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against both pregnancy and STDs. The spermicide in current clinical use, N9, is a detergent that non-specifically kills spermatozoa by disrupting the integrity of the plasma membrane. The non-specific nature of this mechanism-of-action results in collateral damage to the tissues of the lower female reproductive tract, as a result of which women become vulnerable to STDs, including HIV (Van Damme et al., 2002). In contrast, the structures we have identified in this study not only have a therapeutic index orders of magnitude greater than N9, but achieve the instantaneous immobilization of millions of spermatozoa without impairing the viability of the cells. Moreover the mechanism-of-action appears to be selective and to involve a direct attack on the functionality of the sperm flagellum.

Although many of the compounds investigated were intensely redox active and could induce the formation of ROS, this free radical–generating activity was not involved in the spermostatic effect. Rather, this dramatic suppression of sperm movement was achieved by the alkylation of key thiols expressed by AKAP3 and AKAP4 in the human sperm flagellum. The selective nature of this activity was emphasized by the fact that these compounds did not attack the many thiols expressed by McCoy cells, while broad spectrum alkylating agents such as *p*CMBS or N-ethylmaleimide were not spermostatic.

The alkylation of AKAP3 and AKAP4 disrupted the capacity of these proteins to serve as coordination points for cAMP/PKA-dependent signaling pathways essential for motility. Since cAMP is involved in the activation and maintenance of sperm motility (Wade et al., 2003; Bajpai and Doncel, 2003), the net effect of alkylating these AKAPs is to render the axoneme insensitive to the stimulatory effects of this second messenger. The specificity and rapidity of this effect is testimony to the central role that cAMP plays in the regulation of sperm movement, and consistent with the complete lack of sperm motility seen in mutant mice null for sAC (soluble Adenylate Cyclase), the major site for cAMP production in these cells (Hess et al., 2005). Furthermore, targeted disruption of

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the AKAP4 gene in mice leads to male infertility associated with impaired sperm movement (Miki et al., 2002). Similarly, functional deletion of the catalytic subunit of the sperm specific form of PKA, the kinase that targets AKAPs 3 and 4, also results in male infertility associated with the disruption of sperm movement (Nolan et al, 2004). In this case, motility was not completely disrupted, possibly because the targeted PKA catalytic subunit isoform (alpha 2) is not the only form of PKA in mammalian spermatozoa. A recent proteomic analysis of human spermatozoa also found PKA gamma (Baker et al., 2007), which may have provided sufficient kinase activity to drive the low level of motility observed in the PKA Calpha2 knockout mouse.

The ability of spermostatic quinones to disrupt cAMP-dependent signaling pathways in human spermatozoa was demonstrated by assessing their ability to impede the PKA-mediated activation of SRC and subsequent phosphotyrosine expression by these cells. Although this unusual signal transduction pathway represents an ideal means of monitoring AKAP-dependent signaling in the flagellum, it is unlikely that the suppression of SRC and subsequent tyrosine phosphorylation represents the actual mechanism by which motility is disrupted. SRC-mediated tyrosine phosphorylation is associated with the terminal maturation of mammalian spermatozoa in the female tract via a process known as capacitation, but not the activation of sperm movement (Bennetts et al., 2004; Baker et al., 2006; Mitchell et al., 2008). Thus, while AKAP alkylation clearly disrupts PKA-dependent signaling pathways in human spermatozoa, the changes responsible for arresting sperm motility in the presence of quinones, still await resolution. A possible candidate is dynein, since cAMP/PKA dependent phosphorylation of dynein has been associated with axonemal function in a variety of species from protozoa to fish (Hamasaki et al., 1991; Itoh et al., 2003; Salathe, 2007).

Several of the spermostatic compounds identified in this study also possessed anti-microbial activity in an assay that examined the infectivity of Chlamydial elementary

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bodies. This microbicidal action was also associated with the alkylation of key thiols, in this case on the major outer membrane protein of Chlamydia. These results are consistent with previous reports that outer membrane proteins of elementary bodies must be reduced for entry and productive infection (Raulston et al., 2002) and that Chlamydial infection is susceptible to oxidative stress (Hillier et al., 1992). The fact that HIV infection also involves a disulphide switching mechanism that is dependent of the expression of surface thiols (Matthias et al., 2002) is also compatible with an anti-HIV role for these spermostatic compounds in vivo.

Clearly these results are promising and will serve as the foundation for further studies on the development of dual-purpose reagents exhibiting both spermostatic and anti-microbial activity. Several of the electrochemical properties associated with compounds exhibiting strong spermostatic activity were identified in this study and can now be used to refine the synthesis of compounds combining high levels of spermostatic and anti-microbial activity with a minimum of cytotoxicity.

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Footnotes

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Figure legends

Figure 1. Analysis of the mechanisms by which quinones suppress sperm motility. A, dose dependent analysis of the impact of 1,4 *p*-benzoquinone on the motility of human spermatozoa in a Triton-X demembranation, ATP reactivation model. B, similar data for 1,4 *p*-naphthoquinone,. Control motilities are shown for the beginning (0 min) and end (15 min) of the experimental period. Motilities were assessed 20 sec after exposure to a given dose of quinone. With both compounds the dose dependent suppression of sperm movement in this demembranated model was statistically significant ($P < 0.001$). C, Dose-dependent analysis of the effect of 1,4 *p*-benzoquinone on the motility of intact human spermatozoa in oxygen depleted medium using an exposure time of 20 sec. D, similar data for 1,4 *p*-naphthoquinone. Note the dose-dependent suppression of motility (open bars) with both quinones ($P < 0.001$) in the absence of any significant impact on cell viability (closed bars).

Figure 2. Influence of redox cycling and ROS generation on the spermostatic impact of quinones. A, Impact of SOD and catalase and B, a SOD-mimetic, on the motility and viability of human spermatozoa. None of these antioxidant strategies had a significant impact on the spermostatic effect of *p*-naphthoquinone (*p*-NQ). C, Spermostasis was not correlated with the redox cycling activity of the compounds as measured by DHE fluorescence or D, the ratio of 2-hydroxyethidium (2OHEt, a specific product generated by the interaction between DHE and superoxide anion) to ethidium (Et, the two electron oxidation product of DHE). All data points in these scattergrams represent the mean of 3 replicate determinations on independent sperm populations; detailed information to be found in Supplementary Table 4. E, Ratios of 2OHEt:Et for several of the compounds examined in this study. Compounds such as menadione produced a preponderance of

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$O_2^{-\cdot}$ in human sperm cells and yet were not spermostatic, whereas highly spermostatic compounds such as *p*-benzoquinone generated largely Et.

Figure 3. Influence of alkylating activity on the spermostatic effect of quinones. A, Spermostatic quinones such as *p*-benzoquinone (p-BQ) alkylate two major thiol expressing proteins in human spermatozoa that were characterized by mass spectrometry as AKAP3 and AKAP 4 as indicated. B, Spermostatic quinones including *p*-naphthoquinone (*p*-NQ) and bismaleimide (BM) had no impact on patterns of thiol expression in a non-specific cell line (McCoy cells). C, Expression of PKA-dependent activated SRC (SRC phosphorylated at position Y416) in the tail of capacitating human spermatozoa, detected by immunocytochemistry following treatment with dbcAMP and pentoxifylline. D, complete suppression of SRC activation in the presence of a spermostatic quinone (*p*-benzoquinone) after treatment of the cells with the same dbcAMP and pentoxifylline combination as depicted in panel C. E, unstimulated control sample.

Figure 4. Alkylating activity of spermostatic quinones was also correlated with the suppression of tyrosine phosphorylation in human spermatozoa. Spermatozoa were incubated with the test compounds (100 µM) for 15 min, centrifuged for 5 min at 500*g*, and then either sampled immediately (Control) or resuspended in fresh medium BWW supplemented with 3 mM dibutyryl cAMP (dbcAMP) and 1 mM pentoxifylline (Ptx) to induce capacitation. The compounds evaluated were: 1, medium control; 2, *p*-benzoquinone; 3, duroquinone; 4, diamide; 5, *p*CMBS. A, Western blot analysis, including β-tubulin loading controls, revealing complete suppression of phosphotyrosine

expression with the spermostatic quinone, *p*-benzoquinone (lane 2), and significant suppression with diamide (lane 4). B, motility (open bars) and viability (closed bars) following exposure; arrows indicate that only the spermostatic quinone, *p*-benzoquinone, and the thiol oxidant, diamide, induced motility loss. C, Using a large family of related benzoquinones the relationship between spermostatic activity and either the one electron reduction potential or D, the energy of the lowest unoccupied molecular orbital (LUMO), was examined. A highly significant ($P \leq 0.001$) parabolic relationship was observed for both parameters.

Figure 5. Influence of quinones on the infectivity of *Chlamydia muridarum* using McCoy cells as the target. A, ability of quinones and the alkylating agent bismaleimide to neutralize the infection of McCoy cells with *Chlamydia muridarum* elementary bodies at a concentration of 1000 IFU. Pen/strep was used at a concentration of 1 µg/ml; H_2O_2 was used at a concentration of 3% and the test compounds were used at doses that reflected their relative spermostatic activity: *p*-naphthoquinone (*p*-NQ, 5 µM); 2,6 dichloro-*p*-benzoquinone (2,6DCBQ, 5µM); bismaleimide (BM, 4 µM); menadione (Mena, 150 µM) and *p*-benzoquinone (*p*-BQ, 21 µM). The suppression of infectivity was highly significant ($P < 0.001$) for all of the compounds tested and was not significantly influenced by the concomitant presence of human spermatozoa (Spz, 10^7/ml). B, Illustration of Chlamydia-infected McCoy cells (arrowed). C, Investigation of the ability of quinones and bismaleimide to suppress the expression of free thiols by recombinant Chlamydial Major Outer Membrane Protein (MOMP). Upper panel, thiol expression visualized using monobromobimane as the probe; lower panel, a Coomassie blue stain of the same gel demonstrating the equal loading of MOMP in each lane. The spermostatic compounds *p*-benzoquinone (*p*-BQ) and bismaleimide (BM) completely suppressed thiol expression

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at a dose of 25 μM. Conversely the non-spermostatic quinone, 2,6 dimethoxy-*p*-benzoquinone (2,6-DMBQ) had little impact on thiol expression.

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Table1. Spermicidal activity of quinones

	Compound [a]	ED_{50} (μM)	TI[b]
(22)	2,3,7-trichloro-5-hydroxy-*p*-naphthoquinone	0.3±0.1	2.4
(24)	2,3-dichloro-*p*-naphthoquinone	0.5±0.1	2.2
(2)	2,2-ethylendi-*p*-benzoquinone	0.8±0.1	1.9
(53)	2,2-pentylene-di-(5-bromo-*p*-benzoquinone)	1.3±0.1	ND
(54)	2,6-dibromo-3,5-dimethyl-*p*-benzoquinone	1.5±0.0	ND
(55)	2,5-diiodo-*p*-benzoquinone	2.7±0.2	ND
(56)	2,5-dibromo-*p*-benzoquinone	2.8±0.1	ND
(6)	2,5-dichloro-*p*-benzoquinone	3.3±0.2	2.0
(35)	bismaleimide	4.1±0.1	1.7
(9)	*p*-chloranil	4.9±0.6	2.0
(57)	2-bromo-5-chloro-*p*-benzoquinone	5.2±0.3	ND
(58)	2-chloro-5-phenyl-*p*-benzoquinone	5.3±0.2	ND
(12)	2,6-dichloro-*p*-benzoquinone	5.5±0.0	2.2
(59)	5'5'-dimethyl-2,2'-bi-*p*-benzoquinone	6.3±0.7	ND
(8)	*p*-bromanil	6.6±0.7	1.8
(60)	5-benzoyloxy-*p*-naphthoquinone	8.3±0.9	ND
(10)	2-chloro-*p*-benzoquinone	10.2±0.7	1.8
(38)	quinhydrone	10.3±0.7	ND
(27)	2,2'-bis(*p*-naphthoquinone)	11.0±0.0	1.7
(71)	*p*-naphthohydroquinone	18.7±0.7	ND
(13)	*o*-bromanil	19.3±2.6	1.9
(11)	*p*-benzoquinone	20.3±1.5	1.6
(21)	1,4-anthraquinone	20.7±2.4	0.4
(19)	*p*-naphthoquinone	20.7±3.3	0.1
(23)	2-bromo-*p*-naphthoquinone	26.7±2.9	0.3
(34)	*N*-phenylmaleimide	27.3±2.9	0.6
(7)	2-phenyl-*p*-benzoquinone	32.7±0.3	1.0
(16)	*o*-chloranil	32.7±4.8	1.8
(4)	2-methyl-*p*-benzoquinone	37.3±1.8	0.6
(31)	*o*-naphthoquinone	42.3±7.0	1.5
(20)	juglone	52.6±1.3	-0.2
(39)	nonoxynol-9 (N9)[c]	130.0±1.2	0.4

[a]Numbers in parentheses correspond to compound number in Supplemental Table 1

[b]$TI = \log(1/ED_{50}) - \log(1/LD_{50})$

[c]N9 is the current industrial standard and is included for comparative purposes.

ND = Not Determined

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Table 2. Spermostatic activity in seminal plasma

	Compound[a]	Spermostasic[b]	
		50 µM	100 µM
(22)	2,3,7-trichloro-5-hydroxy-*p*-naphthoquinone	-	+
(24)	2,3-dichloro-*p*-naphthoquinone	+	+
(6)	2,5-dichloro-*p*-benzoquinone	-	-
(35)	bismaleimide	+	+
(9)	*p*-chloranil	-	-
(10)	2-chloro-*p*-benzoquinone	-	-
(38)	quinhydrone	+	+
(27)	2,2'-bis(*p*-naphthoquinone)	+	+
(71)	*p*-naphthohydroquinone	+	+
(11)	*p*-benzoquinone	±	±
(19)	*p*-naphthoquinone	+	+
(31)	*o*-naphthoquinone	-	-
(20)	juglone	-	+
(46)	hydroquinone[d]	+	+
(39)	nonoxynol-9 (N9)[c]	-	-

[a]Numbers in parentheses correspond to compound number in Supplemental Table 1

[b]Spermostatic activity measured in the Sander Kramer assay at 2 doses .

[c]N9 is the current industrial standard and is included for comparative purposes. In the presence of seminal plasma the ED_{50} value for N9 was 150 µM

[d]This compound had been weekly active in the absence of seminal plasma with an ED50 value of only >500 µM

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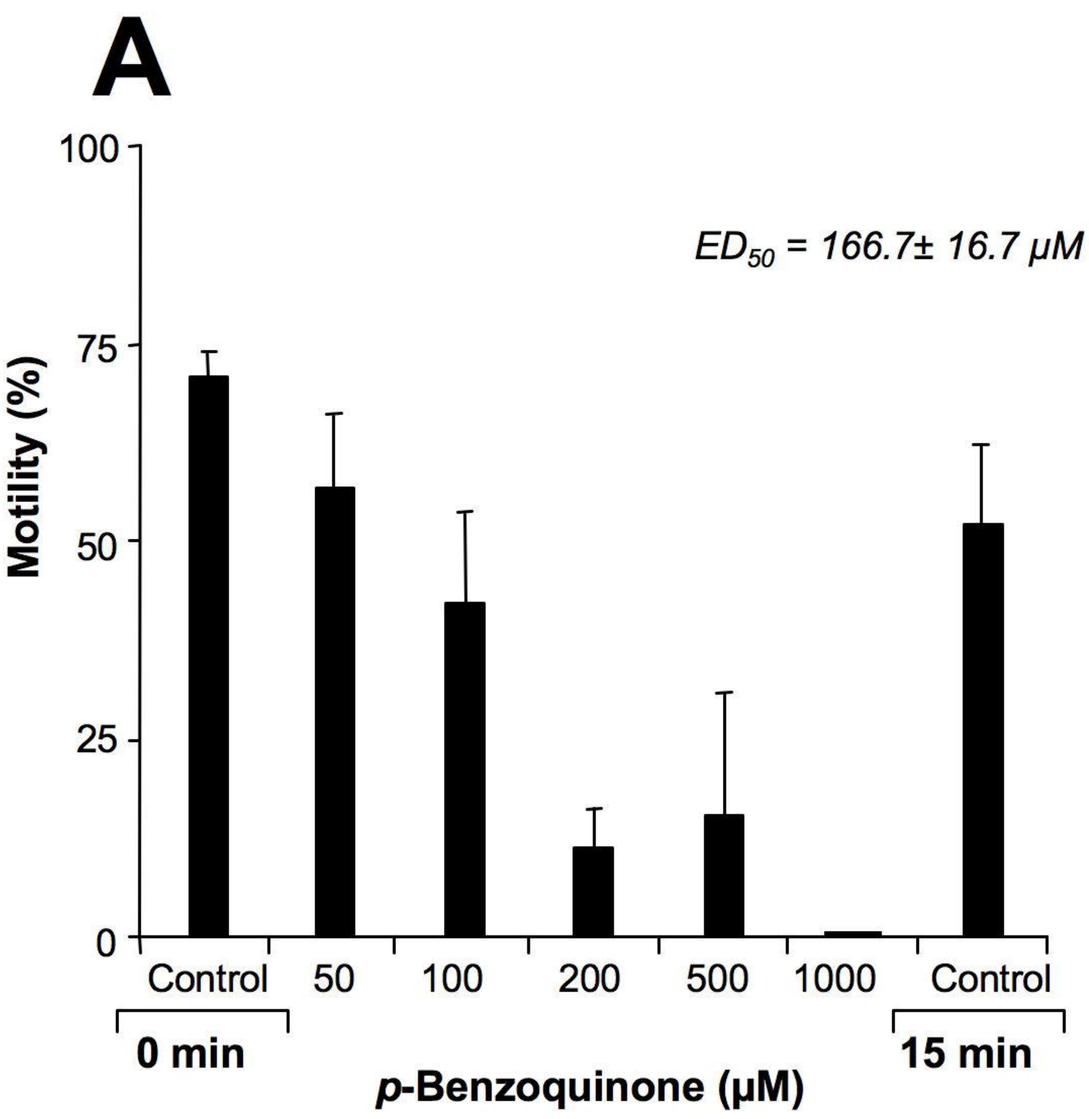
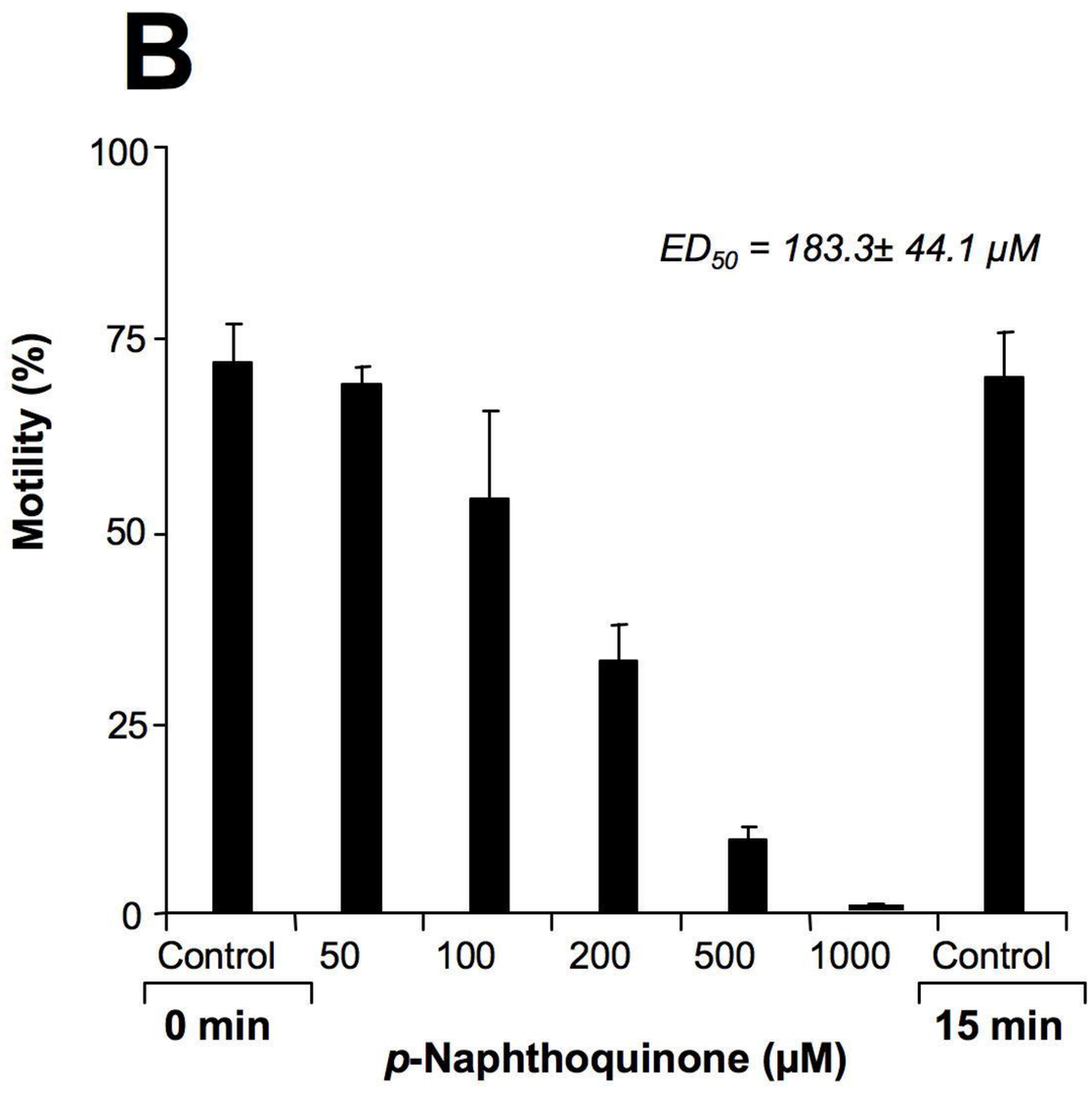
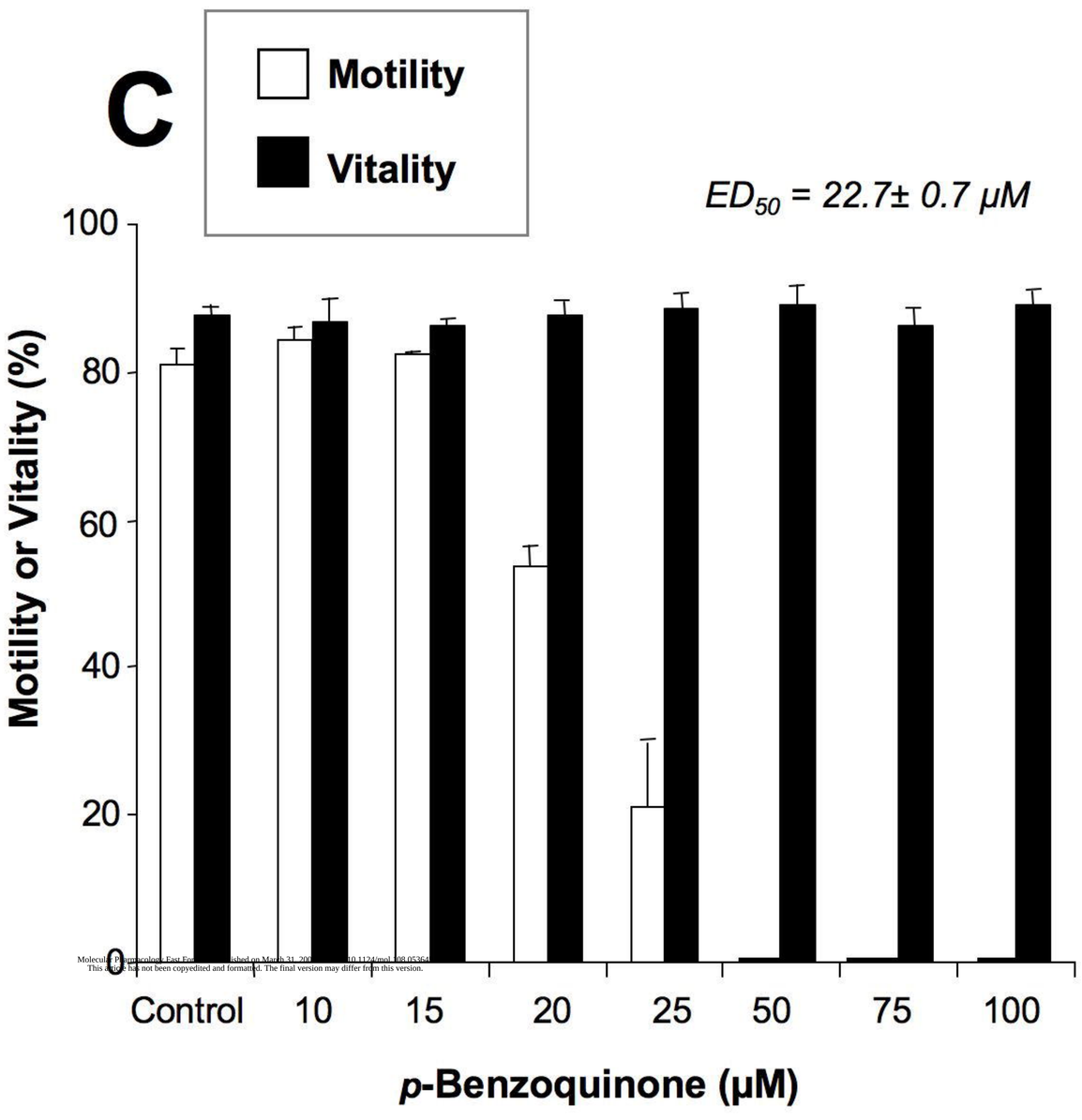
Table 3. Detection of redox activity with dihydroethidium

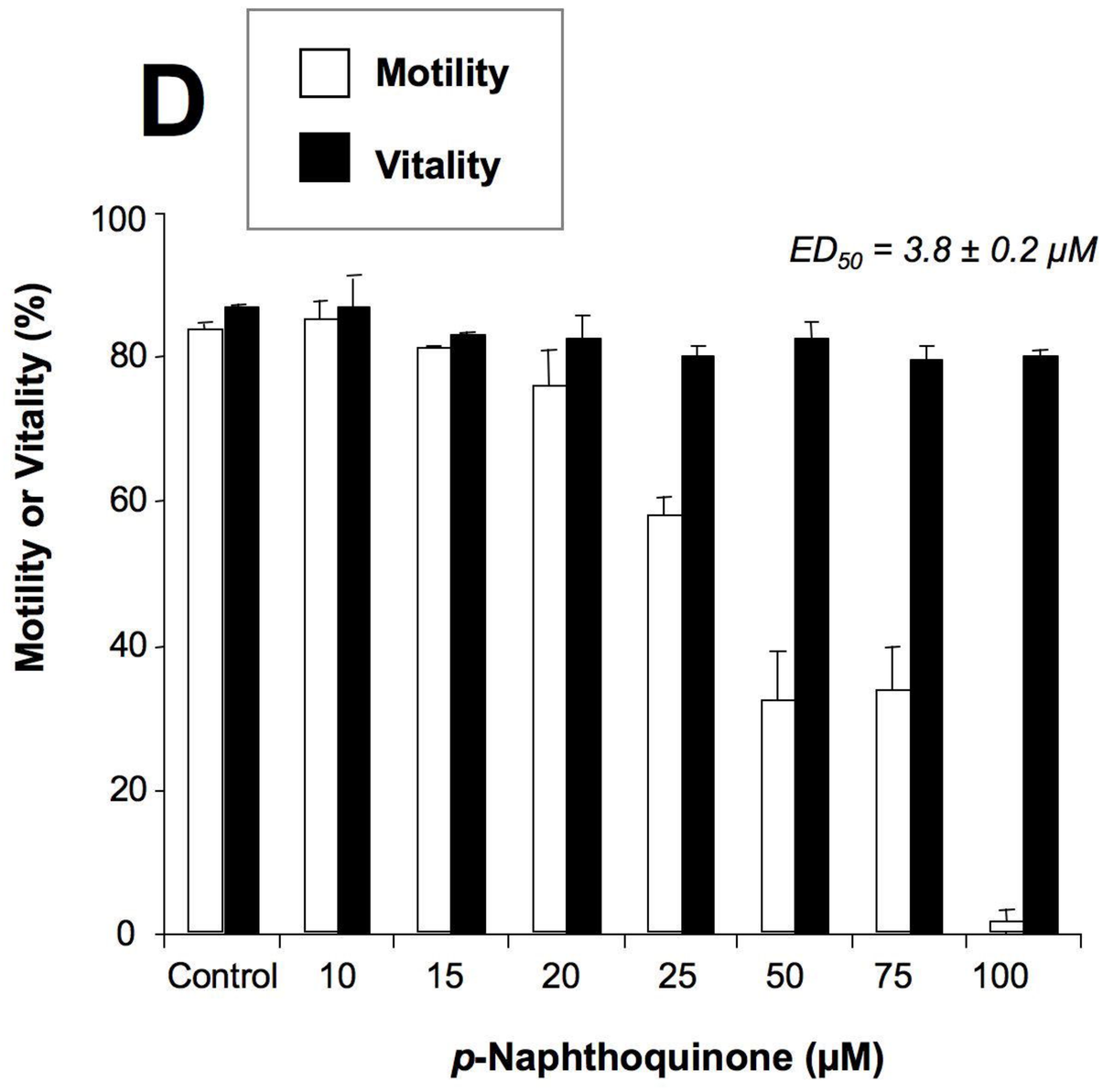
	Compound [a]	DHE (%)	Vitality (%)
(16)	*o*-chloranil	97.0±0.9	99.0±0.3
(1)	5-chloro-2,3-dimethyl-*p*-benzoquinone	93.7±1.0	95.0±0.9
(31)	*o*-naphthoquinone	93.6±0.7	94.4±0.7
(4)	2-methyl-*p*-benzoquinone	93.2±1.4	94.5±1.5
(21)	1,4-anthraquinone	92.0±1.2	93.2±1.3
(26)	plumbagin	91.4±1.5	93.4±1.0
(5)	thymoquinone	90.9±2.4	96.2±1.0
(23)	2-bromo-*p*-naphthoquinone	90.5±1.0	91.8±0.9
(14)	2,5-diphenyl-*p*-benzoquinone	90.1±0.8	92.6±0.6
(27)	2,2'-bis(*p*-naphthoquinone)	89.0±0.6	92.6±0.2
(20)	juglone	88.2±4.2	91.2±3.6
(7)	2-phenyl-*p*-benzoquinone	87.9±2.4	90.4±0.7
(11)	*p*-benzoquinone	86.6±10.5	86.5±5.1
(10)	2-chloro-*p*-benzoquinone	82.3±5.8	87.3±4.3
(3)	2,6-dimethoxy-*p*-benzoquinone	79.8±7.3	86.2±6.0
(12)	2,6-dichoro-p-benzoquinone	79.6±4.0	82.6±4.0
(22)	2,3,7-trichloro-5-hydroxy-*p*-naphthoquinone	79.3±4.7	82.9±3.4
(13)	*o*-bromanil	76.7±6.8	84.3±3.7
(24)	2,3-dichloro-*p*-naphthoquinone	75.7±4.1	79.3±2.6
(19)	*p*-naphthoquinone	73.1±13.6	89.5±1.4
(59)	5'5'-dimethyl-2,2'-bi-*p*-benzoquinone	71.9±6.9	73.5±7.4
(6)	2,5-dichloro-*p*-benzoquinone	52.6±1.3	73.2±3.4
(8)	*p*-bromanil	58.4±3.6	69.1±1.6
(9)	*p*-chloranil	51.7±3.6	57.2±2.0
(35)	bismaleimide	19.0±4.7	68.3±8.7
(36)	maleimide	5.3±1.9	94.2±1.4
(68)	*N*- ethylmaleimide	12.6±1.5	85.7±5.2
(46)	hydroquinone	7.2±3.2	92.2±3.1

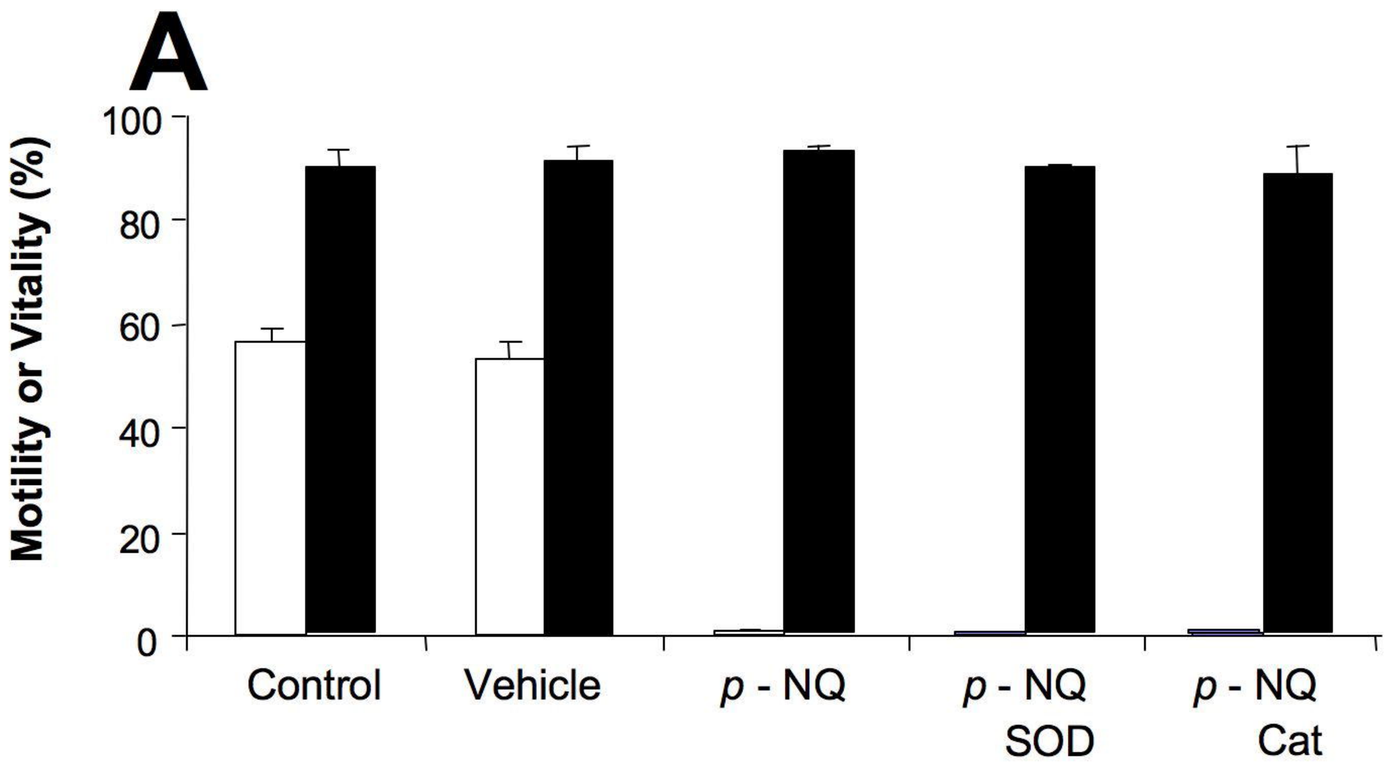
[a]Numbers in parentheses correspond to compound number in Supplemental Table 1

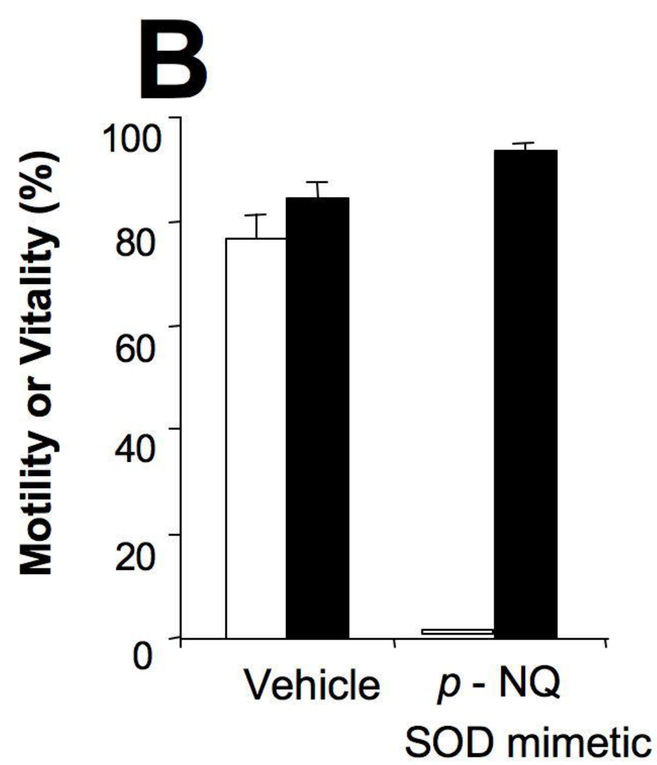


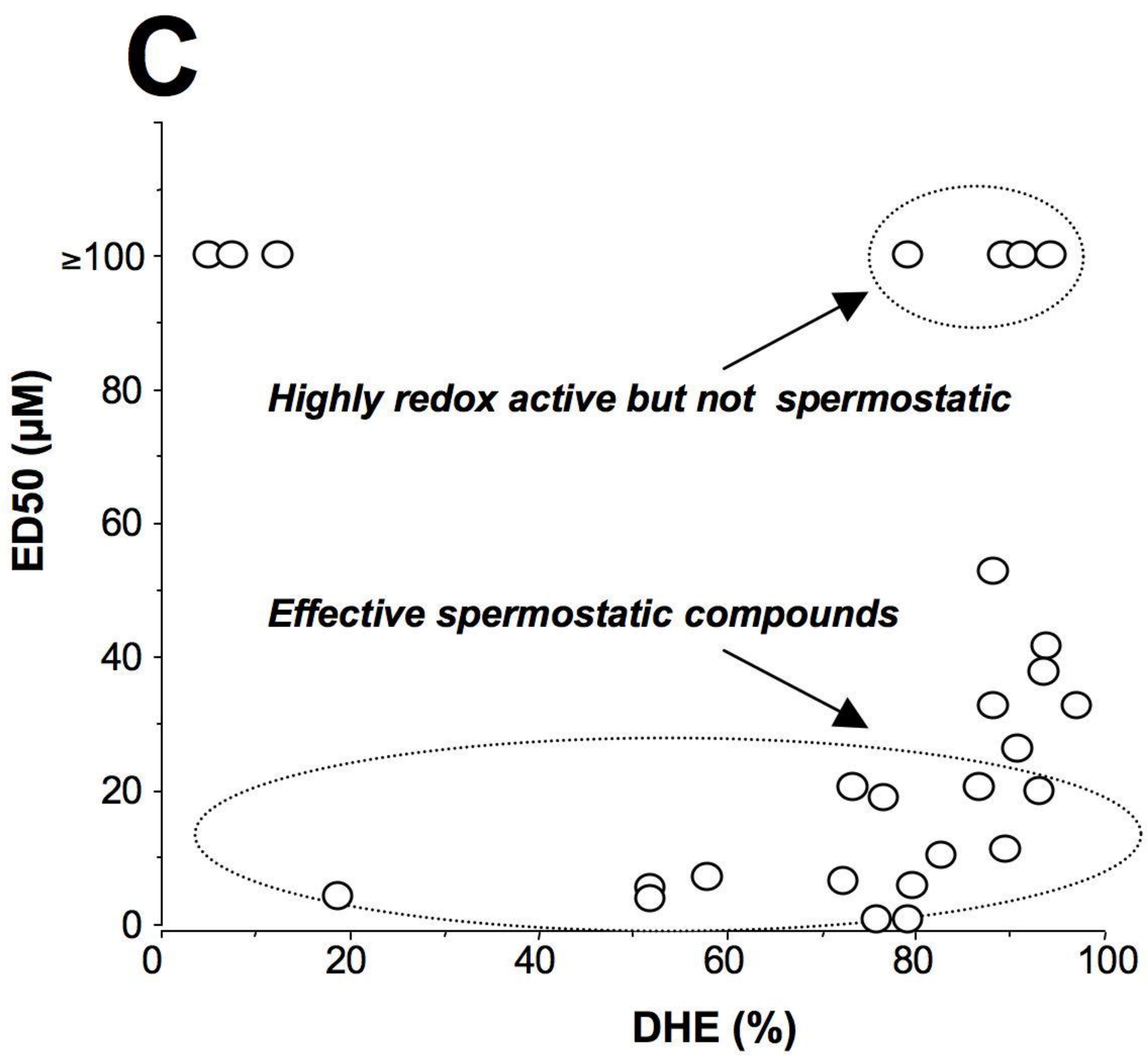


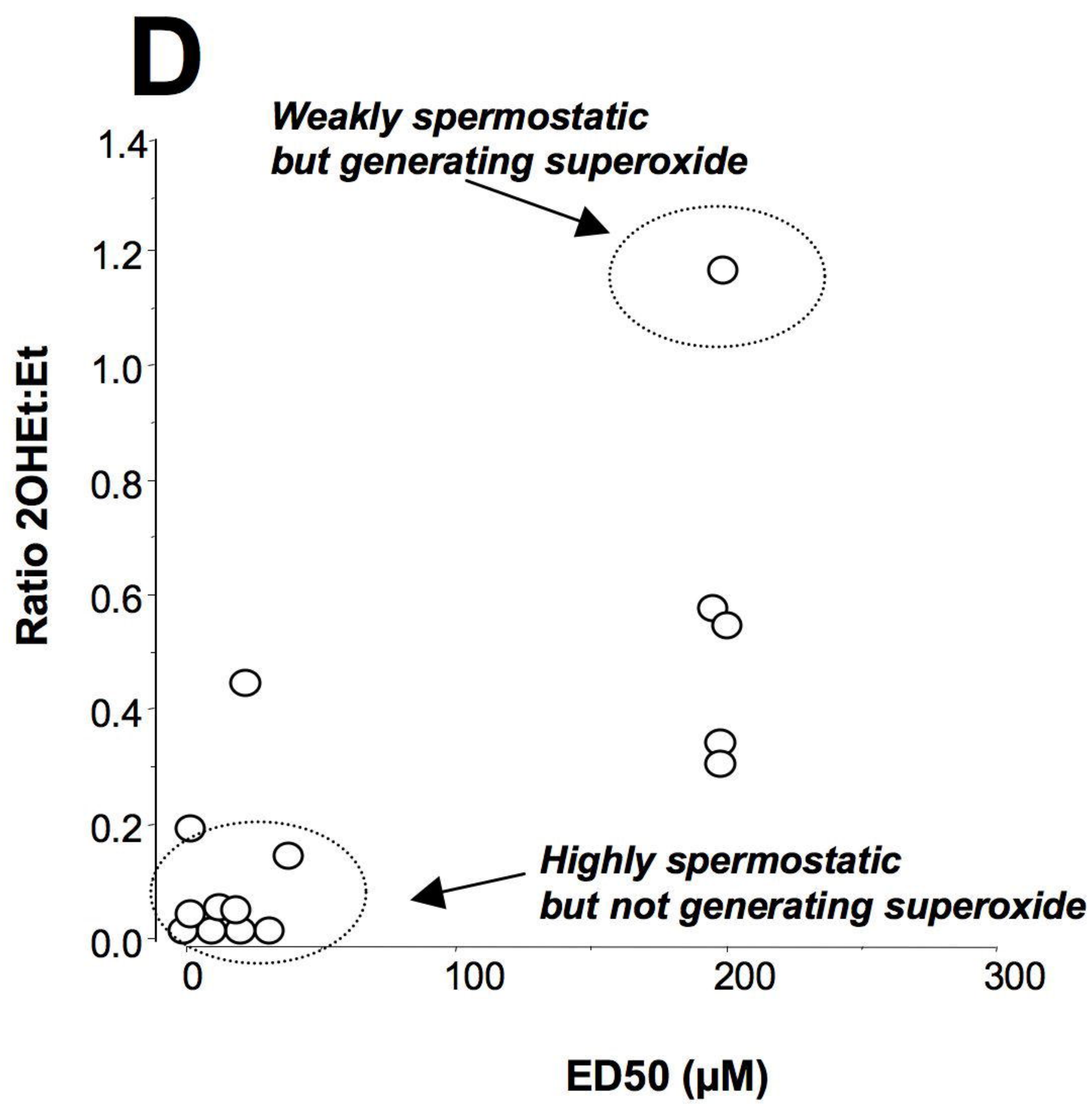


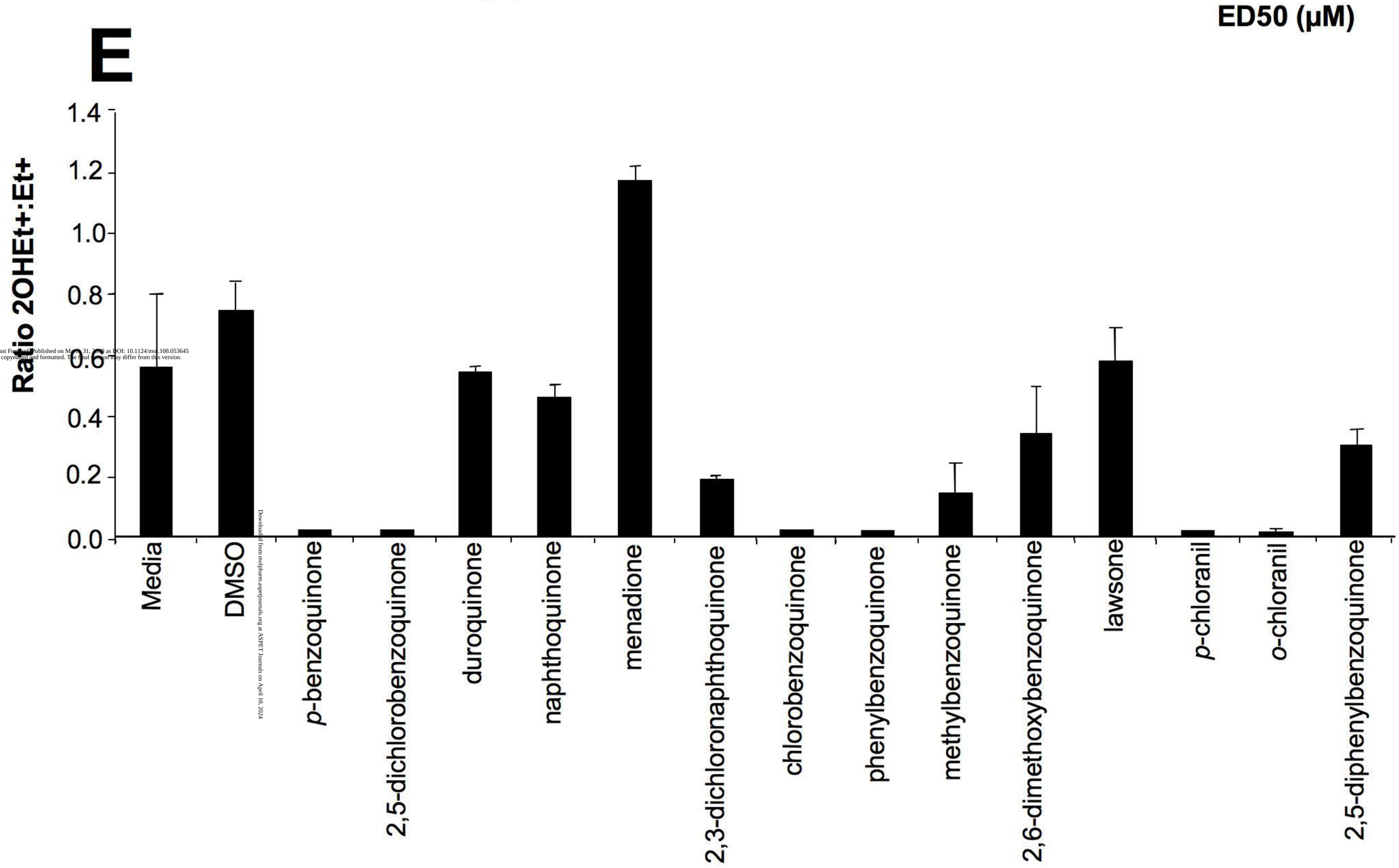


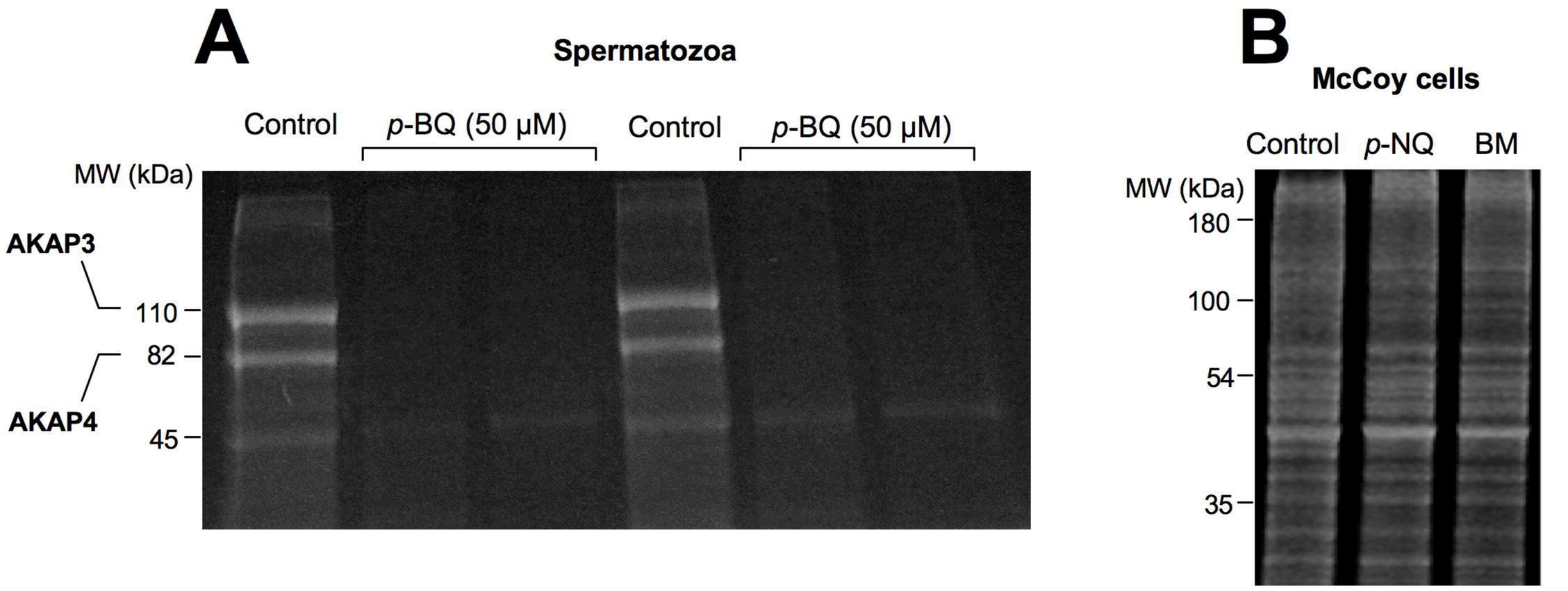












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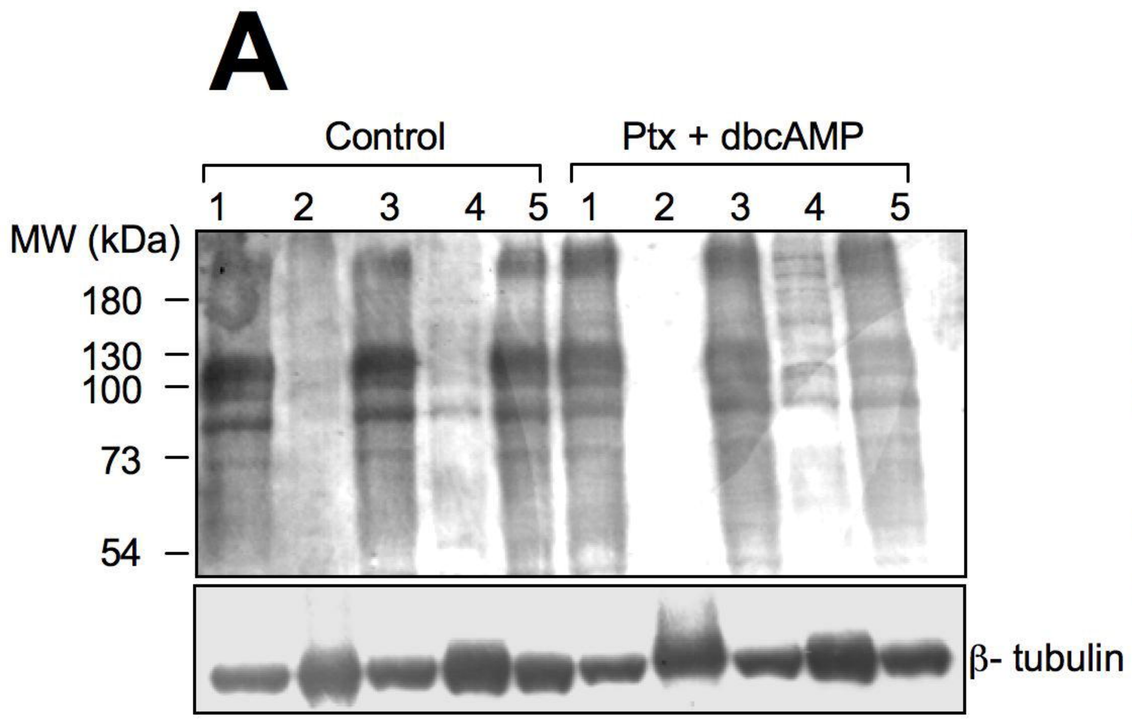

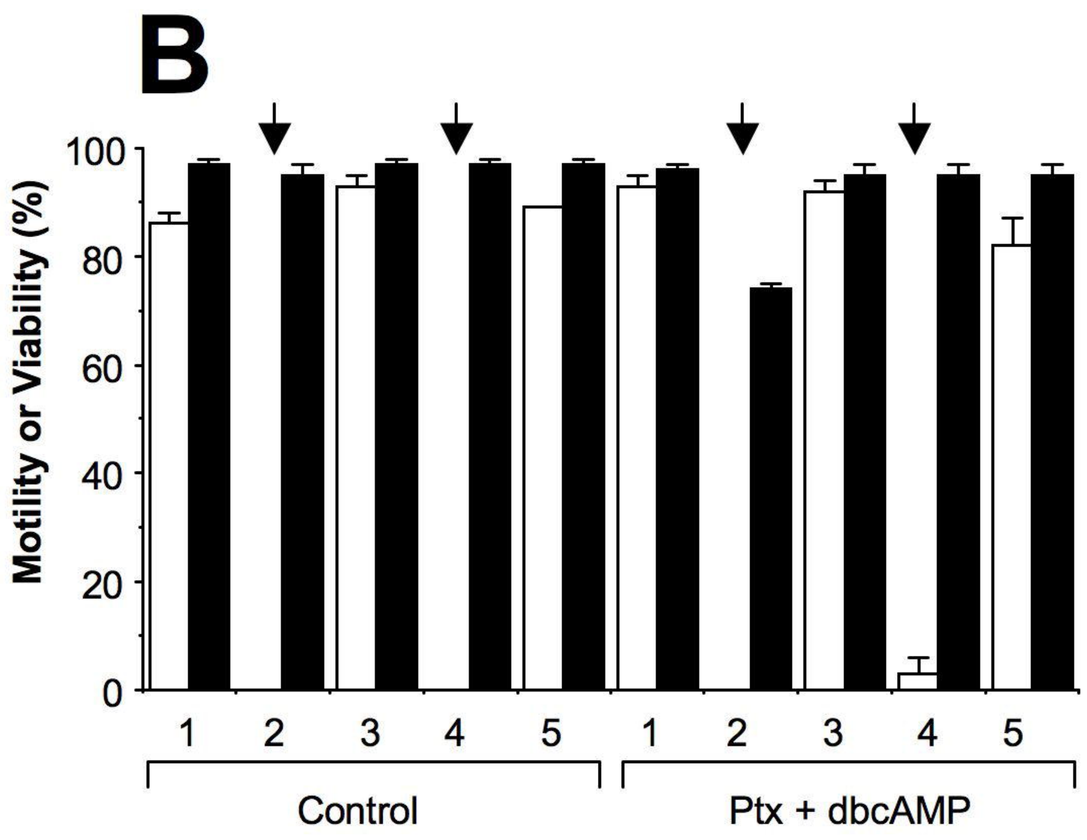

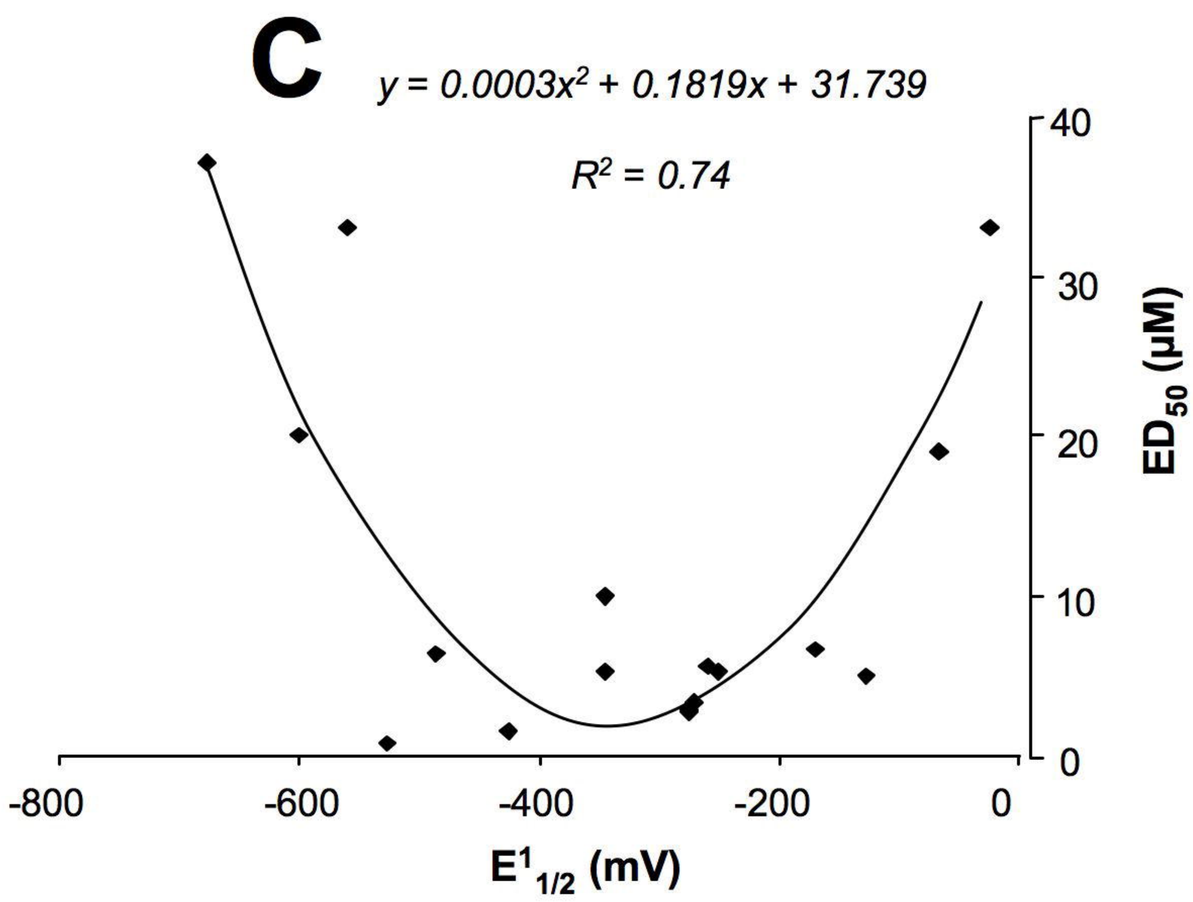

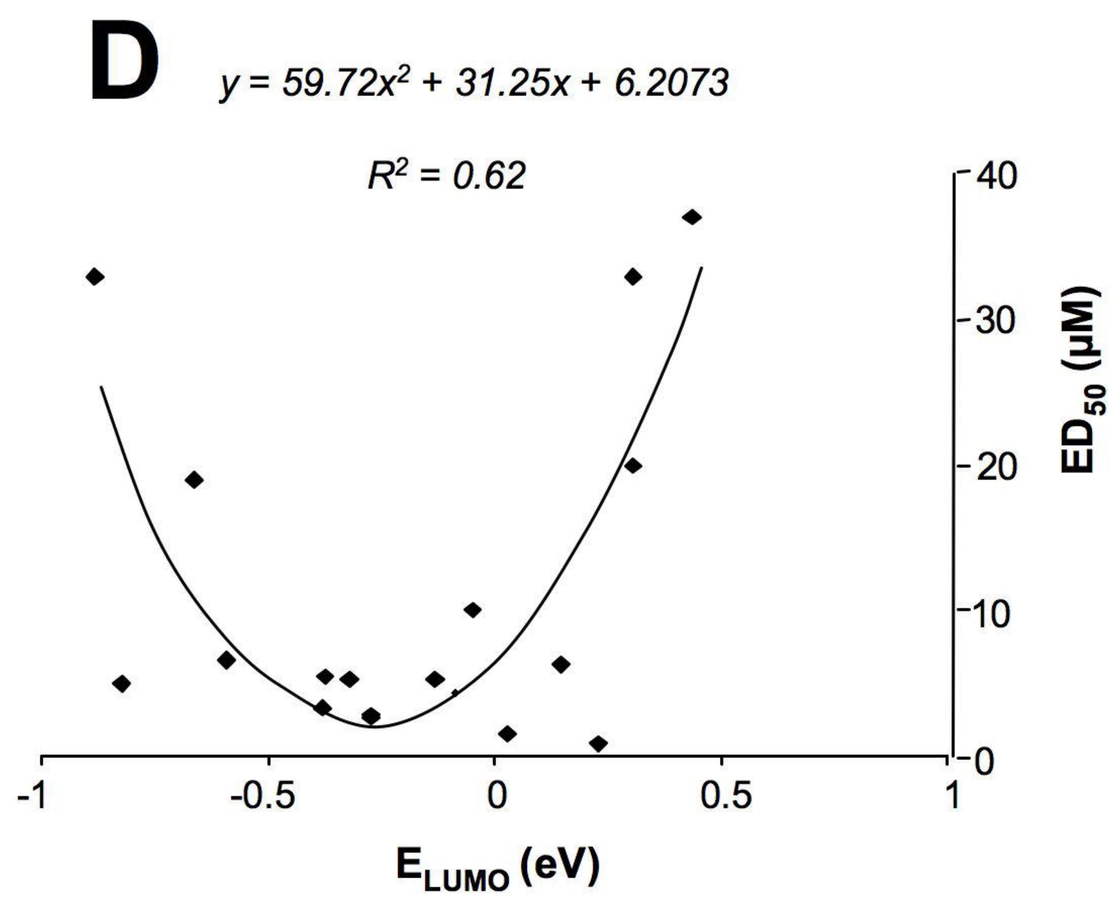

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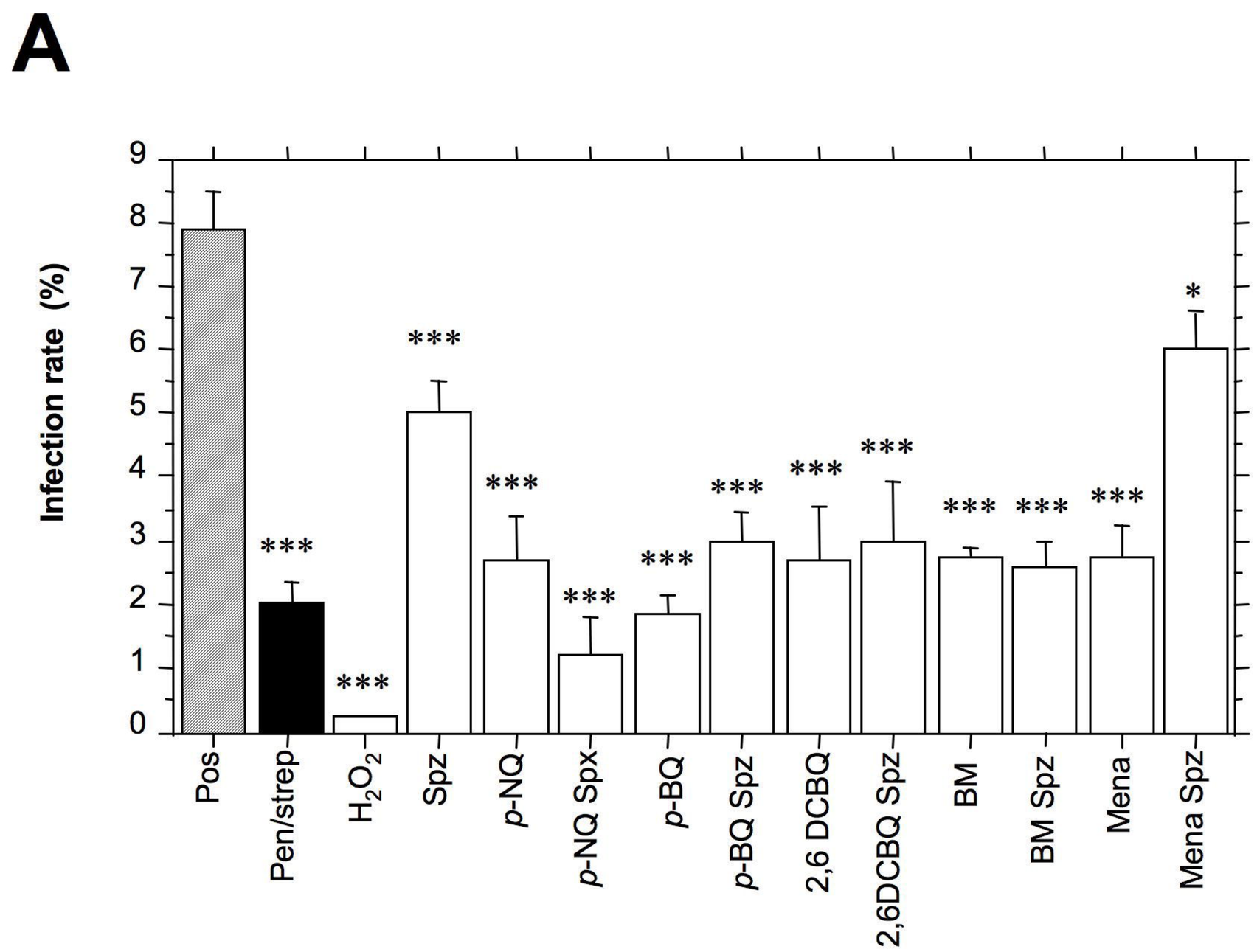

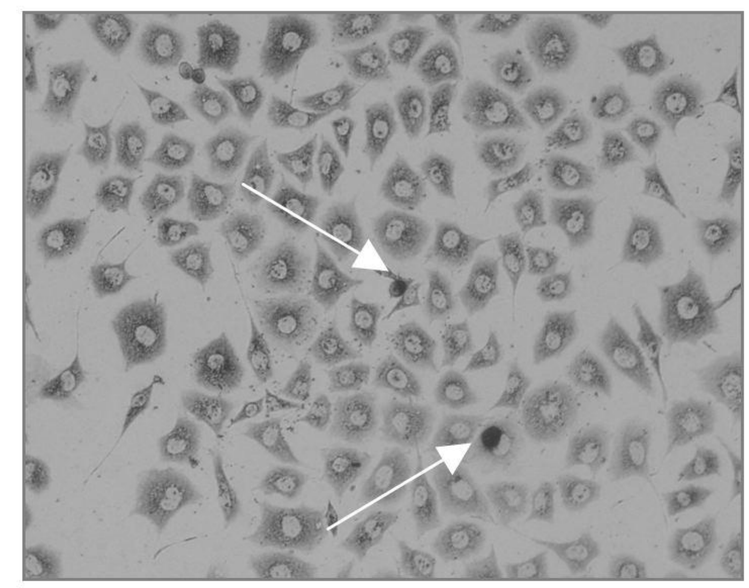

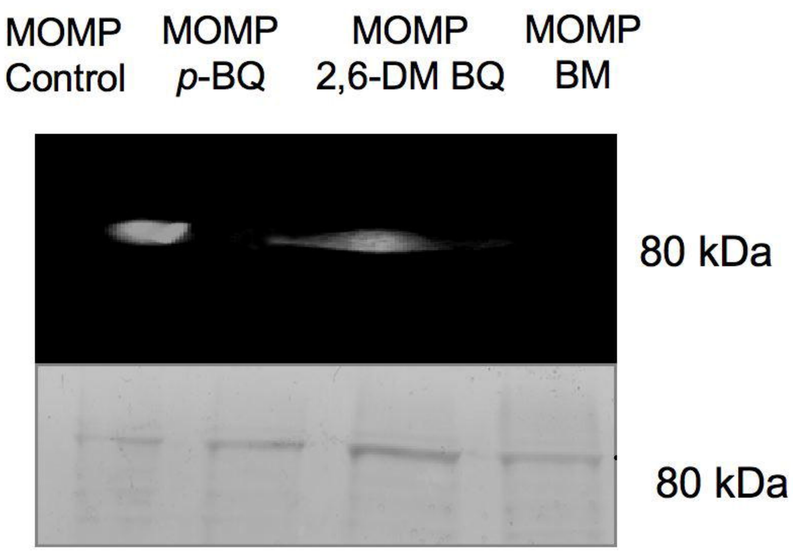

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