

The Spermostatic and Microbicidal Actions of Quinones and Maleimides: Toward a Dual-Purpose Contraceptive Agent[§]

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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 diseases. 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 protein kinase A-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 muridarum* 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 Major Outer Membrane Protein (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.

The World Health Organization has highlighted the need to develop novel, safe, effective, dual-purpose contraceptive agents that combine the prevention of pregnancy with protection against sexually transmitted disease (World Health Organization, 2002). Currently, more than 40 million people are living with HIV, and 18,000 new infections are notified every day (UNAIDS/WHO epidemic update, available at

<http://www.unaids.org>). In 2006 alone, AIDS claimed approximately three 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 approximately 340 million new infections per 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 more than 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

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ABBREVIATIONS: STD, sexually transmitted disease; PKA, protein kinase A; AKAP, A kinase-anchoring protein; MOMP, major outer membrane protein; N9, nonoxynol 9; BWW, Biggers, Whitten and Whittingham medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DHE, dihydroethidium; DTT, dithiothreitol; pCMBS, p-chloromercuribenzenesulfonic 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; TI, therapeutic index; ROS, reactive oxygen species; Et, ethidium; 2OHEt, 2-hydroxyethidium; LUMO, lowest unoccupied molecular orbital; p-BQ, p-benzoquinone; BM, bismaleimide; p-NQ, p-naphthoquinone; RT, room temperature; PAGE, polyacrylamide gel electrophoresis; IFU, inclusion forming unit; SOD, superoxide dismutase.

under unsafe and insanitary conditions, causing the deaths of 68,000 women per 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 nonoxonyl 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 whereas 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 benzyllonium chloride, whereas 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 antimicrobial activity, as exemplified by their suppressive effect on chlamydial infectivity. This group of compounds therefore represents 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 shown in Supplementary Fig. 1 and Supplementary Table 1. Antiphosphotyrosine monoclonal antibody (clone 4G10) and anti-mouse IgG horseradish peroxidase conjugate were from Millipore (Billerica, MA), whereas mouse anti-phosphorylated SRC (pTyr416) monoclonal was from Calbiochem (La Jolla, CA). Fluorescein isothiocyanate-labeled anti-mouse IgG was obtained from Sigma (St. Louis, MO).

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. After 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). Five microliters of the compound to be tested was added to 95 μ l of sperm suspension prepared in BWW. The percentage of sperm motility was assessed after 20-s incubation at room temperature (RT). Sperm were classified 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 dimethyl sulfoxide and 5 μ l of stock solution was added to BWW to prepare working solutions to determine the ED₅₀ 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 was evaluated over time, sperm were incubated with 20 or 50 μ M quinone for 20 s, centrifuged, and resuspended in fresh medium. Sperm 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 Demembrated-Reactivated Sperm. Demembration 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 demembrated 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 ED₅₀ minus the inverse log of LD₅₀: $TI = \log(1/ED_{50}) - \log(1/LD_{50})$.

Dihydroethidium Assay Using Flow Cytometry. Superoxide formation in response to quinone exposure was measured with the fluorescent probe dihydroethidium (DHE), whereas cell viability was simultaneously monitored using Sytox Green (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 (PerkinElmer Life and Analytical Sciences, Wokingham, UK) using a Ag/AgCl reference electrode and platinum counter and working electrodes. The procedure is described in detail in the Supplementary file on Methodology.

Protein Thiol Expression. After incubation with 50 μ M concentration 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 SDS buffer (Ecroyd et al., 2004) and quantified using the bicinchoninic acid assay (Pierce, Rockford, IL) in accordance with the Lowry method. SDS-PAGE was conducted using 7.5% polyacrylamide gels at a 20-mA constant current.

Tyrosine Phosphorylation. Sperm were suspended in BWW at a density of 2.0×10^7 /ml and treated with 100 μ M quinone (compounds **11** and **15**), diamide (compound **50**), *p*-chloromercuribenzenesulfonic acid (*p*CMBS), or dimethyl sulfoxide vehicle control for 15 min at RT. The sperm suspensions were then centrifuged, resuspended at 5×10^6 cells/ml, and divided into two Falcon tubes. One half was centrifuged, the supernatant was removed, and proteins were extracted with SDS extraction buffer. The other half of the sperm suspension was incubated with 3 mM dibutyl cyclic AMP

(dbcAMP) and 1 mM concentration of a phosphodiesterase inhibitor (pentoxifylline; Ptx) for 90 min, after which the sperm suspension was centrifuged, and proteins were 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 μ g of solubilized sperm protein using 7.5% polyacrylamide gels at 20-mA constant current. Immunodetection of phosphotyrosine residues was performed at RT as described previously (Ecroyd et al., 2004) using an anti-phosphotyrosine monoclonal antibody (clone 4G10; Millipore). Phosphorylated proteins were detected using an enhanced chemiluminescence kit according to the manufacturer's instructions (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Immunolocalization of Phosphorylated SRC. Capacitated cells were prepared in BWW without CaCl_2 but supplemented with 3 mM Ptx, and 5 mM dbcAMP. After incubation, the spermatozoa were fixed in 4% paraformaldehyde, washed three times 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% bovine serum albumin for 1 h. Slides were washed three times with PBS for 5 min and incubated in a 1:50 dilution of primary antibody (pTyr416) at 4°C overnight. Slides were then subjected to three 5-min washes with PBS and incubated in a 1:100 dilution of fluorescein isothiocyanate-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 (Carl Zeiss, Thornwood, NY) 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/ionization-time-of-flight mass spectrometer (MALDI-TOF) (GE Healthcare) was performed as described by Baker et al. (2005). MALDI-TOF mass spectrometry protein identifications were also confirmed at the Australian Proteome Analysis Facility using MALDI-TOF tandem mass spectrometry. 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)] 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, MT). The MOMP was produced and purified as described previously (Berry et al., 2004). Recombinant MBP-MOMP was treated with 50 μ M *p*-benzoquinone (compound 11), 2,6-dimethoxy-*p*-benzoquinone (3), and bismaleimide (35) for 20 min at RT. MBP-MOMP was incubated with 1 mM concentration of the fluorescent thiol probe monobromobimane for 10 min before SDS extraction. SDS-PAGE was conducted using 15% polyacrylamide gels at 100 V for approximately 2 h.

Microbicidal Activity. *Chlamydia muridarum* (VR-123; American Type Culture Collection, Manassas, VA), formally the mouse pneumonitis biovar of *Chlamydia 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 μ g/ml streptomycin sulfate, 2 μ g/ml gentamicin, and 20 mM HEPES. Elementary bodies were purified using a discontinuous Renografin gradient. McCoy cells were plated at 5×10^4 cells/well the day before use in 48-well plates. The compounds were diluted to the concentrations that reflected their spermstatic activity: *p*-naphthoquinone (compound 19, 5 μ M), 2,6-dichloro-*p*-benzoquinone (compound 12, 5 μ M), bismaleimide (compound 35, 4 μ M), menadione (compound 25, 150 μ M), and *p*-benzoquinone (compound 11, 21 μ M). Each treatment was made up to a volume of 100 μ l with the com-

pound and/or spermatozoa and had 2000 IFUs *C. muridarum* added. Treatments were incubated for 1 h at 37°C, 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 μ g/ml cycloheximide. They were then incubated for 24 to 30 h at 37°C and 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, 100 μ l of 3% H_2O_2 and 100 μ l of media containing 1 μ g/ml penicillin/streptomycin were used and treated as above.

Statistical Analysis. All experiments were repeated at least three times on independent samples, and the results were analyzed by analysis of variance using the SuperANOVA program (Abacus Concepts Inc., Berkeley, CA) on a Macintosh G5 computer (Apple Computer, Cupertino, CA); post hoc comparison of group means was by Fisher's protected least significant difference test. Differences with a *P* value of <0.05 were regarded as significant.

Supplemental Data. The supplemental data comprise a list of the chemical structures (Supplementary Fig. 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, and thereafter the compound is referred to by its chemical name alone. Supplementary Table 2 gives a detailed description of the spermstatic activities and TI values for all of the compounds assessed in this study, whereas Supplementary Table 3 lists the DHE responses and cell viabilities. Supplementary Table 4 gives details of the data used for the scattergrams reported in Fig. 2, C 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.

Results

Certain Quinoid Structures Possess Spermicidal Activity Log Orders of Magnitude Greater than Nonoxy-nol 9. This study originated in a proteomic analysis of human spermatozoa, which revealed the presence of quinone oxidoreductase (IPI 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. Because 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 Fig. 1 and 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 sulfones (compounds **51** and **52**) that are Michael acceptors incapable of such redox-related activity.

The assay used 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 s. For the purpose of this assay, we recovered highly motile spermatozoa from the base of the high-density region of a two-step discontinuous Percoll gradient and suspended these cells in a modified Tyrode's medium at a standard concentration of $20 \times 10^6/\text{ml}$. Table 1 illustrates the results obtained for the 31 most active compounds analyzed 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 $130 \mu\text{M}$. 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**, and **56**) were equally active (ED_{50} value of approximately $3 \mu\text{M}$). 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 K₁ (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 that could be attacked by nucleophiles. However, the number of unsubstituted positions on the quinoid ring did not correlate with activity. There were six nonspermicidal benzoquinones (compounds **1, 3, 5, 14, 18**, and **62**) that exhibited an unsubstituted position capable of undergoing nucleophilic attack. Furthermore, the benzoquinone, thymoquinone (compound **5**), was not spermicidal despite the presence of two unsubstituted carbons (C₃ and C₆).

Naphthohydroquinone (**71**) was the only spermicidal hydroquinone in this series. This compound is known to autoxidize rapidly in solution compared with other hydroquinones, so its activity was probably due to naphthoquinone formation (Munday, 1997). Benzohydroquinones (compounds **46** and **72-76**) were not spermicidal in the Sander Cramer assay. This observation is in keeping with previous studies indicating that the addition of 2,5-ditertbutylhydroquinone (100

TABLE 1

Spermicidal activity of quinones

Bold numbers correspond to compound numbers in Supplemental Table 1.

Compound No.	Compound	ED_{50} μM	TI
22	2,3,7-Trichloro-5-hydroxy- <i>p</i> -naphthoquinone	0.3 ± 0.1	2.4
24	2,3-Dichloro- <i>p</i> -naphthoquinone	0.5 ± 0.1	2.2
2	2,2-Ethylendi- <i>p</i> -benzoquinone	0.8 ± 0.1	1.9
53	2,2-Pentylene-di-(5-bromo- <i>p</i> -benzoquinone)	1.3 ± 0.1	N.D.
54	2,6-Dibromo-3,5-dimethyl- <i>p</i> -benzoquinone	1.5 ± 0.0	N.D.
55	2,5-Diiodo- <i>p</i> -benzoquinone	2.7 ± 0.2	N.D.
56	2,5-Dibromo- <i>p</i> -benzoquinone	2.8 ± 0.1	N.D.
6	2,5-Dichloro- <i>p</i> -benzoquinone	3.3 ± 0.2	2.0
35	Bismaleimide	4.1 ± 0.1	1.7
9	<i>p</i> -Chloranil	4.9 ± 0.6	2.0
57	2-Bromo-5-chloro- <i>p</i> -benzoquinone	5.2 ± 0.3	N.D.
58	2-Chloro-5-phenyl- <i>p</i> -benzoquinone	5.3 ± 0.2	N.D.
12	2,6-Dichloro- <i>p</i> -benzoquinone	5.5 ± 0.0	2.2
59	5'5'-Dimethyl-2,2'-bi- <i>p</i> -benzoquinone	6.3 ± 0.7	N.D.
8	<i>p</i> -Bromanil	6.6 ± 0.7	1.8
60	5-Benzoyloxy- <i>p</i> -naphthoquinone	8.3 ± 0.9	N.D.
10	2-Chloro- <i>p</i> -benzoquinone	10.2 ± 0.7	1.8
38	Quinhydrone	10.3 ± 0.7	N.D.
27	2,2'-Bis(<i>p</i> -naphthoquinone)	11.0 ± 0.0	1.7
71	<i>p</i> -Naphthohydroquinone	18.7 ± 0.7	N.D.
13	<i>o</i> -Bromanil	19.3 ± 2.6	1.9
11	<i>p</i> -Benzoquinone	20.3 ± 1.5	1.6
21	1,4-Anthraquinone	20.7 ± 2.4	0.4
19	<i>p</i> -Naphthoquinone	20.7 ± 3.3	0.1
23	2-Bromo- <i>p</i> -naphthoquinone	26.7 ± 2.9	0.3
34	<i>N</i> -Phenylmaleimide	27.3 ± 2.9	0.6
7	2-Phenyl- <i>p</i> -benzoquinone	32.7 ± 0.3	1.0
16	<i>o</i> -Chloranil	32.7 ± 4.8	1.8
4	2-Methyl- <i>p</i> -benzoquinone	37.3 ± 1.8	0.6
31	<i>o</i> -Naphthoquinone	42.3 ± 7.0	1.5
20	Juglone	52.6 ± 1.3	-0.2
39	N9 ^a	130.0 ± 1.2	0.4

N.D., not determined; TI, $\log(1/\text{ED}_{50}) - \log(1/\text{LD}_{50})$.

^a N9 is the current industrial standard and is included for comparative purposes.

μM), a Ca^{2+} -ATPase inhibitor, to human spermatozoa had no effect on motility for up to 6 h (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 TI for each of the compounds tested. The TI is a measure of nonspecific cytotoxicity secured using McCoy cells in conjunction with 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 nonspecific cytotoxicity calculated as the inverse log of the spermicidal ED_{50} value, minus the inverse log of LD_{50} [$\text{TI} = \log(1/\text{ED}_{50}) - \log(1/\text{LD}_{50})$]. In this assay, the compound in current clinical use, N9, yielded a TI value of 0.4. Although some quinones were more cytotoxic than N9 (juglone, 2-bromo-*p*-naphthoquinone, and *p*-naphthoquinone), the 12 most spermicidal compounds listed in Table 1 gave TI values of ≥ 1.6 . Because the TI score is a log value, this indicates that the values recorded for these compounds were at least 10 times greater than that recorded for N9 and, in the case of the most active compounds, 100 times greater. The highest therapeutic index was given 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), and 2,3,7-trichloro-5-hydroxy-1,4-naphthoquinone (TI = 1.7), which 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 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-s exposure to 20 or 50 μM *p*-benzoquinone or *p*-naphthoquinone. The cells were then pelleted by centrifugation (500g for 5 min), resuspended in fresh medium BWW, and examined for motility over the ensuing 28.5 h. 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 $20 \times 10^6/\text{ml}$. As indicated in Table 2, a range of benzoquinones, naphthoquinones, and maleimides were assessed in the 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** and **11**) 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 two-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 $>500 \mu\text{M}$ (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 semiquinone 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. 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 demembration-ATP reactivation model was used (Yeung et al., 1988). With this model system, spermatozoa are demembrated with 0.08% Triton X-100 in the presence of DTT (1 mM) and EDTA (0.5 mM) and reactivated by the addition of 1 mM ATP and 50 μM cAMP. Under these conditions, motility is reactivated in approximately 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. 1, A and B). The ED_{50} values were higher than those recorded in intact cells because quinone bioavailability would have been 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 alky-

TABLE 2
Spermostatic activity in seminal plasma

Bold numbers correspond to compound numbers in Supplemental Table 1. Spermostatic activity was measured in the Sander Kramer assay at two doses.

Compound No.	Compound	Spermostatic ^a	
		50 μM	100 μM
22	2,3,7-Trichloro-5-hydroxy- <i>p</i> -naphthoquinone	—	+
24	2,3-Dichloro- <i>p</i> -naphthoquinone	+	+
6	2,5-Dichloro- <i>p</i> -benzoquinone	—	—
35	Bismaleimide	+	+
9	<i>p</i> -Chloranil	—	—
10	2-Chloro- <i>p</i> -benzoquinone	—	—
38	Quinhydrone	+	+
27	2,2'-Bis(<i>p</i> -naphthoquinone)	+	+
71	<i>p</i> -Naphthohydroquinone	+	+
11	<i>p</i> -Benzoquinone	+/-	+/-
19	<i>p</i> -Naphthoquinone	+	+
31	<i>o</i> -Naphthoquinone	—	—
20	Juglone	—	+
46	Hydroquinone ^a	+	+
39	N9 ^b	—	—

^a This compound was weakly active in the absence of seminal plasma with an ED_{50} value of $>500 \mu\text{M}$.

^b 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 .

lating activity. The fact that quinones were still spermstatic in demembrated-ATP reactivation models, in which redox cycling would not be expected to occur because of the loss of oxidoreductases, 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 spermstatic agents in oxygen-depleted media (Fig. 1, C and D). Indeed, *p*-naphthoquinone was more active in oxygen-depleted medium ($ED_{50} = 3.8 \mu\text{M}$) than in normal medium BWB equilibrated with air ($ED_{50} = 20.7 \mu\text{M}$), suggesting that redox cycling actually counteracted the spermstatic activity of such compounds. In keeping with this conclusion, neither superoxide dismutase (SOD; 300 U) nor catalase (3000 U) had any effect on the spermstatic activity of 100 μM *p*-naphthoquinone (Fig. 2A). Likewise, the addition of a membrane-permeant SOD mimetic [Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrins pentachloride] at a dose of 25 mM did not influence the spermstatic activity of 100 μM *p*-naphthoquinone (Fig. 2B).

Further evidence that redox cycling was not involved in the mechanisms by which quinones exhibited spermstatic behavior 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 DHE as the probe and revealed no discernable relationship between the spermstatic activity of a given compound and its redox cycling

activity (Table 3; Supplemental Table 3 for full data set). Figure 2C illustrates this point by plotting the spermstatic 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 spermstatic 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 an ROS response in a majority of cells; the complete data set for this scattergram is contained in Supplementary Table 4.

High-pressure liquid chromatography 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 nonspecific two-electron oxidation product of DHE) (Fig. 2, D and E; Supplementary Table 4). Menadione generated the highest levels of 2OHEt and yet was not spermstatic, whereas 2,3-dichloro-*p*-naphthoquinone, *p*-benzoquinone and 2,5-dichloro-*p*-benzoquinone were extremely spermstatic 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 nega-

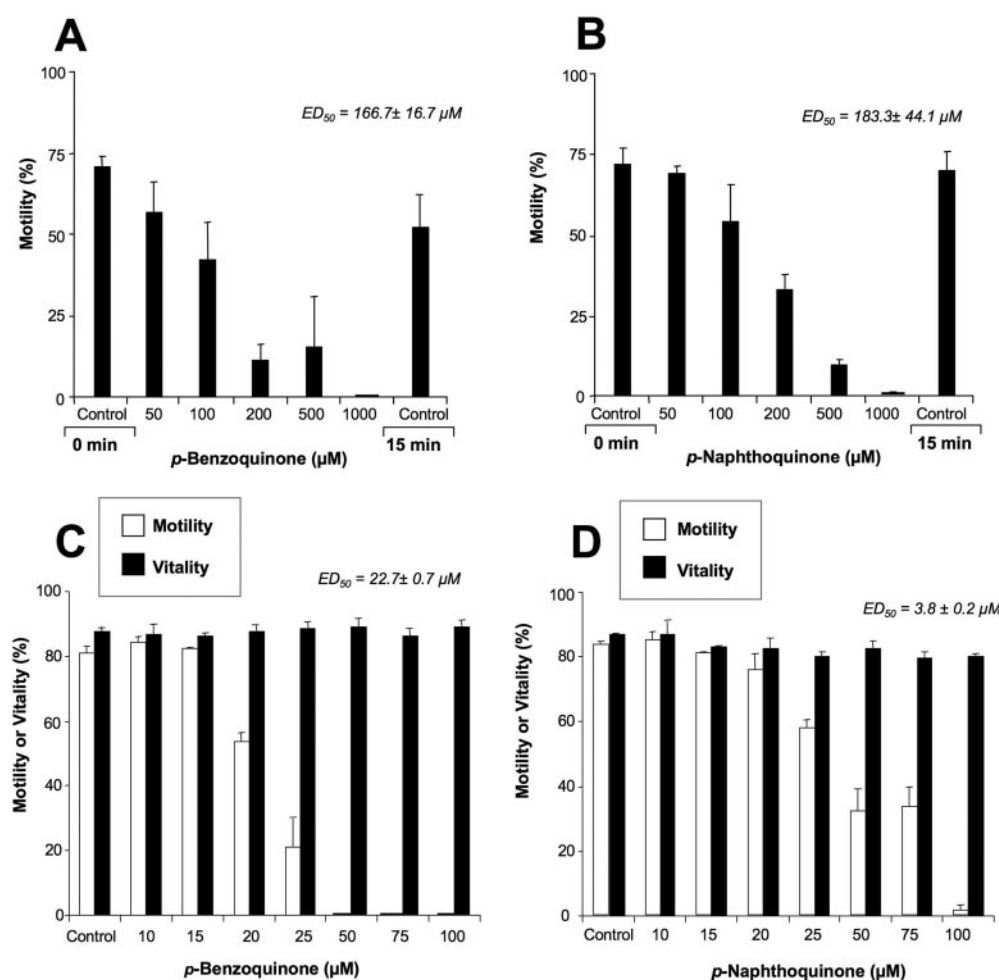


Fig. 1. Analysis of the mechanisms by which quinones suppress sperm motility. A, dose-dependent analysis of the affect of 1,4 *p*-benzoquinone on the motility of human spermatozoa in a Triton-X demembration, ATP reactivation model. B, similar data for 1,4 *p*-naphthoquinone. Control motilities are shown for the beginning (0 min) and end (15 min) of the experimental period. Motilities were assessed 20 s after exposure to a given dose of quinone. With both compounds, the dose-dependent suppression of sperm movement in this demembrated 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 s. D, similar data for 1,4 *p*-naphthoquinone. Note the dose-dependent suppression of motility (\square) with both quinones ($P < 0.001$) in the absence of any significant impact on cell vitality (\blacksquare).

tive, 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. To examine this hypothesis, spermatozoa were exposed to a range of quinones or maleimides followed by DTT to reduce any remaining disulfide 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 spermstatic activity (Fig. 3A). Thus, highly spermstatic quinones such as *p*-benzoquinone (Fig. 3A), *p*-chloranil, and 2,2-bis-*p*-naphthoquinone suppressed thiol expression by human spermatozoa,

whereas inactive quinones such as lapachol or menadione were ineffectual in this regard. Likewise, within the maleimides tested, the only compound to exhibit spermstatic activity (bis-maleimide) suppressed thiol expression, whereas related alkylating agents (*N*-ethylmaleimide and iodocetamide) displaying no spermstatic 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. Likewise, 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 spermstatic agents such as *p*-benzoquinone or *p*-naphthoquinone (Fig. 3B).

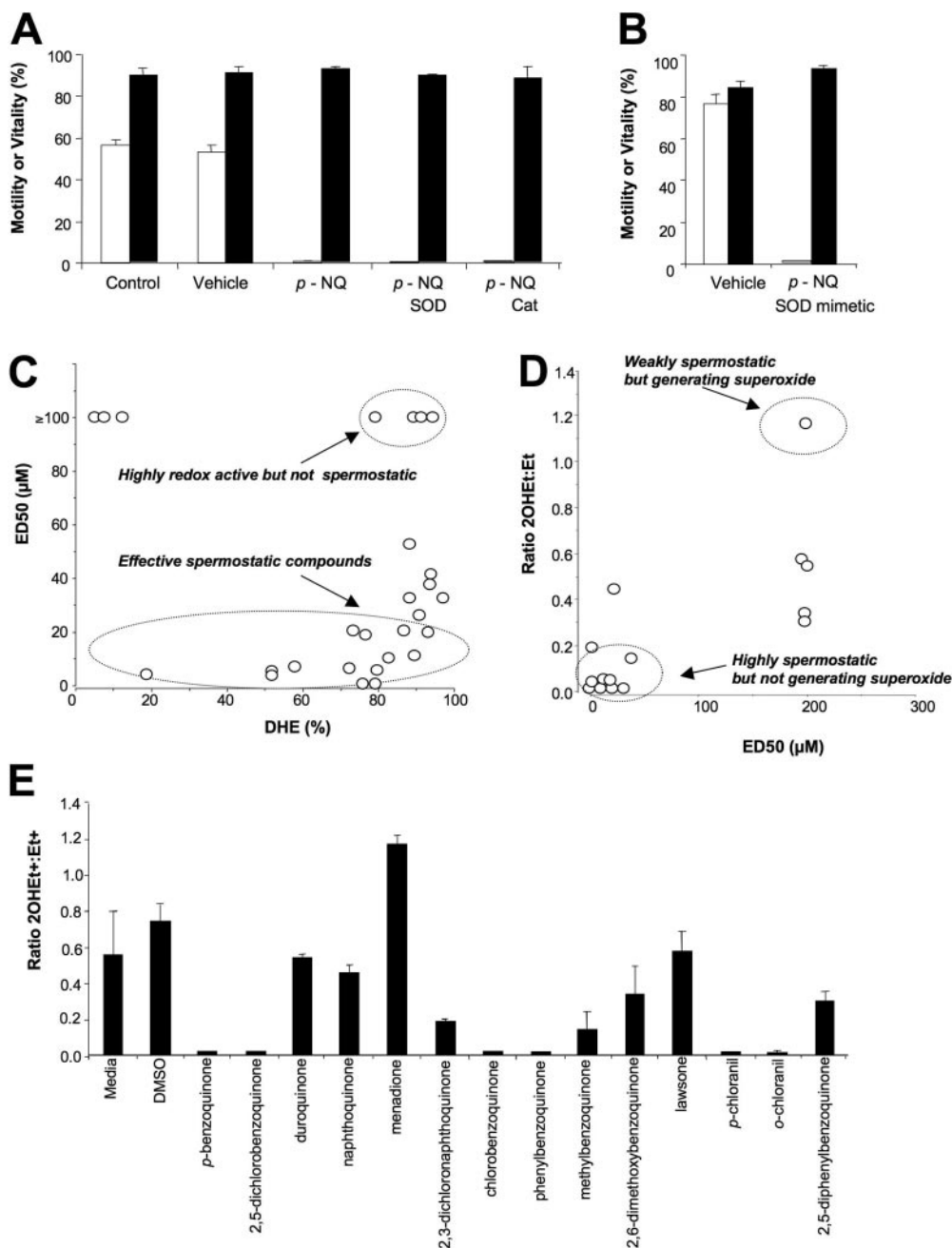


Fig. 2. Influence of redox cycling and ROS generation on the spermstatic impact of quinones. The impact of SOD and catalase (A) and an SOD mimetic (B) on the motility and viability of human spermatozoa. None of these antioxidant strategies had a significant impact on the spermstatic effect of *p*-naphthoquinone (*p*-NQ). Spermstasis was not correlated with the redox cycling activity of the compounds as measured by DHE fluorescence (C) or the ratio of 2OHEt (a specific product generated by the interaction between DHE and superoxide anion) to Et (the two-electron oxidation product of DHE) (D). All data points in these scattergrams represent the mean of three replicate determinations on independent sperm populations; detailed information is shown 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 $O_2^{\cdot -}$ in human sperm cells and yet were not spermstatic, whereas highly spermstatic compounds such as *p*-benzoquinone generated largely Et.

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. 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 tar-

gets 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 up-regulation in phosphotyrosine expression in proteins that are largely located on the fibrous sheath and mitochondria of the sperm tail. If the spermostatic agents identified in this study interfere with the ability of AKAPs to orchestrate cAMP signaling in the flagellum, then we should see an immediate down-regulation in the activation of SRC and consequent suppres-

TABLE 3

Detection of redox activity with dihydroethidium

Bold numbers correspond to compound numbers in Supplemental Table 1.

Compound No.	Compound	DHE	Vitality
		%	
16	<i>o</i> -Chloranil	97.0 ± 0.9	99.0 ± 0.3
1	5-Chloro-2,3-dimethyl- <i>p</i> -benzoquinone	93.7 ± 1.0	95.0 ± 0.9
31	<i>o</i> -Naphthoquinone	93.6 ± 0.7	94.4 ± 0.7
4	2-Methyl- <i>p</i> -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- <i>p</i> -naphthoquinone	90.5 ± 1.0	91.8 ± 0.9
14	2,5-Diphenyl- <i>p</i> -benzoquinone	90.1 ± 0.8	92.6 ± 0.6
27	2,2'-Bis(<i>p</i> -naphthoquinone)	89.0 ± 0.6	92.6 ± 0.2
20	Juglone	88.2 ± 4.2	91.2 ± 3.6
7	2-Phenyl- <i>p</i> -benzoquinone	87.9 ± 2.4	90.4 ± 0.7
11	<i>p</i> -Benzoquinone	86.6 ± 10.5	86.5 ± 5.1
10	2-Chloro- <i>p</i> -benzoquinone	82.3 ± 5.8	87.3 ± 4.3
3	2,6-Dimethoxy- <i>p</i> -benzoquinone	79.8 ± 7.3	86.2 ± 6.0
12	2,6-Dichloro- <i>p</i> -benzoquinone	79.6 ± 4.0	82.6 ± 4.0
22	2,3,7-Trichloro-5-hydroxy- <i>p</i> -naphthoquinone	79.3 ± 4.7	82.9 ± 3.4
13	<i>o</i> -Bromanil	76.7 ± 6.8	84.3 ± 3.7
24	2,3-Dichloro- <i>p</i> -naphthoquinone	75.7 ± 4.1	79.3 ± 2.6
19	<i>p</i> -Naphthoquinone	73.1 ± 13.6	89.5 ± 1.4
59	5'5'-Dimethyl-2,2'-bi- <i>p</i> -benzoquinone	71.9 ± 6.9	73.5 ± 7.4
6	2,5-Dichloro- <i>p</i> -benzoquinone	52.6 ± 1.3	73.2 ± 3.4
8	<i>p</i> -Bromanil	58.4 ± 3.6	69.1 ± 1.6
9	<i>p</i> -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	<i>N</i> -Ethylmaleimide	12.6 ± 1.5	85.7 ± 5.2
46	Hydroquinone	7.2 ± 3.2	92.2 ± 3.1

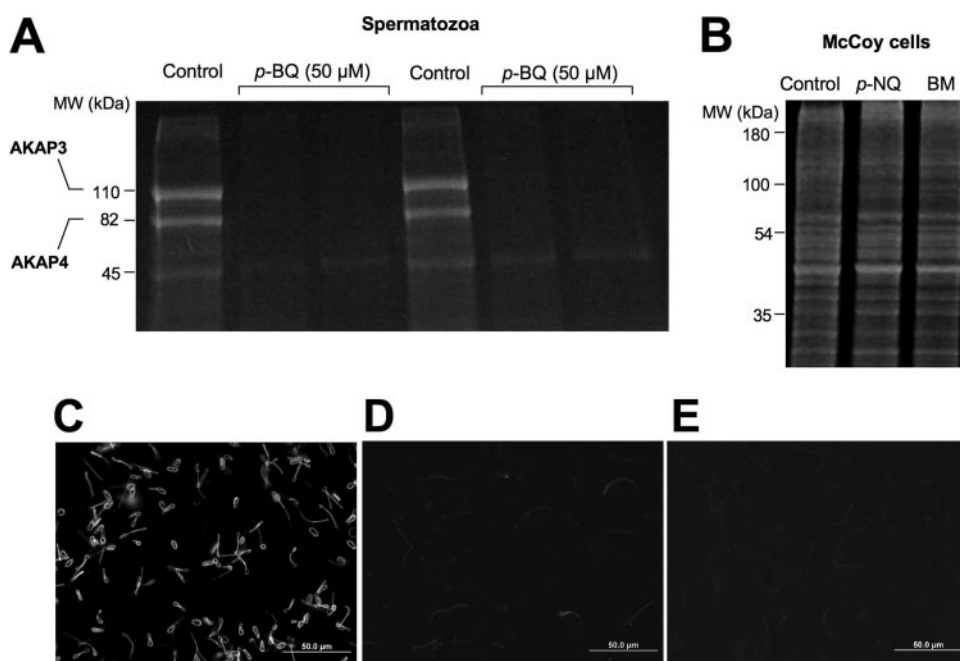


Fig. 3. Influence of alkylating activity on the spermostatic effect of quinones. A, spermostatic quinones such as *p*-benzoquinone (*p*-BQ) alkylate two major thiol-expressing proteins in human spermatozoa that were characterized by mass spectrometry as AKAP3 and AKAP 4 as indicated. B, spermostatic quinones including *p*-NQ and bismaleimide (BM) had no effect on patterns of thiol expression in a nonspecific cell line (McCoy cells). C, expression of PKA-dependent activated SRC (SRC phosphorylated at position Tyr416) in the tail of capacitating human spermatozoa, detected by immunocytochemistry after 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 C. E, unstimulated control sample.

sion of phosphotyrosine expression in the sperm tail. As illustrated in Fig. 3, C to E, the addition of the spermostatic quinone, *p*-benzoquinone, led to a dramatic cessation of phospho-SRC expression in the presence of dbcAMP. Likewise, the addition of this quinone to human spermatozoa completely suppressed both the basal and the cAMP-induced stimulation of tyrosine phosphorylation in human spermatozoa (Fig. 4, A and B; lane 2). The nonspecific thiol oxidant, diamide, also suppressed both motility and tyrosine phosphorylation (Fig. 4, A and B, lane 4). Although this compound was not strictly spermostatic (in that it could not immobilize spermatozoa within 20 s), 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 pK_a of the corresponding hydroquinone, or the lowest unoccupied molecular orbital (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.

4, C and 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 *C. muridarum* elementary 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 white females. Both antibiotics and 3% H₂O₂ 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 chlamydial infectivity at doses as low as 1 μ M ($P < 0.001$). The concomitant presence of spermatozoa had no effect 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, although the H₂O₂ result il-

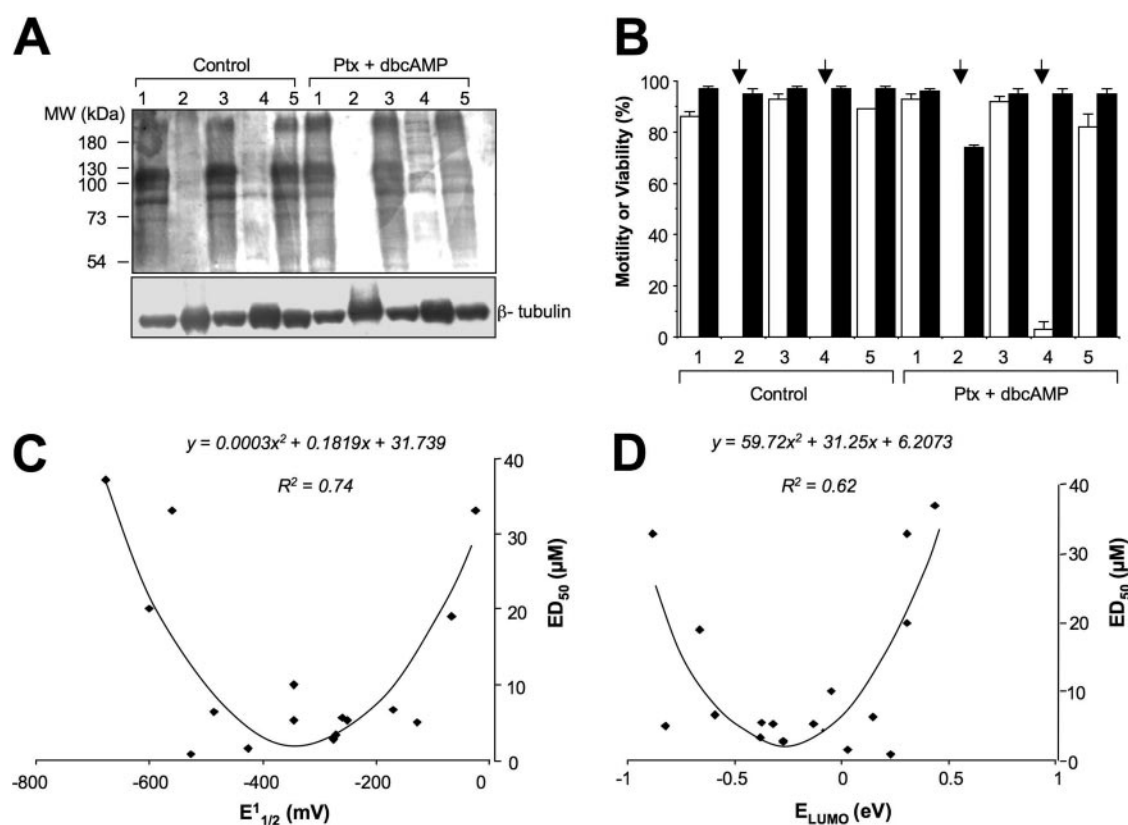


Fig. 4. Alkylating activity of spermostatic quinones was also correlated with the suppression of tyrosine phosphorylation in human spermatozoa. Spermatozoa were incubated with the test compounds (100 μ M) for 15 min, centrifuged for 5 min at 500g, and then either sampled immediately (Control) or resuspended in fresh medium BWW supplemented with 3 mM dbcAMP and 1 mM Ptx to induce capacitation. The compounds evaluated were the following: 1, medium control; 2, *p*-benzoquinone; 3, duroquinone; 4, diamide; and 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 (\square) and viability (\blacksquare) after exposure; arrows indicate that only the spermostatic quinone, *p*-benzoquinone, and the thiol oxidant, diamide, induced motility loss. Using a large family of related benzoquinones, the relationship between spermostatic activity and either the one-electron reduction potential (C) or the energy of the LUMO (D) was examined. A highly significant ($P \leq 0.001$) parabolic relationship was observed for both parameters.

lustrated 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 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 against both pregnancy and STDs. The spermicide in current clinical use, N9, is a detergent that nonspecifically 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 that is 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 seems 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, whereas 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/

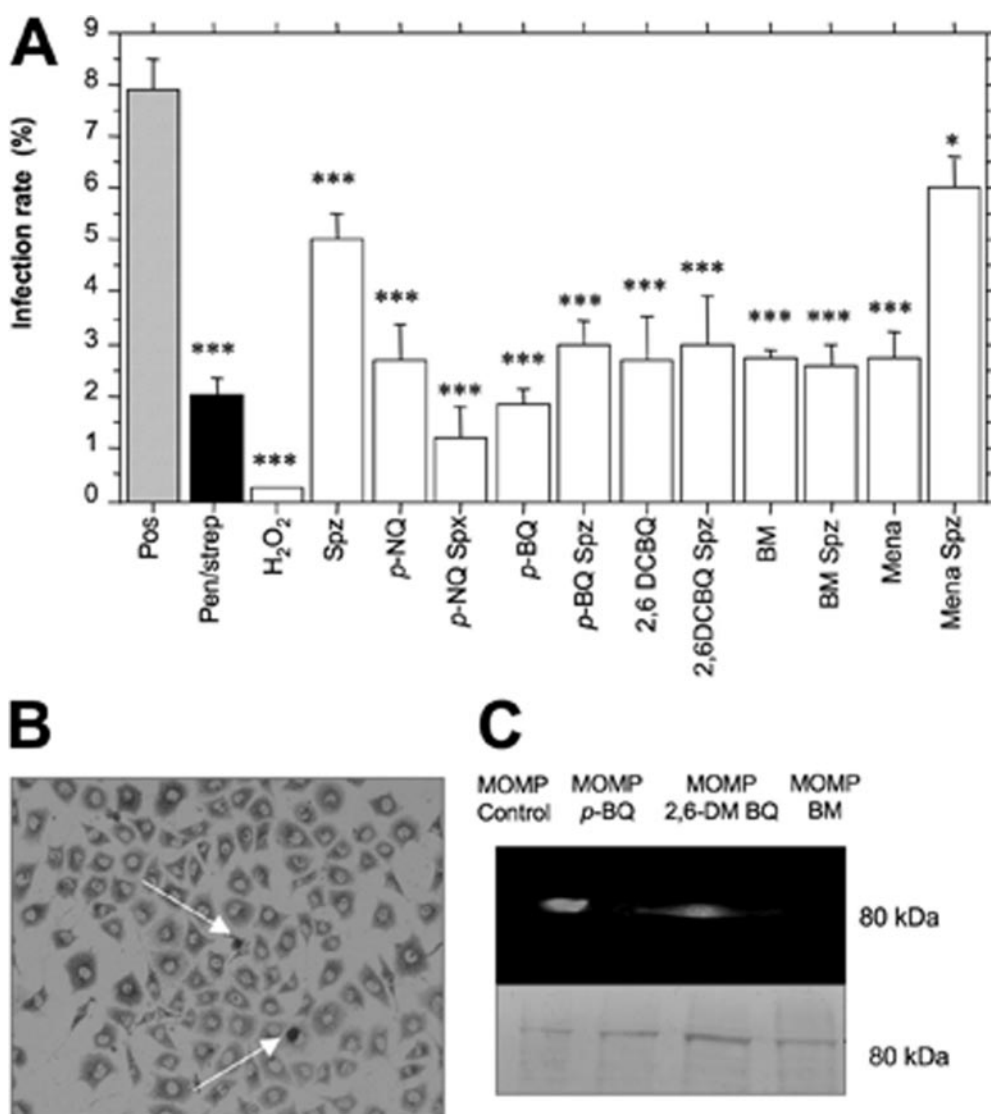


Fig. 5. Influence of quinones on the infectivity of *C. muridarum* using McCoy cells as the target. **A**, ability of quinones and the alkylating agent bismaleimide to neutralize the infection of McCoy cells with *C. muridarum* elementary bodies at a concentration of 1000 IFUs. Penicillin/streptomycin was used at a concentration of 1 μ g/ml; H₂O₂ was used at a concentration of 3%, and the test compounds were used at doses that reflected their relative spermostatic activity: *p*-NQ (5 μ M), 2,6 dichloro-*p*-benzoquinone (2,6DCBQ, 5 μ M), BM (4 μ M), menadione (Mena, 150 μ M), and *p*-BQ (21 μ M). 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 (arrows). **C**, investigation of the ability of quinones and bismaleimide to suppress the expression of free thiols by recombinant chlamydial MOMP. Top, thiol expression visualized using monobromobimane as the probe; bottom, a Coomassie blue stain of the same gel demonstrating the equal loading of MOMP in each lane. The spermostatic compounds *p*-BQ and BM completely suppressed thiol expression at a dose of 25 μ M. Conversely, the nonspermostatic quinone, 2,6 dimethoxy-*p*-benzoquinone (2,6-DMBQ), had little effect on thiol expression.

PKA-dependent signaling pathways essential for motility. Because cAMP is involved in the activation and maintenance of sperm motility (Bajpai and Doncel, 2003; Wade et al., 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 is consistent with the complete lack of sperm motility seen in mutant mice null for soluble adenylyl cyclase, the major site for cAMP production in these cells (Hess et al., 2005). Furthermore, targeted disruption of the AKAP4 gene in mice leads to male infertility associated with impaired sperm movement (Miki et al., 2002). Likewise, 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 γ (Baker et al., 2007), which may have provided sufficient kinase activity to drive the low level of motility observed in the PKA C $\alpha 2$ knockout mouse.

The ability of spermstatic 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, although 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, because 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 spermstatic compounds identified in this study also possessed antimicrobial activity in an assay that examined the infectivity of chlamydial elementary bodies. This microbicidal action was also associated with the alkylation of key thiols, in this case on the major outer membrane protein of *C. muridarum*. 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 disulfide switching mechanism that is dependent on the expression of surface thiols (Matthias et al., 2002) is also compatible with an anti-HIV role for these spermstatic 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 spermstatic and antimicrobial

activity. Several of the electrochemical properties associated with compounds exhibiting strong spermstatic activity were identified in this study and can now be used to refine the synthesis of compounds combining high levels of spermstatic and anti-microbial activity with a minimum of cytotoxicity.

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