THE EFFECT OF 5-BROMO-2-THIENYL-ETHYL KETONE THIOSEMICARBAZONE ON THE INDUCED DECIDUOMAL RESPONSE IN THE RAT

FRANK C. TINSLEY, ROBERT P. RATHMACHER
AND ROBERT L. COCHRANE

*Lilly Research Laboratories, Eli Lilly and Company,
Indianapolis, Indiana 46206, U.S.A.*

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Summary. The effect of 5-bromo-2-thienyl-ethyl ketone thiosemicarbazone (70026) on the decidual response was studied to determine the mode of its antifertility activity. The study utilized intact, ovariectomized and hypophysectomized rats that were made pseudopregnant and/or treated with progesterone, corn oil, prolactin and 70026, either alone or in combination. The results indicated that 70026 partially inhibited the decidual response by a direct action on the uterus, but also had an additional inhibitory effect by a direct action on the ovary. This latter effect indicated that 70026 inhibited progesterone synthesis and/or secretion from the corpus luteum, or interfered with luteotrophin stimulation of the ovary. Compound 70026 had no significant effect on ovarian weight but it did have a highly significant effect on body weight.

INTRODUCTION

Routine screening of compounds for antifertility effects in rats led to the discovery that a series of thiophene thiosemicarbazones possessed antifertility activity. The most promising of the series studied to date has been 5-bromo-2-thienyl-ethyl ketone thiosemicarbazone (70026). Preliminary studies on this compound showed that it was active when administered *post coitum* and inhibited or delayed ovo-implantation. cursory observations indicated that progesterone administration would offset this antifertility effect. The compound caused continued oestrous cycles in pseudopregnant and pregnant rats, as judged by vaginal smear cytology. These preliminary findings seemed to indicate that 70026 was having its antifertility effect by interference with progesterone synthesis, secretion or utilization.

This study was the first in a series to determine whether or not 70026 was, in fact, having these effects, and, if so, the level at which it acted. The decidual reaction was used as the model for studying the antifertility action of 70026, since it provided a means for assessing: (1) the presence of progesterone in the circulation (Astwood, 1939; Rothchild, Meyer & Spielman, 1940), (2) the

utilization of progesterone at the target organ (Astwood, 1939; Rothchild *et al.*, 1940) and (3) the function of many of the factors presumed to be involved in ovo-implantation (Shelesnyak, 1957; Shelesnyak & Kraicer, 1963; Finn & Keen, 1963; Finn, 1966).

MATERIALS AND METHODS

The animals used in these studies were mature female Carworth CFE strain rats which were maintained on Purina Lab Chow and tap water. They were housed separately, or in groups of up to six animals per cage, at a constant temperature (74 to 76° F) and on a regimen of 14 hr light and 10 hr dark. The animals completed at least three normal 4- to 5-day oestrous cycles before being put on the experiment. Pseudopregnancy was induced by uterine cervical stimulation with a glass rod on the morning and evening of the day of oestrus. Surgical procedures were performed under ether anaesthesia using a semi-sterile technique. Ovariectomy was carried out through dorso-lateral incisions, while the parapharyngeal approach was used for hypophysectomy. Uterine trauma was accomplished through a mid-ventral incision by scratching the entire length of the anti-mesometrial side of the left uterine horn with a burred needle. Progesterone and 5-bromo-2-thienyl-ethyl ketone thiosemicarbazone (70026) were each injected subcutaneously (s.c.) at a daily dose of 2 mg in 0.1 ml corn oil. Corn oil alone was administered as a daily 0.1 ml s.c. injection. Prolactin (ovine, 14 i.u./mg) was given as twice daily s.c. injections of 5 i.u. in 0.1 ml of 0.9% saline; except for the first and last day of treatment when it was given only once a day.

All animals were started on the experiment on the last day of oestrus (Day 1). Ovariectomy and hypophysectomy were carried out on the afternoon of Day 1. Pseudopregnancy, likewise, was induced on Day 1. Injections were begun on the afternoon of Day 1 and continued through to the morning of Day 10. All animals had one uterine horn injured on Day 5 and were killed on the afternoon of Day 10. The specific treatment regimens of each group appear in Table 1.

Body weights were recorded at the beginning of the experiment, at the time of uterine trauma and at autopsy. Animals with infections and hypophysectomized animals possessing macroscopic pituitary remnants in the sella turcica were discarded from the experiment. The ovaries were removed and weighed at autopsy. The two uterine horns were, likewise, removed at this time, evaluated grossly for the presence or absence of a decidual response and weighed separately. All uterine horns and ovaries were preserved in Bouin's fluid. Later, the injured uterine horns of four rats from each of Groups 1 and 2, three rats from each of Groups 5, 6, 7, 9 and 10 and eight rats from Group 8 were sectioned, mounted, stained with haematoxylin and eosin, and evaluated histologically. Horns where obvious decidual reactions existed had nine sections cut from the decidual area; horns where the presence of decidual reactions was in doubt had nine sections cut from each of three entirely different areas. In all cases, the uterine horns thought most likely to contain decidual responses were the ones selected from the group for histological study. Four representative sections from each of the left ovaries of forty of the animals were,

likewise, prepared and studied histologically. These forty rats consisted of six rats from Group 8, four rats from Group 1 and three each from Groups 2, 3, 4, 7, 9, 10, 11, 12, 15 and 16.

Comparisons between groups were made using the difference in weight of the injured and uninjured uterine horns as the measure of the decidual response, since uterotrophic effects of the treatment should be reflected in the weight of the uninjured uterine horn. Ovarian weight, expressed as the mean total weight of both ovaries, and change in body weight during the treatment were also compared. Analyses of variance of all data and, in the case of the uterine weight differences and ovarian weights, a further Duncan's Multiple Range Test, were calculated (Steel & Torrie, 1960). The Duncan's Multiple Range Test was modified by using the harmonic mean in calculating the standard error. Because of a positive correlation between means and their respective standard deviations in the uterine weight data, the responses were converted to natural logarithms before analysis. In contrast to this, the absolute values were used in the analysis of ovary weights and change in body weight. In the latter data, there were three observations missing in each of Groups 7 and 8 and one in each of Groups 9 and 12. One additional observation was omitted from Group 8 because it alone caused significant heterogeneity of variance in this data.

Six of the sixteen animals in Group 1 were injected with corn oil daily, while the other ten received no injections at all. These two sub-groups were compared to check for a placebo effect. There was no significant difference in the two

TABLE 1
EFFECTS OF 70026 ON WEIGHT DIFFERENCES BETWEEN THE INJURED AND UNINJURED UTERINE HORNS, OVARIAN WEIGHTS AND CHANGES IN BODY WEIGHTS

Group no.	No. of animals	Treatment	Mean difference in the two uterine horns (mg)	Mean ovarian weight (total of both ovaries in mg)	Mean change in body wt (g)
1	16 (16)*	Psp	1425	77.2	+ 13.3
2	10 (10)	Psp + 70026	56	69.3	+ 4.1
3	10 (10)	Prl	1674	73.3	+ 12.4
4	10 (10)	Prl + 70026	41	74.3	+ 5.2
5	10 (10)	Ovx + Prg	846	—	+ 14.8
6	11 (11)	Ovx + Prg + 70026	179	—	+ 10.4
7	10 (7)	Apx + Prl	748	47.6	- 13.0
8	11 (7)	Apx + Prl + 70026	46	46.6	- 15.4
9	10 (9)	Apx + Prg	697	53.6	- 16.1
10	10 (10)	Apx + Prg + 70026	151	50.8	- 23.0
11	5 (5)	Apx + Corn oil	16	55.5	- 22.6
12	6 (5)	Apx + 70026	12	47.6	- 29.0
13	5 (5)	Ovx + Corn oil	5	—	+ 9.6
14	5 (5)	Ovx + 70026	6	—	+ 6.4
15	10 (10)	Prg	1203	68.7	+ 21.7
16	10 (10)	Prg + 70026	312	66.7	+ 9.6

Psp = pseudopregnant; Apx = hypophysectomized; Ovx = ovariectomized; Prg = progesterone; Prl = prolactin.

* Number in parentheses represents the number of observations involved in the change in body weight.

sub-groups in any of the end-points studied so they were pooled and used as one group.

RESULTS

The results showed that 70026, in all cases except those of the negative controls (Groups 11, 12, 13 and 14), significantly reduced ($P < 0.01$) the decidual response below that of their respective controls (Tables 1 and 2). It was evident from the weight differences in the two uterine horns, as well as gross evaluation of the uteri, that decidual responses had not occurred in any of the negative control groups, thus explaining the lack of effect of 70026 in these groups. The results of the analysis of the decidual response (Table 2) demonstrate quite convincingly that 70026 was acting both at the uterine level and at the ovarian level to reduce or inhibit the decidual response. The effect at the uterine level was shown by the fact that 70026 significantly reduced ($P < 0.01$) the decidual response in progesterone-treated animals compared with animals not receiving 70026 (Groups 6, 10 and 16 versus Groups 1, 3, 5, 7, 9 and 15). An additional effect at the ovarian level was indicated by the fact that 70026-treated animals depending on endogenous sources for their progesterone (Groups 2, 4 and 8) had significantly smaller ($P < 0.01$) decidual responses than 70026-treated animals receiving exogenous progesterone (Groups 6, 10 and 16). This was particularly striking in comparing the hypophysectomized luteotrophin (prolactin)-treated (Group 8) with the hypophysectomized progesterone-treated (Group 10) animals. The other differences that existed between groups in this end-point (i.e. Group 3 versus 9; Group 13 versus 2, 4 and 8; and Group 14 versus 2 and 8) were not so clear-cut and explanations for them are purely speculative. The uterine weight data from the negative controls, both from the standpoint of the individual means and the comparisons of 70026-treated with non-treated groups, demonstrates that 70026 itself did not support the decidual response and, for that matter, had little effect on the response of the uterus to trauma in the absence of progesterone.

The uteri that were examined from the animals in Groups 1, 5, 7 and 9 all contained large, fully-developed decidualmata consisting of both the large, anti-mesometrial, binucleate type of stromal cell and the smaller, mesometrial, vacuolated type of stromal cell, similar to those described by Krehbiel (1937) and Selye & McKeown (1935). By contrast, all uteri examined from animals in Groups 6 and 10 contained very small decidualmata usually consisting of only small sub-epithelial plaques of the anti-mesometrial type of binucleate decidual cell. The uteri that were examined from Groups 2 and 8 contained only small decidualmata, similar to those in Groups 6 and 10, or no decidualmata at all. In the latter cases, there were usually some pycnotic epithelial and stromal cells and a number of erythrocytes and polymorphonuclear leucocytes in the tissue spaces and uterine lumen associated with the uterine trauma. In general, the histological evaluation of the uterus as to the presence or absence and extent of the decidual response roughly parallels the results obtained using the weight difference in the injured and uninjured uterine horns.

Treatment with 70026 did not have any apparent effect on ovarian weight

TABLE 2
DUNCAN'S MULTIPLE RANGE TEST ANALYSIS OF WEIGHT DIFFERENCES BETWEEN THE INJURED AND UNINJURED
UTERINE HORNS OF RATS

	<i>Negative controls</i>						<i>Endogenous progesterone plus 70026</i>			<i>Exogenous progesterone plus 70026</i>			<i>No 70026 treatment</i>								
	Ovx	Ovx	Ovx	Apx	Apx	Apx	—	Apx	Psp	Apx	Ovx	---	Apx	Ovx	—	Psp	—	—			
Operation	CO	70026	70026	CO	CO	CO	Prl	Prl	---	— <td>—<td>—</td><td>—<td>—<td>Prg</td><td>Prg</td><td>Prg</td><td>Prl</td></td></td></td>	— <td>—</td> <td>—<td>—<td>Prg</td><td>Prg</td><td>Prg</td><td>Prl</td></td></td>	—	— <td>—<td>Prg</td><td>Prg</td><td>Prg</td><td>Prl</td></td>	— <td>Prg</td> <td>Prg</td> <td>Prg</td> <td>Prl</td>	Prg	Prg	Prg	Prl			
Treatment	13	14	12	11	11	11	4	8	2	10	6	16	9	7	5	15	1	3			
Group no.																					
Groups not underscored by the same line differ from one another at the 1% level																					

Psp = pseudopregnant; Apx = hypophysectomized; Ovx = ovariectomized; Prl = prolactin; CO = corn oil; Prg = progesterone.

(Table 3). The hormonal-surgical treatments, however, did have a highly significant ($P < 0.0005$) effect. It is evident from Tables 1 and 3 that this effect was caused mostly by the surgical procedure of hypophysectomy reducing the ovarian weight. When the 5% probability level was used as the level of significance, this effect was quite clear-cut. When the 1% level was used, however, some overlap occurred between the intact and hypophysectomized animals and the difference due to this operation was not quite so obvious.

TABLE 3
DUNCAN'S MULTIPLE RANGE TEST ANALYSIS OF OVARIAN WEIGHTS

Treatment	<i>Hypophysectomized</i>						<i>Intact</i>					
	Prl	Prl	—	Prg	Prg	CO	Prg	Prg	Psp	Prl	Prl	Psp
70026	+	—	+	+	—	—	+	—	+	—	+	—
Group no.	8	7	12	10	9	11	16	15	2	3	4	1
Groups not underscoring by the same line differ from one another at the 5% level	_____						_____					
Groups not underscoring by the same line differ from one another at the 1% level	_____						_____					

Psp = pseudopregnant; Prg = progesterone; Prl = prolactin; CO = corn oil.

Subjective evaluation of the ovaries from the animals in this experiment indicated that 70026 treatment had little or no obvious effect on ovarian histology, with the possible exception of Group 8. The corpora lutea from the animals in this group may have contained a few more pycnotic and vacuolated luteal cells than their controls but this was not very clear-cut. By contrast, progesterone treatment seemed to have a definite effect on the corpus luteum morphology. It apparently caused the cytoplasm of many of the luteal cells to be more vacuolated in some cases and contain fewer granules in other cases. As a result, many of the luteal cells from the progesterone-treated animals were very lightly stained. Connective tissue invasion of the corpora lutea was also more evident in progesterone-treated animals than in those which were not treated with progesterone. Hypophysectomy caused a slight reduction in size of the corpora lutea and the luteal cells and also seemed to reduce the number and size of the vesicular follicles in the ovary. These latter structures were almost invariably atretic in all the ovaries examined. Prolactin seemed to increase the size of the corpora lutea and the luteal cells in both intact and hypophysectomized animals; while corn oil had no obvious effect on ovarian histology.

The mean changes in body weight (Table 1) indicated that 70026 treatment

caused a reduction in body weight. An analysis of variance of the data showed that this tendency was highly significant ($P < 0.0005$). This analysis also showed that the other hormonal-surgical treatments considered collectively had a highly significant effect ($P < 0.0005$) on body weight change. There was no significant ($P > 0.05$) interaction, however, between the 70026 treatment and the collective hormonal-surgical treatments. No attempt was made to determine specific group differences, since the purpose of this part of the experiment was only to obtain some crude measurement of the effect of 70026 on body weight.

DISCUSSION

The decidual response in the rat is apparently specific for progesterone (or progestin) conditioning of the uterus; however, oestrogen has a marked quantitative influence on this reaction (Astwood, 1939; Rothchild *et al.*, 1940; Yochim & De Feo, 1962, 1963). The present experiments attempted to utilize the decidual response quantitatively to partition out the level at which 70026 was acting. It was suspected that progesterone was the factor affected; therefore, progesterone alone was used as the exogenous steroid supplied in spite of the known effects of oestrogen. Fortunately, the interaction of endogenous oestrogen with progesterone did not prevent the partitioning of the level of effect of the 70026. Quite probably this interaction was the reason for the high values obtained for the decidual responses in the intact animals not treated with 70026 (Groups 1, 3 and 15). The daily prolactin dose (Malven, Hansel & Sawyer, 1967) given to the hypophysectomized animals seems to have approximated very closely to the effect of the exogenous progesterone in the ovariectomized and hypophysectomized animals. This prolactin effect was assumed to be caused by endogenous progesterone alone without the interaction of oestrogen.

The use of the decidual response as a method of assessing corpus luteum function is certainly nothing new (Evans, Simpson, Lyons & Turpeinen, 1941; Everett, 1954); neither is the use of this same end-point as a method of studying the antifertility effect of numerous compounds (Duncan, Cornette, Lyster, Northam & Wyngarden, 1966; Duncan, Wyngarden & Cornette, 1968; Harper, 1967; Lindsay, Poulson & Robson, 1961; Schlough & Meyer, 1965; Shelesnyak, 1965). Most of the studies on inhibition of the decidual response have demonstrated an action directly at the level of the uterus. Such studies were those on the classical oestrogens (Astwood, 1939; Rothchild *et al.*, 1940; Velardo & Hisaw, 1951; Yochim & De Feo, 1962), some of the newer impeded oestrogens (Duncan *et al.*, 1968; Schlough & Meyer, 1965), oxazolidinethione (Duncan *et al.*, 1966), some of the pregnanes, testosterone and some of the mineralo- and gluco-corticoids (Hisaw & Velardo, 1951). A few of the decidual studies, on the other hand, have shown (Lindsay *et al.*, 1961, 1962), or indicated (Harper, 1967; Shelesnyak, 1957), an action by the inhibitory agents at some level other than that of the uterus (see also Lindner & Shelesnyak, 1967). None of these studies have demonstrated specifically the site of the inhibitory activity of the agents, but most of them suggest an action on the central ner-

vous-pituitary system and/or the ovary. By contrast, the present study has not only demonstrated a site of action directly at the uterine level itself, but has also defined a specific site of action outside the uterus, namely, the ovary.

Compound 70026 seems to be a unique compound. Unlike many of the deciduomata-inhibiting compounds mentioned previously, neither of the activities of 70026 seemed to be attributable to its oestrogenicity. Treatment of hypophysectomized (Group 12) or ovariectomized (Group 14) rats with 70026 did not cause an increase in uterine weight over that caused by corn oil alone (Groups 11 and 13, respectively). The average weights of the uninjured uterine horns of each of the above groups was 67, 105, 92 and 117 mg, respectively. The data in Table 3 suggest, in addition, that 70026 does not act on the deciduomal response by stimulating or depressing the pituitary gonadotrophins. The direct effect of 70026 on the ovary to inhibit deciduomata formation further demonstrates the uniqueness of this compound when compared with previously reported deciduomata-inhibiting compounds. This latter activity would appear to be best explained by an inhibition of progesterone synthesis and/or secretion by the corpus luteum, or interference with the action of luteotrophin at the target tissue. The reports by Schuler & Meier (1951) (cited in Gaunt, Chart & Renzi, 1961) and Gaunt *et al.* (1961) that 5-chloro-2-acetyl-thiophene thiosemicarbazone depresses adrenal cortical function seems to give additional support to this suggested interference with steroidogenesis by 70026.

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