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An alternative interpretation of, "A lifetime cancer bioassay of quinacrine administered into the uterine horns of female rats"

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ABSTRACT

This companion article offers an alternative interpretation for the quinacrine-induced uterine tumors observed in a 2-year bioassay in rats (CaBio, Cancel et al., 2010), and provides additional data from two new experiments that support a different interpretation and analysis. Our major premise is that the design of the Cancel et al. bioassay was flawed, particularly regarding dose selection that allowed for misinterpretation of carcinogenic activity. We feel the totality of the information provided herein dictates that the doses (70/70, 70/250 and 70/350 mg/kg quinacrine) causing uterine tumors in their study clearly exceeded the maximum tolerated dose (MTD) typically administered in chronic cancer studies. Our new data support this conclusion and serve to explain the development of lesions, especially the uterine tumors, they have reported. We argue that the rat uterus is not a valid surrogate for the human fallopian tube. Further, we maintain that quinacrine is not genotoxic *in vivo*, as suggested in their paper. In summary, we believe that quinacrine is not carcinogenic in rats at doses that do not exceed the MTD.

1. Introduction

The purpose of this paper is to offer an alternative interpretation of "A Lifetime Cancer Bioassay of Quinacrine Administered into the Uterine Horns of Female Rats" (Cancel et al., 2010) and provide new data to buttress this opinion. Cancel et al. (2010) report that two doses of quinacrine in methylcellulose (MC), introduced into the uterine horn of rats approximately 21 days apart, resulted in the development of uterine tumors after 2 years at doses of 70/70 and 70/250–350 mg/kg quinacrine, but not at 10/10 mg/kg. We believe that the exposures causing the uterine tumors in their research exceeded the maximum tolerated dose. Our opinion is supported by additional studies and commentaries presented herein.

One of the most important issues in the design of a 2-year rodent bioassay for determining the carcinogenic potential of a chemical is the selection of the doses to be used in the study. A fundamental tenet in this process is to use a dose that meets, but does not excessively exceed, what is accepted, understood and referred to as the maximum tolerated dose (MTD). This is so critical because, when exceeded, the MTD can result in marked tissue and cellular destruction that lead to neoplastic cell growth, therefore confounding the interpretation of the carcinogenic potential of the xenobiotic in the test species and humans.

2. Background

In January of 2007, the 2-year rat carcinogenicity study of quinacrine conducted by Family Health International (FHI), as reported by Cancel et al. (2010), came to our attention. The reported tumors were unusual and rare, including primitive types of both epithelial (mixed Mullerian tumor, carcinosarcoma, squamous cell carcinoma and yolk sac carcinoma) and mesenchymal (granular cell tumor, hemangioma and hemangiosarcoma) origin. These are unexpected and rare observations in a standard 2-year cancer bioassay (CaBio) of a chemical. Another unusual feature is that only a single example of each of these rare tumors was found in the study. In a typical CaBio, an increased incidence of tumors that normally occur from a uterine carcinogen include endometrial adenomas and carcinomas, endometrial stromal polyps and sarcomas, leiomyomas and leiomyosarcomas and schwannomas (Leininger and Jokinen, 1990). In our opinion, the occurrence of the unusual tumors observed in Cancel et al. (2010) would likely result from se-

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vere cell damage to the endometrium and myometrium in which only a few stem cells survived.

In order to reconstruct what may have happened in the rat uterus as a result of exposure to quinacrine, we asked and received permission from FHI to examine the histopathologic slides from a prechronic multidose study (Cancel et al., 2010) involving the same 2-dose procedure employed in the 2-year study and used to help establish doses for the CaBio.

Briefly, FHI's range-finding study #2 (Cancel et al., 2010) used quinacrine doses of 0.7/0.7, 7/7, 14/14, 35/35 and 70/70 mg/kg. The rats were exposed twice (21 days apart) with quinacrine in an MC slurry via transcervical instillation, and were sacrificed 21 days after the second exposure with no interim sacrifices. For example, the 70/70 mg/kg dose represents a dose of 35 mg/kg into each uterine horn and a second dose of 35 mg/kg into each uterine horn 21 days later. Examination of the histopathologic slides from this study by one of us (EEM) confirmed the severe pathology reported by Cancel et al. (2010) at doses of 14/14 mg/kg quinacrine and above, and the absence of lesions at 7/7 mg/kg or 0.7/0.7 mg/kg quinacrine. Moreover, in addition to uterine dilation reported by them, there was chronic purulent inflammation in several animals and complete occlusion of one horn in one rat exposed to 14/14 mg/kg quinacrine. It is probable that other uterine horns were also obstructed because of the high incidence of dilation, but the tissue sampling approach used in the study (described by Fail et al., 2000) would have precluded finding most of the occlusions because they selected three consistent cross-sections (proximal, middle and near the cervix) with no attempt at opening the remaining uterus in search of lesions.

With the above information in mind, two studies were conducted under the auspices of the International Services Assistance Fund (ISAF). The first was designed in an attempt to explain the dilation of the uterine horns. The second, a multidose study, was initiated to better define the early lesions and establish whether the dose levels met or exceeded the definition of MTD and, thus, might not be relevant to the human experience.

3. ISAF study of the effect of ligating a single uterine horn in the rat

To understand the marked uterine dilation noted in the FHI prechronic study, a hypothesis was developed: Could occlusion of a uterine horn, in and of itself, result in dilation and observed pathology as a result of mechanical pressure from the obstruction? To test this hypothesis, one of us (JL) conducted a study at the University of Buffalo, Buffalo, NY. A brief discussion of this research follows.

3.1. Study protocol

The following study was conducted in accordance with the Animal Welfare Act and was approved by the Institutional Animal Care Use Committee. Ten female Sprague Dawley rats, at 10 weeks of age and each weighing about 250 g, were selected for this investigation. Before surgery, the rats were housed in multi-cage units and were fed Harlan 2018 rat diet. Prior to surgery, incision(s) site(s) were shaved, scrubbed with antiseptic soap, wiped with alcohol, and followed by antiseptic paint.

Bupremorphine was injected for analgesia subcutaneously before anesthesia and then post-operatively as oral tablets on the next day at 0.05 mg/kg, and as needed thereafter. Rats were anesthetized with isofluorine, 4% for induction and 2% for maintenance. At 10-min intervals, corneal reflexes, positive toe pinch and the color of mucous membranes were assessed to be sure that the animals were pain free. A midline incision was made to expose the reproductive organs. The left uterine horn was ligated at both the cervical end and the utero-oviductal junction. Great care was taken to place needles with 4–0 Vicryl close to the serosa, avoiding any diminution of the blood supply to the rat uterine horn, as described previously by Lippes et al. (1972). The abdominal incision was closed in anatomical layers using interrupted sutures of 4–0 Vicryl. The skin was closed with a subcuticular running stitch of 4–0 Vicryl. Time elapsed for this surgery was approximately 1 h.

Following surgery, rats were housed individually in open-top polycarbonate caging with aspen bedding. After 21 days, animals were euthanized in a carbon dioxide chamber. Death was assured by cutting the chest open. The uteri were photographed *in situ*, removed, and placed unopened in 10% neutral buffered formalin. A single (5 μm) cross-section from the mid-portion of each uterine horn was made and stained with hematoxylin and eosin. The sections were examined microscopically by one of us (EEM) to determine the extent of damage, if any, from the ligation. The unligated horn served as the control.

3.2. Results

One rat died early in the study (day 7). The ligated horns of all of the surviving rats showed marked dilation (Fig. 1). Most of the uterine glands were not present due to pressure atrophy. Interestingly, the bursa of the ovary was also dilated (Fig. 2). No uterine lesions, especially purulent inflammatory material as seen in the Cancel et al. (2010), were observed in these rats.

3.3. Interpretation

This research demonstrates that ligation (occlusion) alone can result in uterine horn dilation of the severity that was also observed in the CaBio's dose range-finding study #2 and in Fail et al. (2000). And, that such dilation can occur within 21 days of the ligation. But we emphasize there was no evidence of uterine inflammation or cell damage other than atrophy in any of the animals whose uterine horns were ligated.

We believe the explanation for this relates to the anatomy of the rat reproductive tract. Unlike the fimbria of the human fallopian tube which is open to the ovary, and indirectly to the abdominal cavity, the rat ovary is completely encased in a bursa that is an extension of the fallopian tube. Therefore, increased pressure from occlusion would not be released into the abdominal cavity as

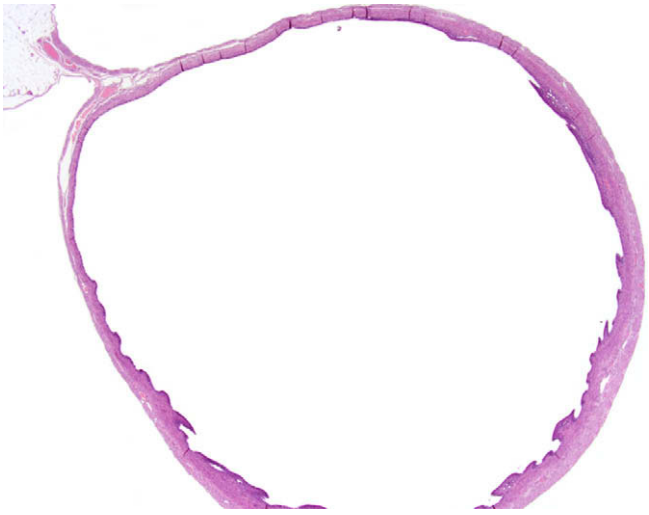

Fig. 1. Marked dilation of a ligated uterine horn. H&E stain, Original magnification 20×.

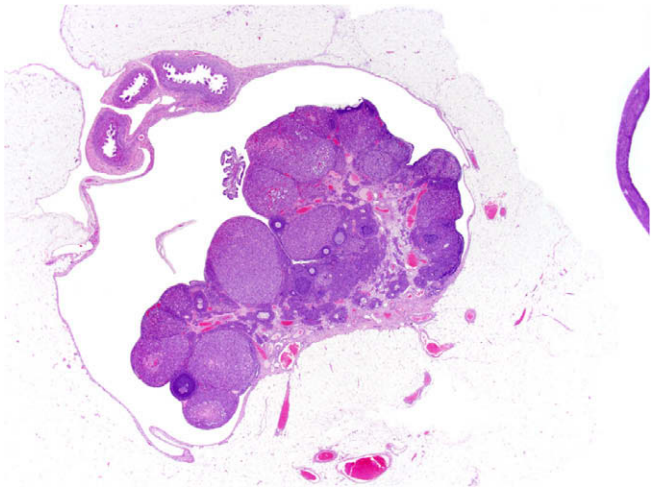

Fig. 2. Dilated ovarian bursa. Original magnification 20×.

would occur in women. The complete encapsulation of the ovary is clearly demonstrated by the dilated bursa in Fig. 2.

It is doubtful if dilation alone can result in tumor formation in a 2-year carcinogenesis bioassay. However, it is known that chronic inflammation can result in tumors in various organs in animals and humans (Kundu and Surh, 2008; Matés et al., 2008). The inflammation seen in the FHI prechronic dose range-finding study #2 (2010) likely resulted from a direct response to quinacrine-induced necrosis, while in this ligation study there was no evidence for necrosis, but rather pressure atrophy.

4. ISAF four-day study of quinacrine in rats

The purpose of this study was to define the early sequential changes in: (1) uterine histopathology; (2) plasma quinacrine concentrations; and (3) presence of residual quinacrine in intrauterine lumen fluids following quinacrine exposure with doses from 10 to 250 mg/kg in female rats. Until this investigation, there were no histologic studies pertaining to the uterus immediately following treatment with quinacrine. The plasma quinacrine concentrations and residual quinacrine findings will be presented in a subsequent publication because the data are not available at this time.

4.1. Materials and methods

Virgin female Sprague–Dawley rats (>66 days of age) were individually housed in stainless-steel wire-bottomed cages with *ad libitum* access to Certified Rodent Diet® #5002 meal (PMI Nutrition International, Inc., St. Louis, MO) and chlorinated deionized water. Groups of 9 or 10 rats were randomly assigned to exposure groups of either 10, 20, 70, or 250 mg/kg quinacrine suspended in 1% MC (4000 centipoise, Spectrum Chemical, New Brunswick, NJ, USA), 1% MC alone, or 0.9% saline alone. Although not employed in dosing humans, methylcellulose was used to mimic the conditions in the Cancel et al. (2010) study. The quinacrine slurry was administered transcervically, half into each uterine horn at a volume of 0.2 mL/horn, during diestrus.

Dosages in the 4-day study were selected on the basis of data from the prechronic and chronic studies reported in this issue by Cancel et al. The highest dose we used was 250 mg/kg quinacrine, chosen because it was the same as the one in the CaBio that caused mortality. The next lowest dose (70 mg/kg quinacrine) was selected because it caused tumors in the CaBio without mortality. The lowest dose (10 mg/kg quinacrine) was chosen because it was the one in the CaBio that did not cause uterine tumors. The 20 mg/kg quinacrine dose was included to better establish a clear dose response curve. Five additional rats were added at 250 mg/kg, because of unexpected mortality of four rats within 1 h after treatment.

Three rats per treatment group were sacrificed at 6-h, 24-h and 96-h post-exposure by carbon dioxide asphyxiation. Necropsies were performed to determine the early changes that occur after quinacrine exposure and time-course events. The uteri were photographed and weighed. The uterine horns were opened longitudinally. Approximately half of the right uterine horn was saved for possible future evaluation. The left horn and one-half of the right horn were pinned (mucosal side up) flat on an index card and fixed in 10% neutral buffered formalin for histologic evaluation. The formalin fixed tissues were submitted to Experimental Pathology Laboratories, Research Triangle Park, NC, where a single 4–5 μm longitudinal histopathologic section of the entire length of the uterine horns was prepared and stained with hematoxylin and eosin (H&E). The sections were examined by one of us (EEM) in a semi-blind manner, i.e. without knowledge of the dosage group, to determine the extent of damage, if any. Lesions were recorded using a severity scale of 0–4 (0 = normal, 1 = minimal, 2 = mild, 3 = moderate and 4 = severe pathology). The lesions were in turn reviewed by another veterinary pathologist (JS – see acknowledgment).

4.2. Results

Four animals in the high dose group died within an hour after exposure to 250 mg/kg quinacrine. No clinical signs were observed in the remaining animals in this group or any in the other dose groups. Histopathologic examination of the H&E stained slides showed various dose- and time-related pathological changes in the endometrium and at times in the myometrium. These changes consisted primarily of necrosis, inflammation and hemorrhage, both in the epithelium and in some cases in subjacent lamina propria stroma (Table 1). Stromal edema was striking in some animals and was correlated with the degree of necrosis. Of note was the fact that in some animals the lesions were of equal severity throughout the length of the horn, while in others it was more focal. While no lesions were observed in any animals in the saline control, minimal changes in the epithelium and glandular portion of the endometrium were observed in a few animals exposed to methylcellulose alone.

4.2.1. Uterine pathology in the 250 mg/kg quinacrine dose group

The uteri of rats that died after exposure to 250 mg/kg quinacrine showed mild to severe necrosis of the endometrium. The uterine lesions in the rats that survived at this dose were more severe than in those that died. At 6-h there was no viable epithelium, but there was superficial inflammation (primarily neutrophils and lymphocytes) and moderate hemorrhage. Diffuse necrosis and severe stromal edema of the lamina propria were observed to the level of the myometrium, i.e. no viable tissue was seen in the endometrium. *Note:* for the purpose of this study we used the term "ulceration" when no viable tissue was found to the level of the myometrium, and recorded whether the ulcers were focal or diffuse. Focal ulcers were macroscopically $\leqslant$ 2 mm diameter, while diffuse ulcers often were a centimeter or more in length and multifocal. Complete epithelial necrosis was still present at 24-h, but accompanied by more intense inflammation. By 96-h, there was significant, but not total, repair in the epithelium while inflammation was less apparent, and there was no evidence of hemorrhage. The glandular portion also showed some degree of repair. Diffuse ulceration was found in 3/3 rats at 6-h, 1/4 at 24-h and 1/3 at 96-h.

Table 1
Histologic findings from female rats exposed to quinacrine: average injury severity per dose group by time exposure, three animals per exposure group/dose.

Dose	h	Epithelium			Endometrium				
		Necrosis	Inflam.	Hem.	Necrosis	Inflam.	Hem.	Edema	Ulcer
Control	6	0	0	0	0	0	0	0	0
	24	0	0	0	0	0	0	0	0
	96	0	0	0	0	0	0	0	0
MC	6	.3	0	1	0	0	0	.3	0
	24	0	0	0	.7	0	0	0	0
	96	.7	0	.7	.7	0	0	.7	0
10 mg	6	3.3	2	2.7	1.7	.7	.7	3	0
	24	3	2.3	.7	1.7	1.7	0	2.7	2 – F
	96	.7	2	0	0	1	0	1.3	0
20 mg	6	2	0	1	1.7	0	1.3	2.7	2 – F, 1 – D
	24	2.3	2.7	.7	.3	1.3	.7	1.7	1 – F
	96	.7	1.3	.3	0	1.3	0	2	0
70 mg	6	3	2.3	1.3	2	2	2	2.3	3 – D
	24	3	3	.7	1	1.7	.7	2.3	1 – F
	96	.3	2.3	.7	.7	1.7	0	.7	0
250 mg	FD	2.3	0	2.3	3	0	3.3	2.3	1 – F, 2 – D
	6	4	.7	2.7	3.3	2	2	3.7	3 – D
	24	3.8	2.3	2	2	1.5	2	2.5	1 – D
	96	1.3	1	.7	2	1	1	1.7	1 – D

F = focal, D = diffuse, FD = found dead.

4.2.2. Uterine pathology in the 70 mg/kg quinacrine dose group

Mild to severe necrosis was observed in the epithelium in rats exposed to 70 mg/kg quinacrine group at 6-h. A minimal to mild amount of inflammation and hemorrhage were also present. Mild necrosis and edema in the lamina propria were observed to the level of the myometrium (Figs. 3 and 4). All three rats had diffuse ulcer development (Fig. 4). At 24-h, the epithelial necrosis was still complete in 2/3 rats, but the necrosis in the subjacent tissues appeared to be slightly less severe. One rat had a focal ulcer. At 96-h, there was evidence of significant epithelial and endometrial repair although inflammation was still apparent in both tissues.

4.2.3. Uterine pathology in the 20 mg/kg quinacrine dose group

At 6-h, mild necrosis of the epithelium and subjacent tissues was observed and was accompanied by a moderate amount of inflammation, edema and some evidence of hemorrhage. Again, necrosis was present to the level of the myometrium and ulcers were noted in all three rats, but only one was diffuse (Fig. 5). At 24-h, the severity of the necrosis of the epithelium was similar to that at 6-h but had decreased in the lamina propria, although the severity of inflammation had increased in both tissues. One rat is this group had a focal ulcer. At 96-h, there was no evidence of necrosis in the lamina propria and only a minimal amount in the epithelium. In some animals, it was nearly normal (Fig. 6).

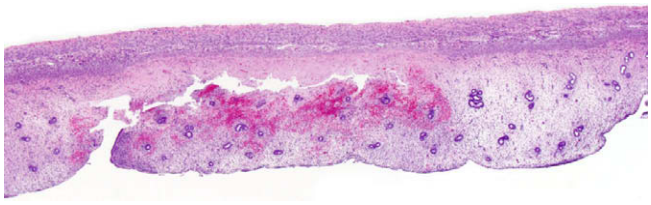

Fig. 4. Full thickness necrosis and edema of the endometrium and incipient ulcer at 6 h. 70 mg/kg. Original magnification 32×.

4.2.4. Uterine pathology in the 10 mg/kg quinacrine dose group

At 6-h, there was mild to severe necrosis of the epithelium, a mild to moderate amount of inflammation and a mild to moderate amount of hemorrhage. There was mild necrosis in the lamina propria, primarily limited to the endometrial glands. A moderate

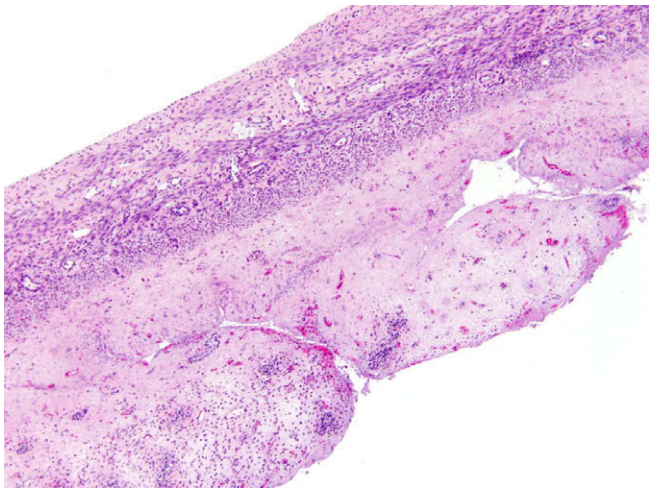

Fig. 3. Full thickness necrosis and edema of the endometrium at 6 h. 70 mg/kg. Original magnification 80×.

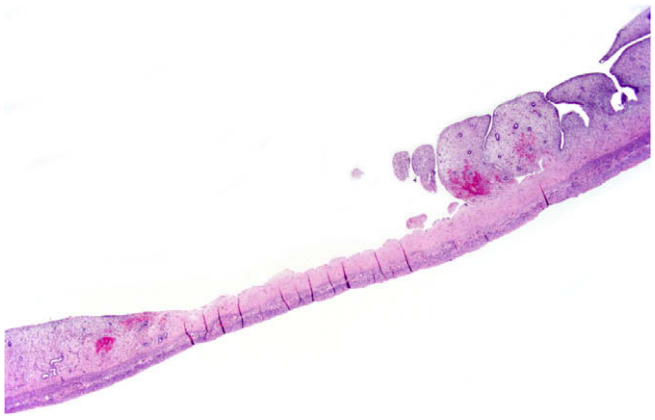

Fig. 5. Ulcer and full thickness necrosis of the endometrium attended by a moderate amount of inflammation, edema and some evidence of hemorrhage at 6 h. 20 mg/kg. Original magnification 20×.

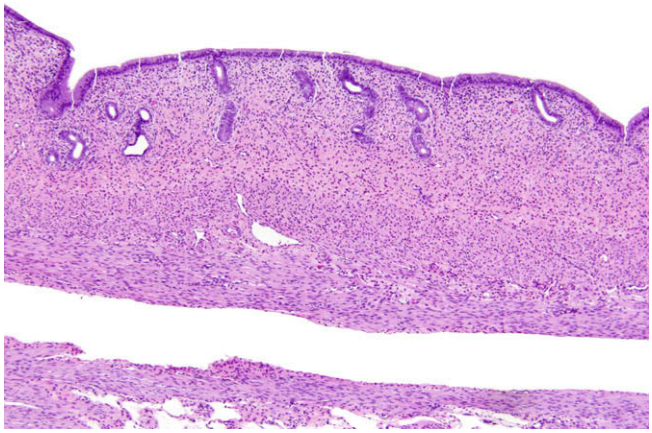

Fig. 6. Almost complete regeneration. 96 h. Original magnification 40×.

amount of stromal edema was also observed. The lesions at 24-h were similar to those found at 6-h (Fig. 7), although 2 of the 3 rats had focal ulcers. At 96-h, almost total repair to the epithelium was observed, although the severity of inflammation in the epithelium was still moderate.

4.2.5. Interpretation

The results of this study show dose- and time-related lesions in the endometrium of rats exposed to quinacrine and a negative association with inflammation (i.e., as the dose decreased the degree of inflammation increased). The major difference in dose response was the incidence and severity of ulceration. While ulceration was found in 2/9 rats at 10 mg/kg and 4/9 at 20 mg/kg, only one rat (20 mg/kg) had diffuse involvement. Again, 4/9 rats at 70 mg/kg showed ulceration, but in 3/4 the ulcers were diffuse. Five of 10 rats at 250 mg/kg showed ulceration and all were diffuse. The presence of ulceration is particularly important because it probably explains the mode of action for uterine occlusions as discussed below. In addition, it is generally believed that the mechanism of action responsible for quinacrine sterilization in humans is initiated via an acute (self-limiting) inflammatory response followed by remodeling and development of a fibrotic scar limited to the uterotubal junction (Lu et al., 2003).

5. Discussion

The quinacrine pellet method of birth control, termed quinacrine sterilization (QS), was developed by Zipper in Chile in 1977 (Zipper et al., 1980) and has to this date been used by more than 100,000 women in many countries (Kessel, 1997). The method requires two transcervical insertions into the uterus (2 doses of 252 mg/dose) of 7 pellets of quinacrine, one month apart, by means of a device similar to an IUD inserter. With a serious complication rate of only 1/50th that of surgical tubal ligation (Lippes, 2002), QS is much safer. Zipper et al. (1980) method placed the pellets in a straight line down the middle of the uterus, beginning at the fundus. The insertion technique described by Hieu et al. (1993), placed all of the pellets at the fundus, improving the efficacy rate to 98% at 4 years (Bilgrami and Shah, 2003). Furthermore, QS is more acceptable to women than surgical techniques (Hieu et al., 2003; Randic, 2000), can be delivered by trained non-physician clinicians as an outpatient service (Hieu et al., 1993), and is inexpensive, estimated to cost $0.53/dose when mass produced (Presource Technologies, Inc., Monmouth Junction, NJ).

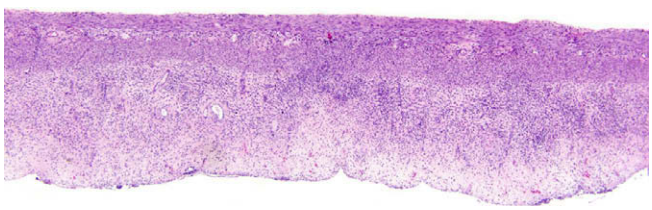

Fig. 7. Complete necrosis of the endometrial epithelium attended by mild to moderate inflammation and a minimal amount of hemorrhage, and a mild amount of necrosis in the lamina propria. 24 h. 10 mg/kg. Original magnification 40×.

Short-term complications and side effects following intrauterine quinacrine insertion have been studied in numerous countries (Agoestina and Kusuma, 1992; Ferreira et al., 2003; Sarin, 1999; Soroodi-Moghaddam, 2003). All such outcomes have been minor except for rare cases of successfully managed allergic reaction. Hospitalization due to a side effect or complication is exceedingly rare. These sequellae are mostly limited to non-infective fever, headache, lower abdominal pain, dysuria, leucorrhea, and back pain. Pain usually lasts for only a few days and is treated with ibuprofen or a similar drug. Notably, chronic pelvic pain is not reported. Menstrual patterns remain the same following insertion, indicating a normally functioning uterus. Permanent damage is limited to the uterotubal junction of the fallopian tube and results in a mature scar filling the intramural portion of the tube (Lu et al., 2003). El-Kady et al. (1991) have described the progression of the development of the scar in the tube from day 7 to day 25. Others (Bhatt et al., 1980; Laufe et al., 1996; Merchant et al., 1986; 1995; Sarin et al., 1998) have undertaken similar prehysterectomy studies and have identified these salient features.

Family Health International has undertaken extensive long-term follow-up studies in Vietnam and Chile, and has recently published three papers on these experiences. In its paper, "Safety of quinacrine contraceptive pellets: results from 10-year follow-up in Vietnam" (Sokal et al., 2008), a study of 2735 women who had quinacrine insertions between 1989 and 1993 compared to 1623 who received the IUD, the cumulative years of follow-up for the quinacrine and IUD cohorts were 28,697 and 17,382 person-years respectively. Losses to follow-up were 6% and 7%, respectively. FHI found that the risk of cancer, hysterectomy, pelvic/gynecologic surgery and death were similar in the two groups. A second FHI study, "Quinacrine Sterilization and Gynecologic Cancers: a case-control study in northern Vietnam," (Sokal et al., in press) was conducted for a 7-year period in 12 provinces in northern Vietnam, where a relatively large number of women had received intrauterine quinacrine. Cases of incident cervical, ovarian and uterine cancer were identified at provincial or referral hospitals in Hanoi. The prevalence of quinacrine exposure was 1.2% among cases and 1.1% among controls. FHI concluded, "We found no evidence of a relationship between quinacrine sterilization and gynecologic cancer." In its third study (Sokal et al., 2010) of women in Chile, where QS had its inception, FHI evaluated a quinacrine cohort from 1977 to 2007 and found their total follow-up time to be 23,894 person-years. FHI discovered that gynecologic cancers are within the range of expected numbers, suggesting that quinacrine does not increase their risk. ISAF currently has completed data collection on larger follow-up studies in Vietnam and China.

In collaboration with the FDA, ISAF mounted a Phase I study which was begun in 2001. Following its successful completion in 2003 (Lippes et al., 2003), ISAF requested permission to conduct a Phase III clinical trial in the United States. An Investigational New Drug (IND) application was approved by the FDA for this purpose in 2006, but was placed on clinical hold in early 2007, when Cancel et al. (2010) reported tumors produced at 70/70 mg/kg quinacrine and above in the CaBio. Therefore, the results of the CaBio are of critical importance and should be interpreted appropriately. In our opinion, the findings in their investigation, along with

their prechronic research, provide data supportive of the conclusion that: the doses causing tumors in their research clearly exceeded the MTD, and were secondary to the tissue destruction caused by the quinacrine doses used and the method of administration. Our rationale follows.

The severe endometrial destruction (diffuse ulceration) was likely responsible for the scarring and subsequent occlusion of the horn observed in the FHI prechronic and 2-year studies. While there was clear evidence of healing and regeneration of both the epithelium and glandular portions of the uterus in most animals, it was not uniform throughout the length of the uterus. Diffuse ulceration with subsequent scarring and chronic inflammation clearly exceed the definition of maximum tolerated dose, as discussed below. Just as importantly, the lesions observed at 10 mg/kg would qualify as a dose that met the definition of the MTD because the necrotic lesions, particularly in the epithelium, were of a moderate severity, accompanied by a significant amount of inflammation in subjacent tissues, but were repairable. If the purpose of this study was to determine the doses for a 2-year rat carcinogenicity bioassay for submission to a regulatory authority, the results clearly suggest that the highest dose level would be 10 mg/kg quinacrine because it did not show diffuse ulceration and therefore would not cause tubal occlusion.

While Cancel et al. (2010) offer their rationale for selection of the doses in their CaBio, their choices exceeded the contemporary definition of MTD, as documented by the United States Food and Drug Administration (United States Food and Drug Administration, 2008, http://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM129112.pdf), the International Conference on Harmonisation, (International Conference on Harmonisation, 2008, http://www.emea.europa.eu/pdfs/human/ich/038395en.pdf), the National Toxicology Program (Bucher et al., 1996; Bucher, 2002; McConnell, 1989) and the Environmental Protection Agency (United States Environmental Protection Agency, 1998, http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Series/870-4200.pdf) for high dose selection. The reasons Cancel and co-authors give for their dose selection are:

> "(1) the need to produce local tissue necrosis and fibrosis in the uterus, similar to that produced in women; (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"

We agree that these criteria are to some degree in concert with the spirit of MTD, but by definition the doses they selected exceeded their criteria. For example, the lesions produced at levels of 20 mg/kg in our study, at 14/14 mg/kg in the FHI prechronic study and at 70/70 mg/kg in the CaBio produced lesions far in excess of what are found in women (see below). Also, local toxicity that could not be repaired, e.g. diffuse ulceration, fibrosis (scarring) and chronic inflammation, would exceed what would be considered "tolerance." Finally, we feel that the dose metric of comparing the animal dose to the human dose on a mg/kg basis is not appropriate for these studies. While a mg/kg comparison is suited to a systemic exposure, this would not be appropriate for our current studies, which are essentially topical applications to the uterine epithelium.

In terms of dose selection, FDA's 2008 guidance, developed in collaboration with the International Conference on Harmonisation (ICH), states, "In the United States, dose selection based on the MTD has traditionally been considered the only appropriate practice" (USFDA & International Conference on Harmonisation, 2008). In fact, it affirms that, "There is no scientific consensus on the use of toxicity endpoints other than the MTD." Sections of this guidance germane to this study include the need to use as the highest dose one that is minimally toxic and is tolerated without chronic dysfunction or pathological changes that would interfere with the interpretation and, therefore, the validity of the study.

Next, the National Toxicology Program (NTP) is widely known for developing guidelines to be used in dose selection for CaBios. Regarding selection of the highest dose group in a CaBio, it makes clear that, "When toxicity limits the top dose selected for a chronic study, attempts are made to use a maximum tolerated dose, or, perhaps more accurately, a *minimally toxic dose* (our emphasis) or minimally toxic exposure to ensure that animals are sufficiently challenged during a chronic study to reveal the carcinogenic potential of the chemical" (Bucher et al., 1996).

Finally, we feel that the Environmental Protection Agency (EPA) guidelines (United States Environmental Protection Agency, 1998) on the dermal route of exposure also apply to dose selection in the case of quinacrine. This is because intrauterine instillation is actually a topical application of the chemical rather than a systemic exposure. The EPA guidelines clearly state that the highest dose used in a dermal study should be one that *does not cause "ulceration"* (our emphasis).

As detailed above, the dose selection criteria used in the Cancel et al. (2010) CaBio are notably different from the FDA/ICH/NTP and EPA Guidelines. The fact that the two high dose groups demonstrated greater than 30% mortality indicates that those doses clearly exceeded the MTD. This also raises the question of whether the 70/70 mg/kg quinacrine dose group also exceeded the MTD. We contend it did for the following reasons:

1. Their prechronic studies showed severe endometrial pathology including occlusive fibrosis at doses of 14 mg/kg quinacrine and higher.
2. The pathology observed in their prechronic investigations (endometrial necrosis, cystic degeneration, ulceration, and inflammation) persisted throughout the 2-year study.
3. The results of our 4-day study reveal pathology at 20 mg/kg quinacrine and above that exceeds the accepted definition of MTD, based on severe endometrial necrosis and diffuse ulceration.

With the above points in mind, we contend that a dose between 7 mg/kg (the one not causing permanent damage in the prechronic study) and 10 mg/kg quinacrine (the dose that caused endometrial necrosis but no diffuse ulceration in our 4-day study), represents the highest scientifically supported dose that can be used in the CaBio for quinacrine given via intrauterine exposure.

Destruction of the uterus (diffuse ulceration), as seen in the rat in these studies, is not found in women using this method of birth control. In the human, quinacrine is administered in the uterus resulting in a well-defined fibrotic scar limited to the uterotubal junction and intramural segment of the fallopian tube, effectively producing a fibrotic scar in the fallopian tubes of > 90% of women after a single dose (Lu et al., 2003). The consistency of this response is well documented in ultrasounds of 128 Brazilian women while undergoing their quinacrine procedures (Ferreira et al., 2003). Most importantly, there is no endometrial ulceration or long-lasting pathology in the uterus.

Furthermore, Laufe et al. (1996) reported on the histopathologic examination of the uteri of women, previously scheduled for a hysterectomy, who then agreed to undergo transcervical intrauterine instillation of quinacrine before their surgery so that possible uterine pathology could be determined. Histopathologic sections of these uteri did not show pathology as a result of quinacrine of a nature different from what would be expected in a normal-cycling

woman. In addition, in a study of 100 women, Agoestina and Kusuma (1992) report that during the month following insertion, women experienced only minor transitory complaints, and rarely, considerable bleeding or pain. In our opinion, if women experienced the extensive damage to the uterus as was seen in rats at 70 mg/kg quinacrine and above, they would be reporting significant bleeding within hours of the insertion, with significant pain continuing, possibly for their lifetime.

Cancel et al. (2010) chose to evaluate quinacrine in the uterine horn of rats at dose levels that produced fibrosis in the fallopian tube in women (Laufe et al., 1996; Merchant et al., 1995). Yet Merchant says, "The sections through the regenerated endometrium, cervix, and myometrium of the uterus in all cases of Groups I [straight quinacrine pellet inserter] and II [curved quinacrine pellet inserter] did not reveal any abnormality, thereby indicating that the chemical has no lasting effect on these structures [in women]." Additionally, Laufe reported no pathology in the endometrium in women from one-month trials. In their research, the permanent damage was considered to be confined to the fallopian tube. Drs. Fail and Zipper, with six studies between them, have the most experience conducting and evaluating the use of quinacrine in the rat uterus. They judge that the requirements of a carcinogenesis study would make the rat uterus an inadequate surrogate model for the human fallopian tube (personal communications).

We agree that if one wishes to examine the carcinogenicity of quinacrine, as used in sterilization, investigators would need to administer quinacrine into the rat uterus and look for neoplasms in the reproductive tract. But, the intent of such a study would be to assess the potential carcinogenicity in the uterus, not the fallopian tube, and most importantly, such a study would not administer dose levels that destroy the endometrium and cause permanent scarring. This would be akin to using an ulcerogenic dose in skin paint or stomach gavage studies which would not be condoned for use in animal studies for both scientific and ethical reasons.

We consider it improper to use the rat uterus as a human fallopian tube surrogate for anatomical reasons. The rat uterus is a complex organ with a thick endometrium consisting of an epithelial lining, numerous subjacent glands and a thick lamina propria. As a proportion, the endometrium is approximately twice as thick as the myometrium. In contrast, the fallopian tube has a very simple structure. It is lined by a single layer of columnar ciliated cells with a scattering of secretory and clear cells and a negligible amount of lamina propria (Leininger and Jokinen, 1990).

In general, the reaction to a given chemical is tissue-specific and depends on a myriad of local tissue and organ-specific chemical/cell interactions. While years of experience support the use of various rat organs as surrogates for the same organs in humans, there is little scientific support for using one organ to predict what would happen in one that is anatomically dissimilar (Quayle, 2002; Pioli et al., 2004; Itoh et al., 2006).

We conclude that the series of events that led to the induction of the tumors in the CaBio are as follows:

(1) Acute severe necrosis with ulceration of the endometrium was followed by scarring and occlusion at dose levels where total uterine length repair was not possible.
(2) Dilation of the uterine horn occurred proximal to the occlusion due to pressure atrophy in this "closed system." As our experiment has shown, dilation, by itself, cannot account for necrosis and inflammation.
(3) Excessive cell necrosis and chronic inflammation at the tissue site allowed for an environment that promoted the formation of reactive oxygen and nitrogen species. The generation of free radicals is known to produce DNA damage and enhanced cell proliferation, which is most likely responsible for the tumor response observed in the CaBio.
(4) The wide range of tumor types found, with different types in different animals receiving the same dose, none of them statistically significant, is not the expected response to a specific chemical insult as would be expected from a genotoxic carcinogen. Instead, such a reaction is strong evidence of the non-specific nature of tumor induction, as would be expected from a response to tissue necrosis and subsequent cell proliferation, coupled with chronic inflammation.

With regard to the Cancel et al. (2010) conclusion – that quinacrine was carcinogenic in the rat CaBio via a genotoxic mechanism because the tumors were seen after only two doses – we offer the following opinion:

They fail to note that the Cancel et al. 2006 neonatal mouse study concluded that the only histopathology of concern consisted of slight increases in benign stromal polyps at the intermediate and high doses, not significantly different from the control. They also omit the fact that the high dose was lethal to 25% of the high-dose animals following the second administration. There is a consensus that the neonatal mouse assay is effective for distinguishing between genotoxic and non-genotoxic carcinogens, and is negative for the latter (Flammang et al., 1997; McClain et al., 2001). In summarizing a collaborative study coordinated by the International Life Sciences Institute (ILSI), it was concluded that "Negative data in [the neonatal assay] are also mechanistically meaningful, indicating that the drug is not likely to be a trans-species carcinogen; that the drug is unlikely to have *in vivo* genotoxic activity; and the drug is unlikely to be a direct-acting carcinogen. In addition, negative data will contribute to weight of evidence that a tumor response observed in the rat is more likely to involve an epigenetic mode of action" (McClain et al., 2001).

The negative carcinogenicity results in the neonatal mouse assay, and the finding that tumors were only seen in rats with massive uterine tissue destruction in the surviving animals, do not suggest a genotoxic mode of action for the uterine tumors.

We contend that massive cell necrosis in the endometrium, along with chronic inflammation resulting in free radical generation, are the most plausible causes for tumor formation in Cancel et al. (2010). There was clear evidence of initial massive uterine necrosis followed by sustained inflammation in both the prechronic and chronic studies under discussion. Chronic inflammation and free radical generation is a common mechanism for tumor initiation and progression, believed to be responsible for approximately 25% of all tumors in humans (Kundu and Surh, 2008). When tumors arise from this process, they are characterized by being organ-specific, as were seen with quinacrine, rather than having multiple organ involvement. The mechanisms for inflammatory-induced tumors, although complicated, are fairly well understood as due to multiple inflammatory mediators produced by infiltrating inflammatory cells. Two of the more important mediators are reactive oxygen and reactive nitrogen species. These are also produced during cell necrosis and cause DNA damage (strand breaks, cross-linking and genomic instability). The other group of important mediators are cytokines, also products of inflammatory cells, and are responsible for enhanced cell proliferation of initiated cells, altered apoptosis, tumor neovascularization and protein modification (e.g., histone modification and hypermethylation).

6. Conclusions

In conclusion, the study by Cancel et al. (2010) clearly shows that there was no evidence of carcinogenic activity of quinacrine at 10 mg/kg, the highest dose that would have been used in their CaBio if they had been guided by the basic tenets for maximum tol-

erated dose. Further, we think there is strong evidence that the dose (70 mg/kg quinacrine) that produced tumors in their study significantly exceeded the MTD. Therefore, the findings at this dose level should be censored from evaluation of the carcinogenic activity of quinacrine in the rat uterus.

Conflict of Interest statement

The authors declare that this work has not been published previously nor is it under consideration for publication elsewhere. The article is approved by all authors. Dr. McConnell is an unpaid science advisor to ISAF and therefore has no conflict of interest. Dr. Lippes has been the principal investigator on the Phase I and Phase III studies. Roger Growe, Drs. Fail, Luster and Zeiger are paid consultants to ISAF.

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