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A lifetime cancer bioassay of quinacrine administered into the uterine horns of female rats

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ABSTRACT

This study investigated if quinacrine can induce a tumorigenic response in rats when administered in a manner similar to the intended human use for female non-surgical sterilization. Young sexually mature female rats received two doses of quinacrine (or 1% methylcellulose control) into each uterine horn approximately 21 days apart, and were observed for 23 months after the second dose administration. Dose levels were 0/0, 0/0, 10/10, 70/70, and 70/250–350 mg/kg (first dose/second dose), which represent local doses in the uterus at approximate multiples of 1×, 8× and 40× the human dose (mg quinacrine/g uterine weight) used for female non-surgical sterilization. Rats were observed for viability, clinical signs of toxicity, and changes in body weight and food consumption. At necropsy, selected organs were weighed, macroscopic observations were recorded, and tissues were collected, fixed, processed, and examined for microscopic pathologic findings. Acute quinacrine toxicity was evident during the dosing period but did not affect long-term survival. Non-neoplastic findings were more common in treated animals than controls, providing evidence of the appropriateness of the bioassay. The incidence of uncommon tumors of the reproductive tract was similar to controls at doses of 10/10 mg/kg but increased with dose level and was significantly greater than controls at ⩾70/70 mg/kg. We conclude that two doses of quinacrine administered approximately 21 days apart into the uterus of young sexually mature rats at a local dose approximately 8 times the human dose used for non-surgical female sterilization increased the lifetime risk of tumor development in the reproductive tract.

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1. Introduction

Much effort has been expended to develop a safe, effective, simple, and inexpensive method of female non-surgical sterilization that would offer advantages over surgical sterilization. One investigational non-surgical sterilization method is quinacrine sterilization (QS) (Zipper and Kessel, 2003). The most current method of administering quinacrine involves two transvaginal administrations (21 days apart) of seven quinacrine tablets (36 mg each, 252 mg total) directly into the uterine fundus. As the quinacrine tablets dissolve, the quinacrine enters the Fallopian tubes, where it induces tubal scarring leading to tubal closure (Lippes et al., 2003). QS has been shown to result in female infertility, with 1-, 5-, and 10-year cumulative pregnancy probabilities of 3.3%, 10.0%, and 12.1%, respectively (Sokal et al., 2008a) and has been promoted as a simple and inexpensive method of female sterilization (Lippes, 2002). However, to date, quinacrine has not received approval from regulatory authorities for use as a non-surgical female sterilization method in any country (Benagiano, 2001, 2003).

The mechanism by which quinacrine induces tubal closure is believed to be similar to the mechanism of internal tissue repair (Holmdahl and Ivarsoon, 1999). The primary effect of quinacrine in tubal closure occurs through tissue damage, due to its cytotoxic properties, leading to inflammation and tissue repair. Tissue repair, resulting in adhesion formation, is a multifactorial process influenced by the extent of tissue damage (a function of level and duration of exposure to the cytotoxic agent), the ability of the epithelium to regenerate, the relative closeness of opposing surfaces, and the kinetics of the fibrinolytic pathway. If sufficient tissue damage occurs, preventing regeneration of the epithelium, fibroblasts are able to proliferate and fibrin deposition occurs in the damaged area. The narrow lumen of the Fallopian tube allows for the opposing surfaces to be joined, through the fibrotic

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response, leading to a functional tubal closure (Eddy and Pauerstein, 1983; el-Kady et al., 1991).

Quinacrine is a DNA-intercalating agent (Davidson et al., 1978) and has been shown to be mutagenic and clastogenic *in vitro* (reviewed by Blake et al., 1983). A more recent report confirmed that quinacrine was positive for mutagenic activity in an Ames assay and a mouse lymphoma assay, positive for clastogenic potential in a chromosome aberration assay in Chinese hamster ovary cells (Clarke et al., 2001). In contrast, results of the *in vivo* mutagenic studies of quinacrine in the bone marrow micronucleus assay indicate that quinacrine is not clastogenic *in vivo* (Clarke et al., 2001).

The high local tissue concentrations of quinacrine in the reproductive tract tissue, the mechanism by which it induces tubal closure, and its mutagenic and clastogenic activity, as well as the identification of a provocative case of a rare uterine sarcoma in a cancer cluster analysis (Sokal et al., 1995), have raised concerns about the tumorigenic potential of quinacrine (Allen, 1998). A published 18-month chronic toxicity study in rats administered quinacrine orally in either a low- or a high-protein diet (Fitzhugh et al., 1945) did not address the potential tumorigenic effect of quinacrine administered by the intrauterine route. Quinacrine showed equivocal tumorigenic activity in a 52-week neonatal mouse carcinogenicity study (Cancel et al., 2006), in which mice received intraperitoneal doses of quinacrine on postpartum Days 8 and 15. This study found that two doses of quinacrine administered early in life at $\geqslant$50 mg/kg led to an increase in the incidence of uterine endometrial hyperplasia and of benign endometrial stromal polyps, both of which are common background lesions in mice.

In short term tumor inhibition models quinacrine has been shown to have anti-carcinogenic activity (NCI, 1994); but these studies do not address the long-term safety concerns of direct administration of quinacrine into the uterus that result in potentially high local quinacrine concentrations and local tissue damage. To examine the long-term safety concerns of quinacrine, we investigated the potential tumorigenic effect of quinacrine in the rat uterine horn model. This study reports the results of a lifetime cancer bioassay where quinacrine was administered into the uterine horns of young sexually mature rats in two doses, approximately 21 days apart, to mimic the intended human use of quinacrine. We used the rat uterine horn model because it is an accepted and routinely used animal model to evaluate efficacy of investigational drugs for female non-surgical sterilization (Fail et al., 2000; Jensen et al., 2004; Zipper et al., 1968, 1973), and has been used in previous studies to evaluate tumor induction after direct intrauterine administration of drugs (Kitamura et al., 1995; Takahashi et al., 1995; Tanaka and Mori, 1983).

2. Materials and methods

2.1. Animals and animal husbandry

Nulliparous and non-pregnant female Albino Rats (Outbred) VAF/Plus®, Crl:CD®(SD)IGS BR were obtained from Charles River Laboratories, Raleigh, North Carolina at approximately 43 days of age. More rats than required for the study were purchased and acclimated. Animals considered suitable for study, and determined to be in diestrus, were randomized daily into dose groups. Randomization was stratified by animal weight to limit weight variation among groups to <20%. A total of 60 females per group were allocated into each study group with an average weight per group of 190 g. Each rat was then identified with a metal ear tag bearing an assigned unique animal number.

A total of 245 females were placed on the main study and an additional 40 rats were included for health monitoring purposes. Animals were acclimated for at least 14 days prior to the study. All animals were examined during the acclimation period to confirm their suitability for entry into the study.

Animals were individually housed in elevated, stainless steel, wire mesh cages. A 12-h light/dark cycle controlled via an automatic timer was provided. Temperature was monitored and maintained between 20.2 °C and 26.1 °C. The relative humidity was monitored and maintained between 30% and 70%. A few excursions outside the specified humidity range occurred (range 18.3–74.7%) but were not considered to have affected the integrity of the study. Cage racks were rotated periodically to avoid bias from the microenvironment in a given area of the room. Certified Rodent Diet, No. 5002 (Meal) (PMI Nutrition International, St. Louis, Missouri) and water (Elizabethtown Water Company, Westfield, New Jersey) were available *ad libitum*. No known contaminants were identified in the feed or water that might be expected to interfere with the results of the study.

2.2. Test materials and dosing suspensions

Two lots of quinacrine dihydrochloride dihydrate were used in these studies. The first and second dose range-finding studies were conducted using quinacrine manufactured by Vipor Chemicals, India and supplied by SiPharm Sisseln AG, Switzerland. Quinacrine used for the third dose range-finding study and for the lifetime cancer bioassay was manufactured and supplied by Ricerca Biosciences (Ohio, USA) and was of 92.8% purity (excluding water as hydration). Quinacrine, in the form of quinacrine dihydrochloride dihydrate, a yellow powder, was stored in a sealed brown glass container in a controlled environment, at room temperature (10–30 °C).

Methylcellulose was obtained as a white powder, manufactured and supplied by Fisher Scientific (New Jersey, USA). The vehicle was prepared by suspending methylcellulose in 0.9% saline.

Quinacrine dosing suspensions were prepared under yellow fluorescent light by suspending quinacrine in 1% (w/v) methylcellulose in 0.9% saline. Dosing suspensions were stored refrigerated in sterile amber bottles with a stir bar. On days of dosing, dosing formulations were removed from the refrigerator and brought to room temperature (approximately 1 h) by continuous stirring. Dosing formulations were stirred at least 5 min prior to dosing and continuously during dosing.

All dosing formulations were analyzed for quinacrine concentration using a Huntingdon Life Sciences validated High Performance Liquid Chromatography–UV Detection Method for the determination of quinacrine in 1% methylcellulose solution. Samples were analyzed on the day prior to use by taking duplicate samples on the day of preparation. Measured quinacrine concentrations in dosing suspensions were within 95% and 106% of the nominal concentration.

Homogeneity and stability of quinacrine in dosing suspensions used in this study were established prior to initiation of the study, using test batches of the dosing suspensions. Refrigerated stability was conducted on Day 0 and Day 14 from the date of preparation. In addition, room temperature stability was established at time 0, 4, and 8 h following preparation. Quinacrine was uniformly distributed and stable in dosing suspensions under the conditions of storage and use during the study.

2.3. Dose administration

Doses were administered through the uterine cervix into each uterine horn during diestrus. Each rat was treated twice. Administration of the first dose was staggered over a period of approximately 2 weeks due to the large group sizes and particular dosing procedure. The second dose was given approximately

21 days after the first, although the interval sometimes varied to ensure that the second dose was administered during the desired stage of the estrus cycle.

Rats were anesthetized with isoflurane mixed with oxygen. A pediatric otoscope (Welch-Allyn #21700, 3.5 V halogen) with a disposable speculum (3 mm Keenspec) was used to visualize the cervix through the vagina. A single dose (0.7 mL/kg/uterine horn) was injected into each uterine horn at the appropriate dose level using a 22-gauge spinal needle with a blunted end fitted on a syringe, and then inserted through the vagina and the cervix. Leakage of the uterine dose was minimized by injecting 3 μL of 3% methylcellulose at the end of each uterine horn.

2.4. Experimental design and dose selection

Table 1 summarizes the experimental design of the lifetime cancer bioassay. The experimental design of the lifetime cancer bioassay was intended to mimic the dosing regimen used in the human clinical situation as closely as possible. Therefore dosing frequency was limited to two intrauterine instillations of quinacrine administered 21 days apart. Since the mechanism of action of quinacrine-induced sterilization in humans relies on the production of tissue necrosis and fibrosis in the Fallopian tube, the choice of dose level in the rat was based on (1) the need to produce fibrosis as observed in women (Merchant et al., 1995); (2) the need to test dose levels that provided exposures that were equivalent to and greater than those occurring routinely in clinical, non-surgical sterilization programs in women; and (3) the tolerance of the rat to any local or systemic toxicity produced by the quinacrine instillation.

Three dose range-finding studies were conducted to identify doses for the lifetime bioassay.

2.4.1. Range-finding study #1

Groups of six young sexually mature female rats were given intrauterine doses of 2% methylcellulose (control group) or quinacrine suspended in 1% methylcellulose at dose levels of 175 and 350 mg/kg. The dosing volume was 0.5–0.7 mL/kg/uterine horn. Twenty-one days later, half the rats in each group were euthanized. The remaining rats were dosed again at the same dose level and observed for another 21 days, and then euthanized and necropsied. No animal mortality occurred during the study and quinacrine treatment did not cause a significant decrease in body weight gain compared with controls. No gross lesions were observed in the uterine horns examined from the three rats euthanized 21 days after a single administration of 175 mg/kg quinacrine. Lumen closure, fibrosis and inflammation were observed 21 days after the first dose of 350 mg/kg and after a second dose of 175 or 350 mg/kg.

Administration of the first and second dose of the vehicle was successful in the rats from the control group, but administration of the second dose of quinacrine proved unreliable at either dose level of quinacrine. This resulted in only three of the six uterine horns at each dose level receiving a second administration. The inability to administer the second dose was assumed to be due to fibrosis of the horn or technical problems associated with fibrosis of the contralateral horns caused by the first dose, despite the fact that fibrosis was not identified by histopathologic examination in the three rats examined after a single administration of 175 mg/kg quinacrine. These results suggested that the initial dose of quinacrine should be <175 mg/kg, in order to allow a second dose to be administered consistently.

2.4.2. Range-finding study #2

The main purpose of this range-finding study was to identify a dose of quinacrine that allowed a second intrauterine dose. The highest dose of 70 mg/kg was selected from unpublished data, where a single dose of 70 mg/kg quinacrine resulted in no remarkable uterine histopathology findings (8/12 uterine horns were scored as normal and 4/12 uterine horns scored with luminal dilation); and a single dose of 140 mg/kg resulted in fibrosis (i.e., potentially not allowing a second dose) and was not considered adequate for the purpose of this study. Groups of six young sexually mature female rats were given two intrauterine doses (approximately 21 days apart) of quinacrine suspended in 1% methylcellulose at dose levels of 0.7/0.7, 7/7, 14/14, 35/35, and 70/70 mg/kg (first dose/second dose). All animals in the study were dosed in both uterine horns a second time. No deaths occurred during the study and quinacrine treatment did not cause a significant decrease in body weight gain compared with controls. Examination of the stage of the estrus cycle in vaginal smears collected at necropsy showed that all stages of the estrous cycle were represented, suggesting that animals were cycling at the end of the study and that quinacrine did not cause a permanent disruption

Table 1
Experimental outline of the lifetime cancer bioassay.

Test material	Doses[a]					Number of animals (females)[c]			
	Dose (mg/kg)		Dosing volume (mL/kg)	Concentration (mg/mL)		Dose administration	Observation period	Survival to terminal sacrifice	Microscopic pathology
	Day 0	Day 21		Day 0	Day 21				
1% methylcellulose	0	0	1.4	0	0	60	50	13	50
1% methylcellulose	0	0	1.4	0	0	60	50	14	50
Quinacrine	10	10	1.4	7	7	60	50	13	50
Quinacrine	70	70	1.4	50	50	60	50	17	50
Quinacrine	70	250	1.0	50	250	35	24	5	24
Quinacrine	70	350[b]	1.4	50	250	33	21	10	21

[a] Doses represent active ingredient (quinacrine dihydrochloride dihydrate). Each animal was dosed with ½ dose per uterine horn. Dosing was staggered due to the time required to dose the required number of animals via the intrauterine route of administration. An equal number of animals/group were dosed at each interval.

[b] Twelve of 33 rats administered quinacrine at a dose level of 350 mg/kg (Day 21) died or were euthanized shortly after dosing. Due to this high mortality rate (36%), it was decided to not dose the remaining 27 rats left in this group with 350 mg/kg. Instead, these rats (which had been given a first dose of 70 mg/kg) were combined with eight reserve rats that had also been given a first dose of 70 mg/kg, to form a new group that was given a second quinacrine dose at 250 mg/kg. Thus, the high-dose group comprised 21 rats surviving from the 70/350 dosing regime and 35 rats reallocated to the 70/250 mg/kg dosing scheme. A further 11 rats died from the 70/250 mg/kg group leaving 24 rats to enter the observation phase (also see footnote c).

[c] Ten extra animals/group were dosed to ensure a minimum of 50 dosed animals/group with the exception of groups given second doses of 250 and 350 mg/kg. In these two groups, a total of 35 and 33 rats were dosed, respectively. Only those rats surviving 1 month after the second dose were designated to enter the 2-year observation phase of the study for eventual necropsy and microscopic examination. For the high-dose group, this constituted a total of 45 rats (21 females and 24 females given 250 and 350 mg/kg, respectively). Those extra dosed rats not utilized as spares were removed from the study 4 weeks after the second dose.

of the estrous cycle. The greatest severity of the findings occurred in females dosed with 14, 35, and 70 mg/kg. In the 70 mg/kg dose group 5/12 uterine horns were scored as normal, 1/12 with minimal acute inflammation, and 7/12 with dilation. The increased dilation was not universal in any dose group, or within an animal. When it did occur, it was sometimes limited to one uterine horn or one portion of the uterine horn. Lumen closure was observed in only one uterine horn of an animal treated with 14 mg/kg quinacrine. The lack of significant lumen closure, even after two doses of 70 mg/kg, suggested that a second dose of quinacrine could be administered into a uterine horn that had received an initial dose of ⩽70 mg/kg 21 days previously.

2.4.3. Range-finding study #3

The main purpose of this dose range-finding study was to assess the tolerability of a two-dose regimen of quinacrine when administered directly into the uterus of female rats. The high doses for the first and second administration were selected based on the results of the range-finding studies #1 and #2 and subsequent doses were selected as multiples of the high dose (first and second dose). Groups of 10 young sexually mature female rats were given two intrauterine doses (approximately 21 days apart) of quinacrine suspended in 1% methylcellulose at dose levels of 50/250, 50/300, 50/350, 70/250, 70/300, 70/350 mg/kg (first dose/second dose). The second dose was successfully administered at all dose levels, except one rat in the 50/250 mg/kg dose group. Mortality was greater in rats given a first dose of 70 mg/kg (5 of 30) than in rats given a first dose of 50 mg/kg (1 of 30). All deaths occurred during or following the second dose administration and were considered procedural in nature, secondary to inadvertent perforation (3 of 5), or related to acute quinacrine toxicity due to leakage into the peritoneal cavity (2 of 5). From these data it was concluded that young sexually mature female rats could tolerate an initial dose of up to 70 mg/kg and a second dose of up to 350 mg/kg.

On the basis of practical considerations arising from tissue damage at the site of administration the top dose selection for the first administration was guided by the ability to dose animals a second time, as well as general signs of toxicity (mortality and body weight). A dose of 70 mg/kg reliably allowed administration of a second intrauterine dose, and was selected as the top dose level for the first dose in the lifetime cancer bioassay. A low-dose level of 10 mg/kg was selected to be equivalent to twice the human dose on a mg/kg basis.

For the second dose, the top dose level was limited by systemic tolerability, as well as by the desire to produce local tissue damage similar to the mechanism of action of quinacrine in humans. Based on the results of the dose range-finding studies, the top dose selected for the second dose, was 350 mg/kg. During the lifetime cancer bioassay itself, however, this dose produced unacceptable acute mortality and the dose was reduced to 250 mg/kg. A mid-dose level of 70 mg/kg was selected as a dose that produced no uterine closure; and 10 mg/kg was selected as the low-dose level.

The most common QS regimen in women is to give two doses of seven 36-mg pellets 28 days apart. Each quinacrine dose is a total of 252 mg, or approximately 4.2 mg/kg in a 60 kg woman. Since the average uterine weight in a woman is 95 g (Singh et al., 2004), and the estimated average rat uterine weight was 0.8 g (based on rat uterine weights of control animals in the range-finding study #1), the doses selected for the first dose in rats were approximately 1× and 8× the human dose on a mg quinacrine/g uterus basis, and the dose levels for the second dose in rats were approximately 1×, 8×, and 40× the human dose on a mg quinacrine/g uterus basis. Table 1 summarizes the final experimental design of the lifetime cancer bioassay.

2.5. Lifetime cancer bioassay

The lifetime cancer bioassay was designed in two phases: a dose-administration phase that mimicked the QS method used in women and a 2-year observation phase to evaluate tumorigenic potential.

2.5.1. Dose-administration phase

During the dose-administration phase, daily vaginal smears were performed and evaluated for each animal for at least 1 week prior to initiation of dosing and on each dosing occasion. A microscopic evaluation determined the predominant cell type (epithelial cell, cornified cells or leukocytes) and used to determine the stage of estrus, which was recorded.

Dosing was attempted in all 60 rats per group. In the event that a rat could not be given the second dose due to blockage of both uterine horns, the rat was removed from the study. If the rat had blockage of one uterine horn only, it was given the second administration and observed, but included in the study only when necessary to achieve 50 animals per group. Also, two control groups were included in the study because few historical control data were available on tumor incidence in rats dosed via intrauterine administration. One month after the second dose administration each group was culled to 50 rats for the 2-year observation phase. Table 1 summarizes the number of rats that entered the 2-year observation phase.

2.5.1.1. Morbidity, mortality, clinical signs, and physical examinations. During the dose-administration phase, each rat was observed for signs of toxicity twice daily on the day of dosing and for 5 days after each dose.

2.5.1.2. Body weight and food consumption. Non-fasted body weights were recorded twice before initial dosing and then weekly. Food consumption was measured for all animals during the week prior to treatment initiation and then weekly.

2.5.2. Observation phase

2.5.2.1. Morbidity, mortality, clinical signs, and physical examinations. Observations for mortality and general condition were made at least twice daily, in the morning and in the afternoon. Observations for general condition, palpable masses, skin and fur, eyes, nose, oral cavity, abdomen, mammary glands and external genitalia as well as evaluations of respiration were made twice pretest and weekly for the duration of the study.

2.5.2.2. Body weight and food consumption. Non-fasted body weights were recorded weekly for the first 16 weeks and every 4 weeks thereafter during the study. Terminal fasted body weights were obtained just prior to necropsy. Food consumption was measured for all rats weekly for the first 16 weeks and every 4 weeks thereafter.

2.5.3. Necropsy and postmortem observations

Rats found dead were necropsied as soon as possible after discovery to reduce loss of tissues through autolysis. Rats showing signs of severe debility were euthanized, exsanguinated and necropsied. Rats that survived to the scheduled terminal necropsy were fasted overnight, euthanized, exsanguinated, and necropsied.

The necropsy consisted of an external examination, including identification of all clinically recorded lesions, as well as detailed internal examination. All abnormal observations noted at necropsy were recorded. Organ weights were recorded for adrenal glands, brain, kidneys, liver, ovaries, uterus (body/horns) with cervix for all rats surviving to terminal necropsy. Tissue samples were

collected and fixed in 10% neutral buffered formalin or in modified Davidson fixative (eyes only).

2.5.4. Histopathologic evaluation

Fixed tissues were sampled, processed into paraffin wax, sectioned at 4–5 μm, stained with hematoxylin and eosin and then examined by light microscopy. For each uterine horn, 3 mm transverse sections were sampled from three levels: proximal (near the cervix), middle, and distal (near the oviduct). Remaining tissue from each horn was identified by horn and saved for possible future evaluation. The three sections from each horn were embedded in a single paraffin block with the anatomic location identified. The uterine body and cervix were sectioned longitudinally and both halves embedded in a single paraffin block. Any uterine or cervical macroscopic abnormalities noted during necropsy or during histological sampling were also sampled if present at a site other than that in the routine sample and examined microscopically. The uterine horns, body and cervix of every animal were specifically examined for dilation, fibrosis, acute inflammation and histiocyte infiltration, and these four findings were recorded using a 4 point severity grading system. Lumen closure was also scored as present or absent in every uterus. Additional lesions were recorded and graded for severity when present. Histopathologic observations for all tissues and organs were recorded directly into a computerized pathology data capture system (Xybion).

The investigators followed the "Best Practice Guidelines" for histopathologic evaluation of toxicity studies that are provided by the Society of Toxicologic Pathologists (Crissman et al., 2004). Tissue sections for a given animal were examined together, but the order in which animals were examined was randomized with respect to treatment group to minimize the possibility of diagnostic drift. The number of samples taken and examined from each uterine horn and uterine body was standardized across all animals. No additional samples were taken unless there was a gross abnormality noted at necropsy.

For rats that died or were euthanized early, the study pathologist reached a conclusion about the cause of death or morbidity based on clinical, macroscopic and histopathologic findings. If no cause of death or morbidity was apparent, then the cause was listed as "undetermined".

A formal peer review of the histopathological findings was conducted by an external, board-certified pathologist. All tissues from 10% of the animals in the control groups and 10% of animals in the high-dose groups (70/250 and 70/350 mg/kg) were reviewed, as were all neoplasms and proliferative lesions from animals in all groups. The final histopathologic data reflected the consensus view of the study pathologist and the peer-review pathologist.

2.5.5. Regulatory compliance statement

The study was conducted in compliance with GLP regulations and subjected to Quality Assurance audits. Study animals were housed and maintained according to IACUC regulations and the study design was approved by an IACUC Committee.

2.5.6. Statistical analyses

2.5.6.1. Mortality and tumor incidence. Mortality rates between groups were analyzed by logrank tests for trend across the groups. Tumor rates between the groups were analyzed using the method of Peto et al. (1980), which adjusted for time of death and cause of death. For statistical analysis purposes, the two control groups were combined for the analysis.

The statistical analysis for individual tumor types was performed when at least two animals with comparable tumors were found in the high-dose group or at least four animals with comparable tumors were found in the combined intermediate- and high-dose groups. The analysis was performed separately for the two groups of tumors classified as fatal or incidental and the results were combined using the method of Peto et al. (1980). The combined Peto one-sided test for trend of increasing tumor rates over dose levels as performed by SAS procedures was the primary statistical indicator for the incidence of tumors. A positive linear trend (dose–response relationship) was considered not to occur by chance variation alone if the *p*-value was less then 0.005 for a common tumor or 0.025 for an uncommon tumor (i.e., a tumor that occurs with an incidence of less than 1% based on data from concurrent and historical controls). In cases where the Peto test showed a significant trend, a pairwise analysis between the pooled control and individual treated groups was performed using the Peto one-sided test for individual group or the Fischer exact test. If this optional analysis was performed, no correction for multiple comparisons was done. The level of statistical significance for pairwise comparisons was a one-sided *p*-value of less than 0.01 for common tumors and 0.05 for uncommon tumors. Analyses were performed on the incidence of benign and malignant tumors and the combined incidence of certain benign and malignant tumors as recommended by McConnell et al. (1986). The number of tissues examined was used as the denominator to calculate tumor incidences and percentages.

2.5.6.2. Bodyweight, feed consumption, and organ weight data. Evaluation of equality of group means was made by the appropriate statistical method, followed by a multiple comparison test if needed. Bartlett's test (Snedecor and Cochran, 1967) was performed to determine if groups had equal variances. For all parameters except organ weights, if the variances were equal, parametric procedures were used; if not, non-parametric procedures were used. Organ weight data were analyzed only by parametric methods. The parametric method was the standard one-way analysis of variance (ANOVA) using the *F* ratio to assess significance (Dunlap and Duffy, 1975). If significant differences among the means were indicated, additional tests were used to determine which means were significantly different from the control: Dunnett's (Dunlap et al., 1981; Dunnett, 1955, 1964) or Cochran and Cox's modified *t*-test (Cochran and Cox, 1959). The non-parametric method was the Kruskal–Wallis test (Kurskal and Wallis, 1952, 1953) and if differences were indicated, pairwise comparison with Bonferroni correction (Games and Howell, 1976) was used to determine which means differed from control. Bartlett's test for equality of variance was conducted at the 1% significance level; all other statistical tests were conducted at the 5% and 1% significance levels.

3. Results

3.1. Mortality

3.1.1. Dose-administration phase

Differences in mortality among groups were observed only during the dose-administration phase of the study. Rats tolerated administration of the first dose. Administration of the second dose at 10 and 70 mg/kg was also tolerated, but mortality after the second dose at 350 mg/kg was greater than anticipated based on the previous range-finding studies; 12 of the first 33 rats given 350 mg/kg quinacrine died or were euthanized shortly after dosing. Based on this result, no further animals were given this dose and the remaining 27 rats in this group, along with eight reserve rats that had also received a first dose of 70 mg/kg, were combined to create a new group that received a second quinacrine dose of 250 mg/kg. Of these 35 rats, 11 died during the dose-administration phase.

Necropsy of rats that died revealed uterine perforation in one rat, obvious signs of yellow staining in the peritoneal cavity of

three rats and enteromegaly in 10 rats. Death associated with acute toxic effects of quinacrine or due to enteromegaly has been observed previously in animals dosed with quinacrine into the abdominal cavity (Ciaccio et al., 1978; Keeler et al., 1966).

3.1.2. Observation phase

Overall mortality rate among groups in the 2-year observation phase, analyzed by the logrank trend test, was not statistically significant ($p = 0.297$) (Table 2). The major causes of death were pituitary tumors and mammary/skin tumors, and the incidence was similar in control and quinacrine-treated animals. Tumors of the reproductive tract accounted for 11/63 (18%) of unscheduled deaths in rats treated with 70/70 and 70/250 or 350 mg/kg, compared to 1/37 (3%) and 2/73 (3%) in the 10/10 mg/kg and control groups, respectively.

3.2. Clinical signs

3.2.1. Dose-administration phase

During the dose-administration phase, the only clinical signs related to quinacrine administration were yellow anogenital staining due to leakage of the dosing solution (which is bright yellow in color) from the vagina and uterus, which was commonly observed at all dose levels, and reddish brown stains on the forelimbs and snout, which were more frequent in rats receiving a second dose of quinacrine $\geqslant$250 mg/kg.

3.2.2. Observation phase

During the 2-year observation phase, clinical signs and observations were similar to those seen and documented normally in laboratory rats in studies of this duration and generally occurred with similar incidence in control and quinacrine-treated groups.

3.3. Body weight and food consumption

Rats gained weight throughout the dose-administration phase of the study, but transient decreases in body weight gains and food consumption occurred after a second quinacrine dose at $\geqslant$250 mg/kg. As a consequence, body weight remained slightly lower in the high-dose group during the 2-year observation phase of the study.

3.4. Organ weight

Quinacrine did not affect mean organ weight at any dose level. Mean uterus weight was greater in rats given a second quinacrine dose at 250 mg/kg, but this was due solely to two rats that had severely enlarged uteri filled with abnormal contents.

3.5. Macroscopic findings

The only macroscopic findings related to quinacrine were in the uterus at the site of quinacrine administration. The only macroscopic findings considered to be related to quinacrine treatment in the group given two doses of 10 mg/kg were cysts (which were also present in the controls but at a lower incidence), and abscesses. Abscesses showed a dose-related increased incidence in all groups of quinacrine-treated rats. Microscopically, cysts correlated with either dilated/cystic endometrial glands, segmental dilation of the uterine lumen, and/or endometrial cystic degeneration (discussed in more detail below).

Several uterine findings were more common in rats given an initial dose of 70 mg/kg than in the control groups or in rats given two doses at 10 mg/kg. These included distension, enlargement, or thickening; thinning; abnormal contents (clear, red/black, brown/tan, yellow, green, white fluids); nodules, polyps, or masses; and adhesions. Uterine distension, enlargement, and/or thickening correlated with dilation of the uterine lumen, endometrial fibrosis and/or dilated/cystic endometrial glands. Abnormal uterine contents frequently correlated with intra-lumenal eosinophilic material admixed with inflammatory cells/cell debris.

Other macroscopic findings in the uterus and in other organs and tissues occurred with comparable incidence and severity in quinacrine-treated and control groups or they occurred sporadically. These incidental findings have been seen in untreated control rats of this strain and age used in other studies conducted in the testing facility and were considered unrelated to quinacrine.

3.6. Histopathologic neoplastic findings

The only neoplasms that showed a relationship to quinacrine administration were in the reproductive tract; i.e., uterus, cervix, and vagina (Table 3). Although the total number of tumors of the reproductive tract was similar between control and quinacrine-treated groups, the types of tumors were different. Benign

Table 2
Summary of mortality and probable cause of death for rats dying during the observation period.[a]

Dose Group[b] (mg/kg)	0/0	0/0	10/10	70/70	70/250	70/350
Total number of rats	50	50	50	50	24	21
No. of deaths prior to scheduled termination[a]	37	36	37	33	19	11
No. of survivors	13	14	13	17	5	10
Percent mortality	74	72	74	66	79	52
Probable Cause of Death[a]						
Pituitary gland: neoplasm	22 (44)	23 (46)	25 (50)	17 (34)	8 (33)	2 (10)
Mammary gland/skin: neoplasm	7 (14)	8 (16)	6 (12)	7 (14)	3 (13)	1 (5)
Reproductive tract: neoplasm	1 (2)	1 (2)	1 (2)	4 (8)	3 (13)	4 (19)
Uterus/cervix	1 (2)	0	1 (2)	3 (6)	2 (8)	4 (19)
Vagina	0	0	0	1 (2)	1 (4)	0
Ovary	0	1 (2)	0	0	0	0
Reproductive tract: other lesions	0	0	1 (2)	1 (2)	3 (13)	0
Other causes	6 (12)	2 (4)	2 (4)	3 (6)	2 (8)	3 (14)
Undetermined	1 (2)	2 (4)	2 (4)	1 (2)	0	1 (5)

[a] Data represent number of findings and percentages (presented in parentheses).
[b] Doses administered on Days 0 and 21 of study. Each animal was dosed with ½ dose per uterine horn.

Table 3
Number and percentages of neoplasms in the cervix, uterus, and vagina.[a]

Dose Group[b] (mg/kg)	0/0	0/0	10/10	70/70	70/250	70/350
Total number of rats	50	50	50	50	24	21
Cervix: number examined	49	49	50	49	23	21
Benign fibroma	0	1 (2)	1 (2)	0	0	0
Benign squamous cell papilloma	0	0	0	1 (2)	1 (4)	0
Malignant squamous cell carcinoma	0	0	0	0	1 (4)	2 (10)
Malignant basal-squamous cell carcinoma	0	0	0	0	1 (4)	0
Malignant Schwannoma	0	0	1 (2)	1 (2)	0	0
Uterus: number examined	50	50	50	50	24	21
Benign endometrial stromal polyp	5 (10)	9 (18)	4 (8)	1 (2)	2 (8)	1 (5)
Benign hemangioma	0	0	0	1 (2)	0	1 (5)
Benign granular cell tumor	0	0	0	1 (2)	0	0
Benign mixed mullerian tumor	0	0	0	1 (2)	0	0
Malignant endometrial carcinoma	0	0	0	1 (2)	0	2 (10)
Malignant carcinosarcoma	0	0	0	0	1 (4)	0
Malignant stromal sarcoma	0	0	0	1 (2)	1 (4)	0
Malignant squamous cell carcinoma	0	0	0	1 (2)	0	0
Malignant yolk sac carcinoma	0	0	0	1 (2)	0	0
Malignant leiomyosarcoma	0	0	1 (2)	0	0	1 (5)
Malignant Schwannoma	1 (2)	0	0	0	0	2 (10)
Malignant hemangiosarcoma	0	0	0	0	0	1 (5)
Vagina: number examined	50	49	48	49	24	18
Malignant squamous cell carcinoma	0	0	0	1 (2)	4 (17)	0
Total uncommon neoplasms[c]						
Number of tumors	1	1	3	10[*]	9[*]	9[*]
No. of tumor/no. of tissues examined (%)	0.7	0.7	2	6.8	13	15

[a] Data represent number of findings and percentages (presented in parentheses).
[b] Doses administered on Days 0 and 21 of study. Each animal was dosed with ½ dose per uterine horn.
[c] Any neoplasm with an incidence below 1% based on the historical control data of the performing laboratory. (Excludes endometrial stromal polyps, which are common in rats.)
[*] Statistically significant, $p < 0.001$.

endometrial stromal polyp of the uterus, which is a common incidental tumor for this strain of rat, was the main tumor type present in the control groups, with an incidence of 10% and 18% in the two control groups. The only other tumors present in the reproductive tract of control animals were a single benign fibroma of the cervix and a single malignant Schwannoma of the uterus. In contrast, rats that had been dosed with quinacrine at doses of ⩾70/70 mg/kg had reduced numbers of benign endometrial stromal polyps but increased number of tumors considered uncommon, in the reproductive tract of rats. These included malignant squamous cell carcinomas of the cervix, uterus and vagina, malignant yolk sac carcinoma, malignant carcinosarcoma and benign Mullerian gland tumor of the uterus, as well as a variety of other benign and malignant tumors that are generally considered uncommon (tumors with less than 1% historical background incidence). While the incidence of each individual type of tumor was low, collectively, they occurred more frequently in quinacrine-treated groups, and the incidence increased with dose levels at ⩾70/70 mg/kg. The overall incidence of these "uncommon tumors" (i.e., summation of all tumors excluding benign endometrial stromal polyps) was 0.7% among the controls, 2% at 10/10 mg/kg, 6.8% at 70/70 mg/kg, 13% at 70/250 and 15% at 70/350 mg/kg. The incidence of uncommon tumors in the recent historical control database from the laboratory conducting the studies (Huntingdon Life Sciences) was 2% (15/672) in this strain of rat. Thus, the incidence and types of tumors seen in the reproductive tissues of the 10/10 mg/kg group (2%) were considered within normal range.

The incidence of tumors in all other organs and tissues was similar in control and quinacrine-treated groups and the tumor profile was typical for this strain of rat. The total number of primary tumors (benign and malignant) and the numbers of animals with one or more tumors were also similar between groups (Table 4).

3.7. Histopathologic non-neoplastic findings

The only non-neoplastic findings considered related to administration of quinacrine were present in the reproductive tract, i.e., uterus, cervix, and vagina (Table 5).

Findings that occurred only in quinacrine-treated groups were edema (slight to moderate), necrosis (slight to marked), cystic degenerative change (minimal to moderate), and myometrial hypertrophy (slight to moderate) (Fig. 1). Overall, the incidences and/or severities of these findings were greater at ⩾70/70 mg/kg than at 10/10 mg/kg, suggesting a dose-related trend. Cystic degenerative change was one of the most prominent and consistent changes seen in quinacrine-treated uterine horns. It was characterized by a fine meshwork of histiocytic like cells replacing the normal endometrial lining of the uterus.

In addition there were a number of findings that showed an increased incidence and/or severity in rats administered ⩾70/70 mg/kg compared with controls or with rats administered 10/10 mg/kg. These included vascular congestion (minimal to moderate), hemorrhage (minimal to moderate), and acute-to-chronic inflammation (minimal to marked). A small number of these rats also had endometrial erosions/ulcers (minimal to marked).

Table 4
Overall incidence of neoplasms in all tissues.

Dose Group[a] (mg/kg)	0/0	0/0	10/10	70/70	70/250	70/350
Total number of rats	50	50	50	50	24	21
Total primary neoplasms	115	115	91	97	58	52
Animals with one or more	49	48	44	47	22	18
Percent with one or more	98	96	88	94	92	86

[a] Doses administered on Days 0 and 21 of study. Each animal was dosed with ½ dose per uterine horn.

Table 5
Percentages of non-neoplastic histopathologic findings in the uterus, cervix, and vagina, by group.

Dose Group[a] (mg/kg)	0/0	0/0	10/10	70/70	70/250	70/350
Total number of rats	50	50	50	50	24	21
Vagina: number examined	50	49	48	49	24	18
Congestion	8	4	8	10	17	6
Distended	10	4	2	14	17	17
Inflammation, chronic active/ chronic	0	4	6	14	29	6
Necrosis	0	0	0	2	8	0
Erosions/ulcers	0	0	0	0	9	6
Inflammation, acute/subacute	0	2	0	0	4	6
Cervix: number examined	49	49	50	49	23	21
Inflammation, chronic active/ chronic	8	6	4	18	43	19
Atrophy	2	2	2	0	9	0
Necrosis	0	0	2	0	4	0
Edema	0	0	2	0	0	0
Inflammation, acute/subacute	6	2	2	0	4	0
Uterus: number examined	50	50	50	50	24	21
Lumen dilated	62	72	76	78	79	81
Atrophy	10	14	26	60	71	81
Hemorrhages	0	2	4	6	8	10
Necrosis	0	0	4	12	8	14
Hypertrophy, myometrial	0	0	2	4	4	10
Congestion	20	18	20	32	38	38
Edema	0	0	0	2	4	0
Cystic degeneration, endometrial	0	0	0	36	29	43
Erosions/ulcers	2	2	2	8	13	5
Inflammation, acute/subacute	2	2	2	10	13	10
Inflammation, chronic active/ chronic	16	12	10	42	58	29

[a] Doses administered on Days 0 and 21 of study. Each animal was dosed with ½ dose per uterine horn.

Other findings in the reproductive tissues were typical of those seen as common age-related background changes and were present at similar incidences in control and quinacrine-treated animals.

4. Discussion

The choice of dose levels is one of the most important decisions to be made for a 2-year carcinogenicity study. Our approach to selecting dose levels was aligned with the recent ICH Guidance for Industry S1C(R2) that recommends a flexible approach to dose level selection. We intentionally chose to evaluate quinacrine in the uterine horn of rats at dose levels that produced fibrosis, as observed in women (Laufe et al. 1996; Merchant et al., 1995). This could be interpreted as a deviation from one of the S1C(R2) guiding principles that states that the dose level chosen "is tolerated without significant chronic physiological dysfunction and is compatible with good survival". In these regards, ICH S1C(R2) purposely links chronic physiological dysfunction and survival because an adverse effect on survival is a sensitive indicator of chronic physiological dysfunction. Since survival over the 2-year observation period was not affected and was similar among groups, we conclude that quinacrine did not produce significant chronic physiological dysfunction. The fact that quinacrine at the high-dose level (70/350 mg/kg) resulted in high mortality during the dose-administration phase is irrelevant to the interpretation of the study, as these are well known acute toxic effects of quinacrine (Ciaccio et al., 1978; Keeler et al., 1966), without much influence on animal survival during the observation period of the bioassay.

Based on the results of this study, we conclude that two doses of quinacrine administered approximately 21 days apart into the uterus of young sexually mature rats affected the incidence of reproductive tract tumors over the lifetime of the rats. Specifically, quinacrine reduced the incidence of benign endometrial stromal polyps and increased the incidence of different types of uncommon tumors in a dose-related manner. While the incidence of most of the types of uncommon tumors was low, collectively, they occurred more frequently in groups given an initial quinacrine dose of 70 mg/kg than in control rats or rats given an initial quinacrine dose of 10 mg/kg.

The variety of tumor types that arose after two doses of quinacrine in the uterus might have come from a variety of non-specific mechanisms. Confounding factors include: (1) the high local concentration and long half life of quinacrine in the reproductive tract tissues and (2) the chronic tissue inflammation with its potential to promote and/or potentiate neoplastic risk (Coussens and Werb, 2002).

The possible effect of quinacrine-related local toxicity arising as a result of the high local concentration in the rat uterine horn cannot be discounted. The rat uterine horn wall is thinner than the human uterine wall and the high local quinacrine concentration could result in more extensive exposure to multiple somatic cells and stem cells, which coupled with a direct quinacrine genotoxic effect, could explain the increase in uncommon tumors observed.

The increased tumor development could also be attributed to the physiological or pathological perturbations underlying the non-neoplastic findings that might have made the rats more sensitive to tumor development; for example, by generation of free radicals as part of the chronic inflammation or by enhanced cell proliferation associated with healing and repair of tissue damage, which would in turn increase the chances that DNA damage would become fixed as a mutation (Coussens and Werb, 2002; Kundu and Surh, 2008). In this situation, the increased tumor incidence could have been secondary to the quinacrine-related tissue injury and repair, rather than being a direct effect of quinacrine itself. If so, then the increased tumor incidence in rats would be irrelevant in situations where the same changes did not occur. Even if this were the case, however, it does not make the results of the current study irrelevant for women because, in the QS method, quinacrine is administered to women at doses intended to produce local toxicity very similar to that produced in the reproductive tract of rats in the carcinogenicity study. Consequently, it is not necessary to distinguish whether the quinacrine-related increase in tumor incidence in rats was a direct effect of quinacrine, secondary to quinacrine-related local toxicity, or (as seems most likely) a combination of both direct and secondary effects, in order to interpret the results of the study.

Regardless of the mechanisms of action, direct DNA damage as a consequence of quinacrine's genotoxic effect resulting in mutations is a mechanism that cannot be excluded. The fact that only two doses of quinacrine early in life resulted in a lifetime increase in tumor incidence suggests a genotoxic mechanism of action.

The results of this lifetime cancer bioassay in rats identify a potential risk of increased incidence of reproductive tract tumors once quinacrine is administered directly into the uterus. Epidemiologic studies of women that have undergone quinacrine sterilization failed to detect an increase in gynecological cancer incidence (Sokal et al., 2008b, in press-a); and a more recent case-control study of quinacrine sterilization and gynecologic cancers failed to detect an association between quinacrine use and uterine cancer incidence (Sokal et al., in press-b). While these studies have certain limitations, the case-control study results suggest that the incidence of uterine cancers is not increased by exposure to intrauterine quinacrine. A careful risk assessment needs to be completed to make an adequate determination of

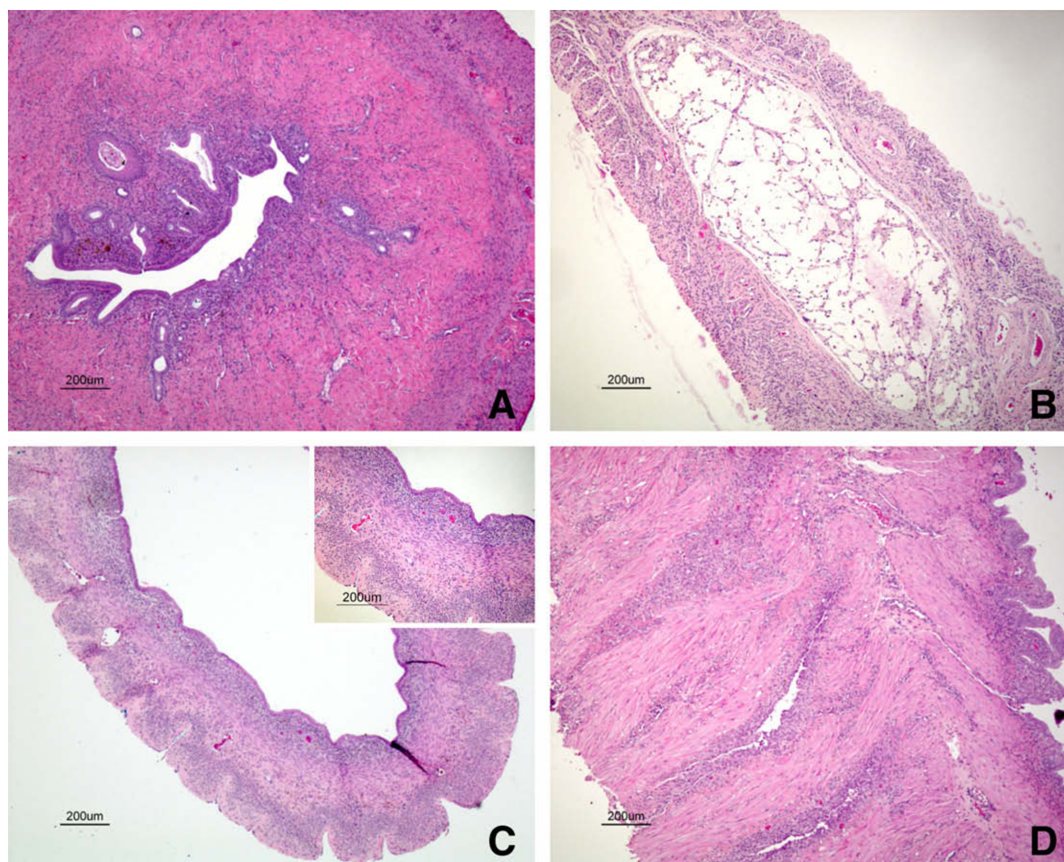

Fig. 1. Examples of common non-neoplastic changes in the uterus of rats administered quinacrine. (a) Representative uterus from a control rat showing endometrium with slight stromal fibrosis and slight focal squamous metaplasia of an endometrial gland; (b) uterus from a rat dosed with 70/70 mg/kg quinacrine showing moderate cystic degeneration of the endometrium; (c) uterus from a rat dosed with 70/70 mg/kg quinacrine showing marked luminal dilation, moderate atrophy and moderate subacute/chronic inflammation of the endometrium (inset shows detail of inflammatory infiltrate); and (d) uterus from a rat dosed with 70/70 mg/kg quinacrine showing moderate myometrial hypertrophy.

the relevance of the results of this lifetime cancer bioassay of quinacrine to women.

5. Conclusions

We conclude that two doses of quinacrine administered approximately 21 days apart into the uterus of young sexually mature rats at dose levels $\geqslant$70 mg/kg increased the lifetime risk of tumor development in the reproductive tract. The types of tumors that developed were mostly uncommon for this strain of rat. The incidence of these tumors was dose-related and was significantly increased at a local quinacrine dose that was a small multiple (8$\times$ based on a mg quinacrine/g uterus basis) of the human dose of quinacrine used for non-surgical female sterilization.

Conflict of interest statement for authors

This study was sponsored by Family Health International (FHI) and was monitored by FHI staff. The authors alone are responsible for the content and writing of the paper and the views expressed in this publication are not necessarily those of FHI or the other organizations mentioned above. The results described in this publication were presented and discussed at a WHO Technical Consultation titled "The Safety of Quinacrine for Non-Surgical Sterilization in Women," Geneva, October 2008. Some of the authors in this publication are involved in research programs to develop other methods of female non-surgical sterilization.

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