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Preliminary Pharmacokinetics and Metabolism of Novel Non-steroidal Antiandrogens in the Rat: Relation of their Systemic Activity to the Formation of a Common Metabolite

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The non-steroidal antiandrogens, RU 58841 and RU 56187 are amongst the most active of a new series of *N*-substituted aryl hydantoins or thiohydantoins. Their pharmacokinetics and principal metabolic profiles have been evaluated in rat plasma after intravenous administration of a 10 mg/kg dose. Both compounds disappear relatively rapidly from the plasma (elimination half-life of the order of 1 h), but they form a common metabolite, the *N*-desalkyl derivative, RU 56279, which is eliminated much more slowly. The percentage transformations of each into RU 56279, estimated from the AUCs of the metabolite compared with the AUC obtained after administration of RU 56279 itself, were respectively 1% and 77%. In parallel, their *in vivo* activity, as well as that of their metabolites, was determined with respect to parameters related to systemic antiandrogenic effects (prostate and seminal vesicle weights). The results showed that: (1) the common metabolite, RU 56279, is clearly antiandrogenic; (2) there appears to be a relationship between the percentage formation of this metabolite and the systemic antiandrogenic activity of the compounds. Thus, the pharmacological profile of RU 58841 which displays a potent local antiandrogenic activity without systemic effects can be related to its very low propensity to form the *N*-desalkyl metabolite.

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INTRODUCTION

The two non-steroidal antiandrogens, RU 56187 and RU 58841, were selected from a series of Anandron-derived compounds designed for the potential treatment of androgen-dependent disorders. These compounds are hydantoin (RU 58841) or thiohydantoin (RU 56187) derivatives characterized by the presence of an alkyl group on the nitrogen in position 3 (Fig. 1); hydroxybutyl in the case of the former and methyl in the latter. Although closely related structurally they differ pharmacologically. RU 58841 is very active by topical route on the hamster flank organ, but shows little effect on either weights of accessory sex organs such as prostate, seminal vesicle or levels of testosterone in both hamster and rat by oral, subcutaneous or topical route [1]. By contrast, RU 56187 displays very strong systemic antiandrogen activity whatever the model studied and the route of administration [2]. In order to explain this apparent dissociation and possibly to relate the extent of the systemic effects of these compounds to their respective metabolic profiles, a preliminary study of their pharmacokinetics and metabolism was undertaken. The study was carried out in the rat by intravenous route, which by definition eliminates all absorption phases and allows a direct comparison of metabolism. The relatively high dose used (10 mg kg) was chosen so that after a single administration the compounds and their metabolites could readily be measured using HPLC with UV detection. The antiandrogenic activity of RU 56187 and its metabolites was subsequently evaluated after oral administration, this being the route of choice in the case of the use of this compound for the treatment of prostate cancer in man. Concerning RU

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58841 which is destined to be used topically for the treatment of acne, alopecia and hirsutism, its activity as well as that of its metabolites was evaluated using the subcutaneous route in order to mimic its complete passage through the skin.

MATERIALS AND METHODS

Chemicals

The hydantoin derivative, RU 58841, the thiohydantoin derivative, RU 56187 (structures: Fig. 1) and the putative metabolites, RU 56191, 56279, 58336 and 59416, were synthesised in the Chemistry Department at Roussel Uclaf (structures of metabolites: Fig. 2).

All solvents and reagents used were of HPLC or analytical grade. Acetonitrile (low UV) was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Methanol and tetrahydrofuran were obtained from Merck (Darmstadt, Germany). Water used for the preparation of buffers was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Stock solutions were prepared in methanol (40 μg/ml) and stored at 4°C.

Animals

Pharmacokinetic studies. Male Sprague–Dawley rats (4 in each group), weighing 200 g on average were used; they received a single i.v. (tail vein) dose in PEG 300 (2 ml/kg). They were killed by decapitation and the blood was collected in heparinized tubes (Li) and rapidly centrifuged (3000 *g*, 4°C, 15 min). Plasma analysis was carried out immediately (without congelation). Concerning the cutaneous treatment, the animals were shaved on the back, using an electric shaver, then they received a single cutaneous administration of the product dissolved in ethanol 99.9% (50 μl/rat).

Pharmacological studies. Male Sprague–Dawley rats weighing 100 g were purchased from Iffa Credo (L'Abresle, France), housed in our central animal facilities under a 12 h dark/light cycle and given food and water *ad libitum*. The animals were castrated under anesthesia with Imalgene 500 (Rhone Mérieux), 24 h later (day 1) they received 0.5 mg/kg of testosterone propionate (TP, Roussel Uclaf) alone or together with the compounds at a dose of 1 mg/kg, or vehicle. Animals were treated daily, from day 1–4 and from day 7–10. TP was administered by s.c. route in germ corn oil (Mayoland, Benedicta, France) containing 10% ethanol and the compounds either by oral route, in aqueous solution containing 0.5% methylcellulose (Colorcon) or by s.c. route, in germ corn oil containing 10% ethanol. The animals were sacrificed by exsanguination under anesthesia 24 h after the last treatment. The prostates and seminal vesicles were removed and fixed for 72 h in demineralized water containing 10% formol (Merck); they were then dissected and weighed.

Each group consists of 5 animals/dose. The antiandrogenic activity was expressed as a percentage of inhibition of the TP effect (TP-treated animals were arbitrarily assigned a value of 0% and vehicle-treated animals a value of 100%).

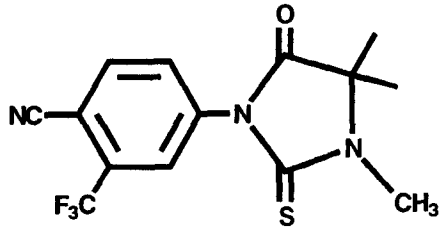
Statistical analysis

The means of the values and the standard error of the mean (SEM) were calculated for each dose. Statistical analysis were performed using Dunnett's [3] test. $P(\star) < 0.01$ were considered to indicate a significant difference between the groups.

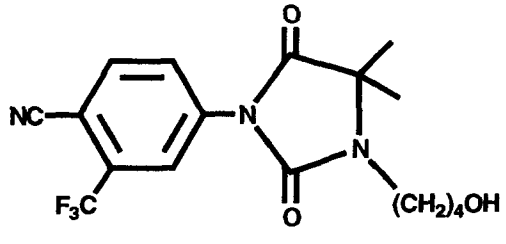
Analytical methods

Chromatography. The high-performance liquid chromatographic (HPLC) system consisted of a Hewlett Packard ternary pump model HP 1090 operated at a flow rate of 1 ml/min, a Spectra Physics SP 8780 autosampler equipped with a 20 μl loop, a Spectra Physics SP 8490 variable-wavelength Spectromonitor operated at 260 nm or a Perkin Elmer LC-235 diode array detector and a Spectra Physics SP 4290 integrator.

For RU 58841 studies, an Ultrabase Kromasil octyl analytical column (150 × 4.6 mm, 5 μm particle size) from Shandon Scientific-SFCC (Eragny, France) was used. In order to separate RU 58841 and its two metabolites, a mobile phase consisting of a 60/40 (v/v) mixture of 50 mM potassium dihydrogenophosphate



RU 56187



RU 58841

Fig. 1. Chemical structures of RU 56187 and RU 58841.

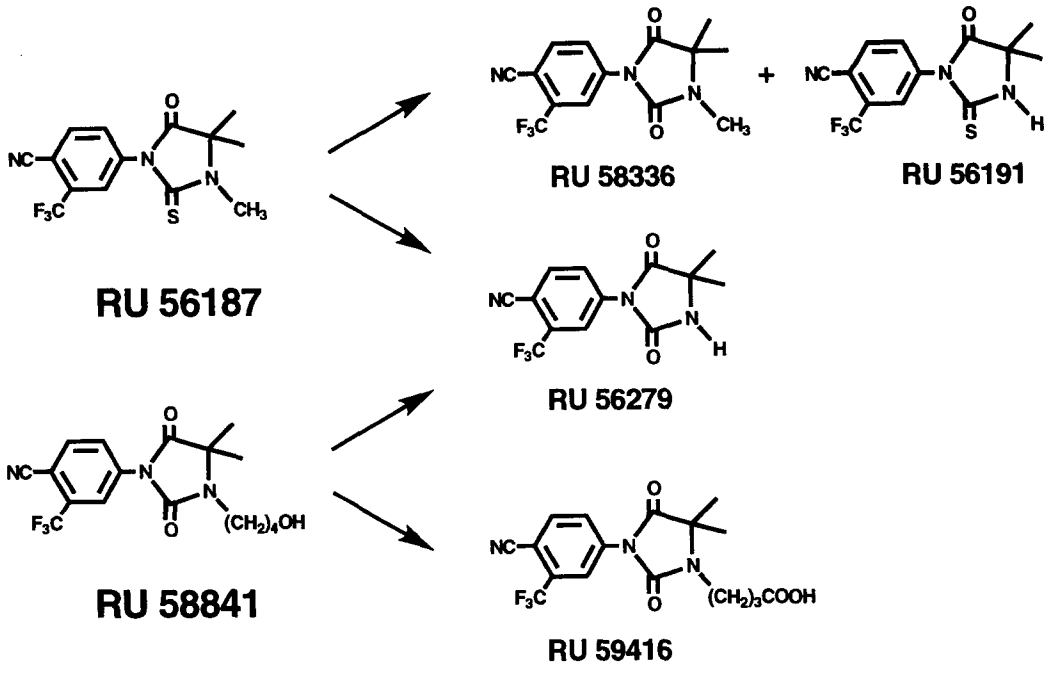


Fig. 2. Proposed principal metabolism scheme of RU 56187 and RU 58841 in the rat.

adjusted to pH 5.2 and tetrahydrofuran (= System I) was used.

RU 56187 and its metabolites were separated on a Nucleosil Cyano analytical column (150 × 4.6 mm, 5 μm particle size) from Colochrom (Gagny, France), with a mobile phase composed of acetonitrile and 0.1 M potassium phosphate buffer, pH 7.0; 35/65(v/v) (= System II).

Plasma sample extraction. A Vac Elut processing station SPS 24 (Analytichem) was used for extracting plasma samples. The complete method is described for RU 58841; for RU 56187, only the internal standard differs (compound of the same chemical series).

To 1 ml of plasma was added 0.8 μg (20 μg of a methanolic solution) of internal standard (RU 57073, compound of the thiohydantoin series). The mixture was vortex-mixed for 10 s, transferred onto octadecylsilane-bonded silica micro-columns (Bond Elut C18, 1 ml capacity, Analytichem International, Harbor City, CA, U.S.A.) and pretreated successively with 1 ml methanol and 1 ml purified water. Endogenous components in plasma were removed by washing the columns once with 1 ml of 1 M NaCl and 3 times with 1 ml purified water. The bound compounds were eluted with 1 ml methanol and 20 μl aliquots of this direct methanolic extract were injected into the chromatographic system. All the metabolites described here were extracted using this method.

Calibration and calculation. For both compounds and their metabolites, the limit of detection was evaluated as 0.005 μg/ml. Calibration curves were constructed by addition of known amounts of RU 58841 and its metabolites (0.010–8 μg/ml), together with the internal standard, to aliquots of control plasma (1 ml). The calibration graphs were found to be linear over the range used, with correlation coefficients >0.998 and y-intercepts close to zero. Unweighted least-square regression lines were generated using peak-height ratios (RU compound/internal standard).

When plasma samples were spiked to final concentrations of 0.5 μg/ml, RU 58841 and its metabolite RU 56279 were totally recovered, the absolute recovery of the acid metabolite RU 59416 was 80%. The recovery of RU 56187, in the same conditions of concentration was 91%.

Identification of metabolites: analysis by mass-spectrometry/thermospray. The principal metabolites of RU 58841 and RU 56187 were tentatively identified by HPLC-SM (thermospray) as well as by the comparison of the peaks on the chromatograms with those of reference compounds.

All experiments were carried out on a Finnigan Model 4600 single quadrupole mass spectrometer equipped with a Data general Nova 4S computer using Superincos Software and fitted with a modified TSP II thermospray ionisation source (Finnigan Mat, San Jose, CA. U.S.A.). Typical operating conditions were as follows:

—Jet (source block) temperature, 230°C (Aerosol 245°C); vaporizer temperature, 105°C; repeller voltage, 10 V.

—For compounds tested, the sensitivity was found to be better when focusing on negative ions instead of positive ions. The negative thermospray profiles of standards and metabolites are relatively simple, with just one major ion predominating. The molecular ion ($M-H^-$) is the base peak of the spectra and no

fragments of lower mass are observed. It is the mode used for the two metabolites of RU 58841 and for the two N–H metabolites of RU 56187. For RU 58336 (containing no mobile proton), the positive ionization mode was used (formation of $M + NH_4^+$. The structures of the 3 metabolites were confirmed by using a discharge electrode, to form M^- (1 kV).

—Data were collected over the mass range of 150–500 amu using 1 s scans in the centroid mode.

—The HPLC system connected to the inlet of the thermospray interface consisted of a Model SP 8700 pump (Spectra Pysics), the solvent system in both cases was ammonium acetate 0.02 M in water/acetonitrile; the UV 260_{nm} elution profile was obtained on a Spectraflow 757 Kratos detector equipped with a special high pressure resistant cell to permit on line coupling with TSP mass spectrometer.

RESULTS

Pharmacokinetics and metabolism of the thiohydantoin, RU 56187, in the rat after i.v. administration of 10 mg/kg

Unchanged RU 56187. 5 min after injection, the plasma concentrations of RU 56187 are of the order of 2.4 μg/ml, they then decrease rapidly; at 6 h the mean value is 0.030 μg/ml. The experimental AUC (0–6 h) is equal to 1.58 μg/ml × h and the terminal elimination half-life is of the order of 1.4 h [Fig. 3(a)].

Principal metabolites (see Fig. 2). The first metabolite identified results from the oxidation of the thio function to give the corresponding hydantoin, RU 58336. This metabolism is very rapid; the maximum concentration (1.6 μg/ml) being observed at approx. 5 min. After 6 h its concentration is 0.4 μg/ml and it is no longer detected beyond this time.

The principal metabolite is the *N*-demethylated hydantoin, RU 56279 (resulting from both oxidation and *N*-dealkylation): from 5 min, it is present at a mean concentration of 0.5 μg/ml and the maximum appears at 6 h (mean concentration = 6 μg/ml). It decreases very slowly afterwards: 24 h after administration the mean concentration is 2.6 μg/ml and after 72 h it is still present at a concentration of 0.7 μg/ml.

The *N*-demethylated thiohydantoin (RU 56191) was detected but not quantified; its concentrations at 4, 6 and 24 h were below or equal to 0.05 μg/ml.

The mass spectra of the three metabolites from a plasma extract are shown in Fig. 4 (a, RU 56279; b, RU 56191; c and d, RU 58336). They are in good agreement with those of the reference compounds.

Pharmacokinetics and metabolism of the hydantoin, RU 58841, in the rat after i.v. administration of 10 mg/kg

Unchanged RU 58841. 5 min after injection the plasma concentrations of RU 58841 are of the order of 6 μg/ml, they then decrease rapidly; at 6 h the mean value is 0.015 μg/ml. The experimental AUC (0–6 h) is equal to 3.61 μg/ml × h and the terminal elimination half-life is of the order of 1 h [Fig. 3(b)].

Principal metabolites (see Fig. 2). The principal metabolite is the acid (RU 59416) resulting from oxidation of the primary alcohol function on the butyl side-chain. It is present from the first sampling time (5 min) at concentrations higher than those of RU 58841 (around 9 μg/ml). They continue to rise up to 2 h (mean value 32 μg/ml) then decrease. At 24 h it is no longer detectable.

The *N*-desalkyl metabolite (RU 56279) appears later. It is only detected after 4 h and shows a maximum at 6 h (mean value 0.125 μg/ml). At 24 h its

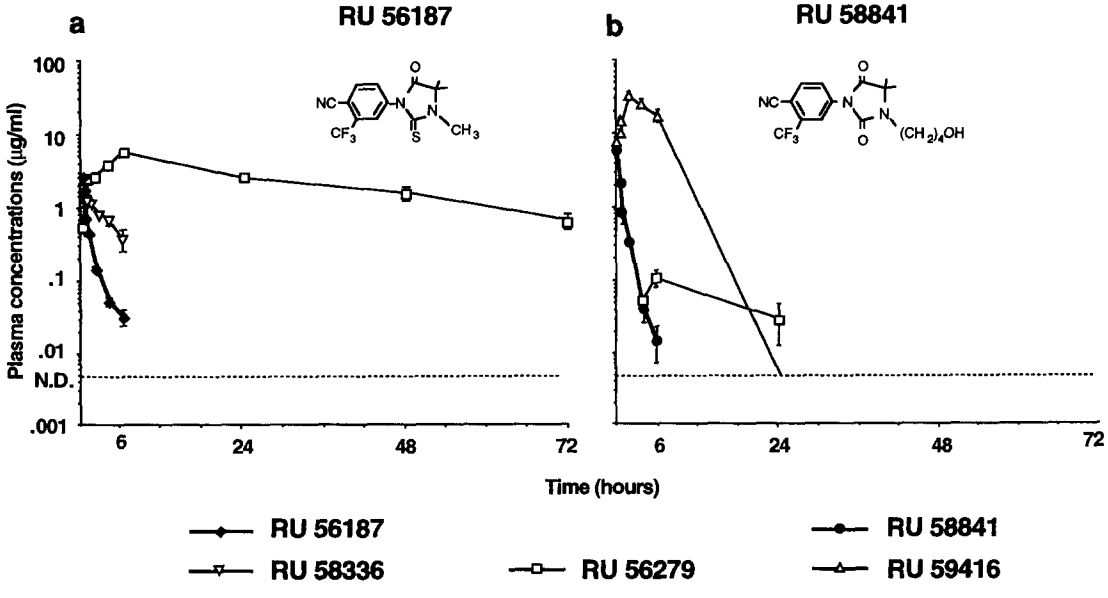


Fig. 3. Plasma concentration–time curves of parent compound and metabolites after i.v. administration of 10 mg/kg of RU 56187 (left) or RU 58841 (right). Each point represents the mean ± SEM of 4 animals.

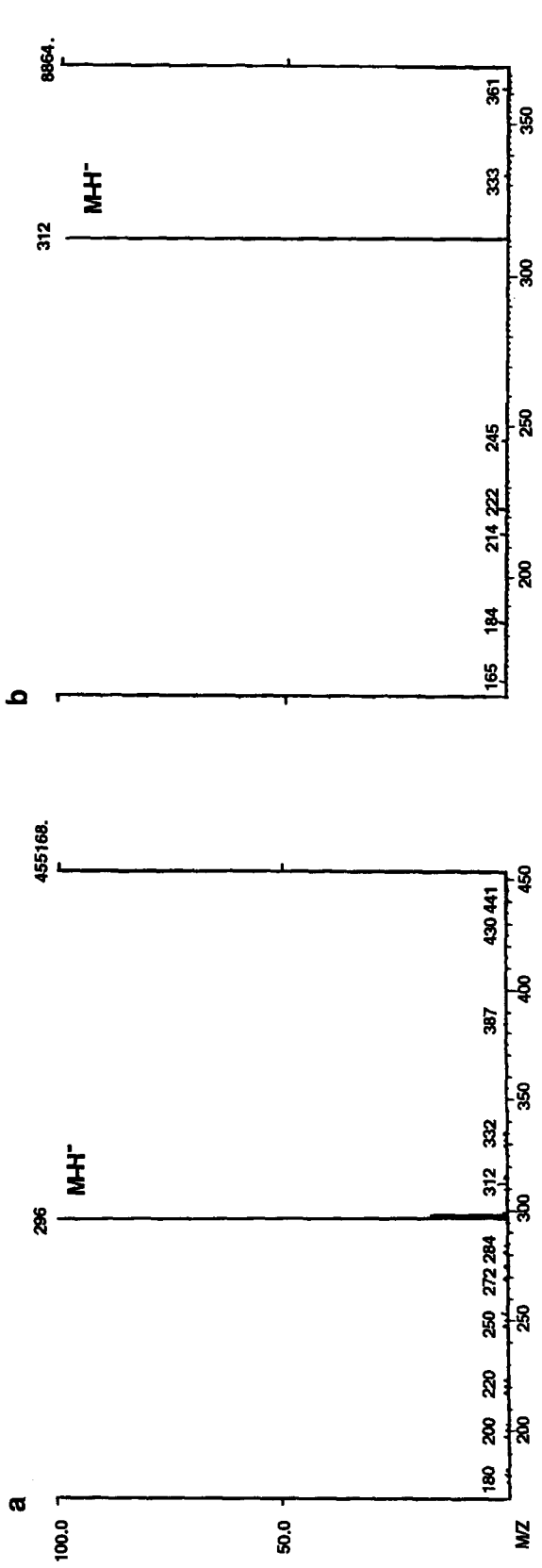


Fig. 4. Mass spectra of RU 56187 metabolites in a plasma extract: (a) RU 56279; (b) RU 56191 (both by thermospray ionisation, mode (−)); (c) RU 58836 (thermospray ionisation, mode (+)); (d) RU 58836 (thermospray discharge, mode (−)).

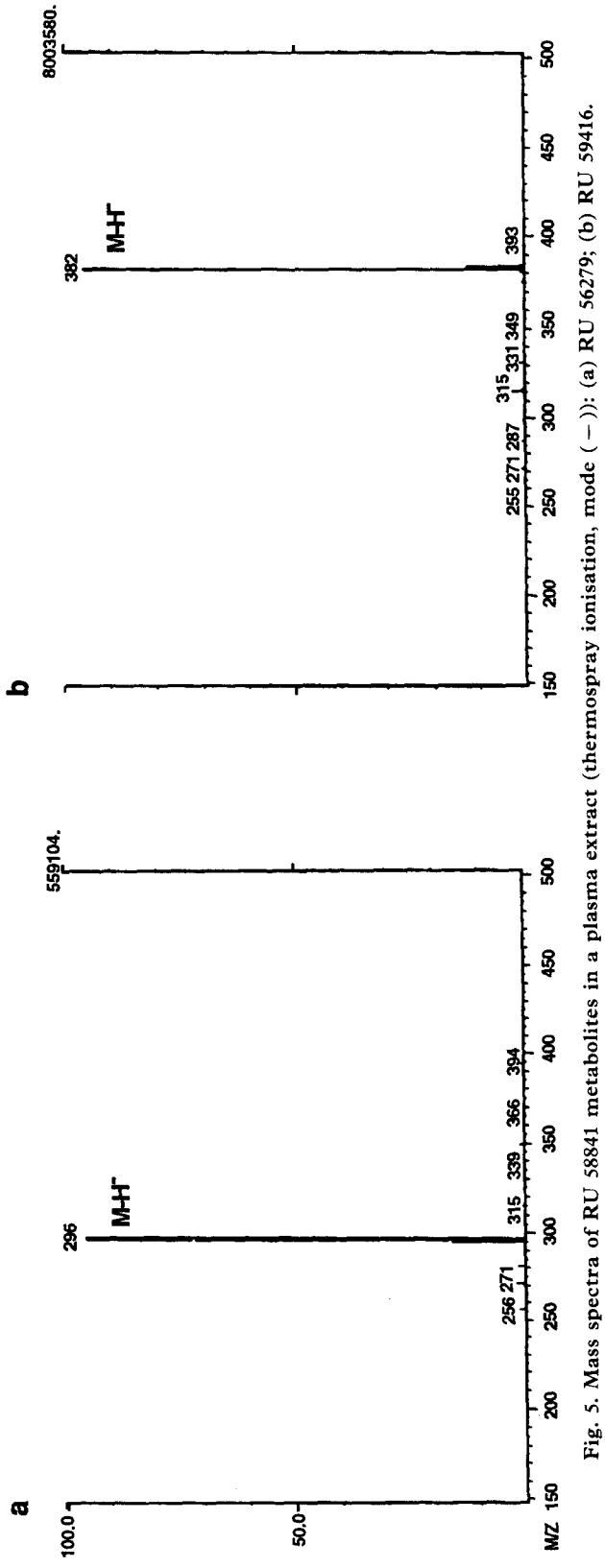


Fig. 5. Mass spectra of RU 58841 metabolites in a plasma extract (thermospray ionisation, mode (−)): (a) RU 56279; (b) RU 59416.

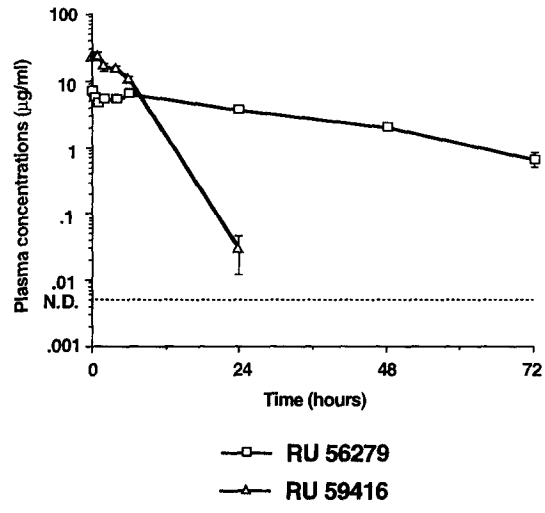


Fig. 6. Plasma concentration–time curves of RU 56279 or RU 59416 after i.v. administration of 10 mg/kg of RU 56279 or RU 59416. Each point represents the mean ± SEM of 4 animals.

concentration is about 4 times lower (0.03 µg/ml) and it is no longer detected at 48 h.

Fig. 5 represents the mass spectra of the two metabolites from a plasma extract, which are in good agreement with those of the reference compounds (a, RU 56279; b, RU 59416).

Quantification of the fraction of the common N-desalkyl-hydantoin metabolite (RU 56279) formed from RU 58841 or RU 56187

The proportion of metabolite (RU 56279) formed from RU 58841 or RU 56187 was determined by comparison of the AUCs obtained after administration of the two parent compounds or of RU 56279 itself [4].

I.v. administration of RU 56279 (10 mg/kg). The curve corresponding to the mean concentrations is shown in Fig. 6. The experimental AUC, from 0 to 72 h, is equal to 230.0 µg/ml × h and the terminal elimination half-life is of the order of 20 h.

After i.v. administration of RU 56187, the experimental AUC corresponding to RU 56279 is equal to 176.4 µg/ml × h; therefore, the proportion of metabolite formed, given by the ratio of the AUC of RU 56279 after administration of RU 56187 and the AUC of RU 56279 after administration of the metabolite itself, is equal to 77% (taking into account the difference in M.W.).

After i.v. administration of RU 58841 (10 mg/kg), the experimental AUC corresponding to RU 56279 is of the order of 2.26 µg/ml × h, thus, the proportion of metabolite formed, calculated in the same way, is about 1%.

Quantification of the fraction of acid metabolite (RU 59416) formed after administration of RU 58841

I.v. administration of RU 59416 (10 mg/kg). The curve corresponding to the mean concentrations is shown in Fig. 6. The experimental AUC from 0 to 24 h, is equal to 190 µg/ml × h and the terminal elimination

half-life is of the order of 2 h (the latter was determined approximately using only the 3 points at 4, 6 and 24 h, since the concentrations decrease very rapidly between 6 and 24 h).

The proportion of metabolite RU 59416 formed from RU 58841, given by the AUC ratios as above, is 93%.

Pharmacokinetics and metabolism of RU 56187 and RU 58841, in the rat after cutaneous administration of 10 mg/kg

Limited curves of the parent compounds and of their principal metabolites, at the same dose of 10 mg/kg, are shown in Fig. 7. Following cutaneous application of RU 56187, plasma concentrations were determined at 4, 6 and 24 h. Unchanged compound was recovered at all 3 times at very low concentrations (maximum equal to 0.014 μg/ml after 6 h); RU 56279 reached concentrations equal to 1 μg/ml after 24 h; RU 58336 was not detected. For RU 58841 and its metabolites, assays were only performed 6 and 24 h after cutaneous application: the parent compound was quantified only at 6 h (mean concentrations equal to 0.012 μg/ml); RU 56279 concentrations at 6 h are about half those obtained after i.v. administration; RU 59416 was only detected at 6 h, at concentrations equal to 1 μg/ml.

Antiandrogenic activity of RU 56187 and its metabolites by oral route

The oral activity of RU 56187 was compared with that of its metabolites RU 56279 and 59416 characterized after i.v. administration. Castration leads to a strong decrease in sex organ weight which is prevented by TP. After a chronic treatment at a dose of 1 mg/kg, RU 56187 induced 61 and 78% decreases in prostate and seminal vesicle weights respectively compared with castrated animals supplemented with TP. Concerning RU 56191, 56279 and 58336, their administration reduces prostate weights by 67, 57 and 53% and seminal vesicle weights by 89, 88 and 86% respectively (Table 1).

Table 1. Oral antiandrogenic activity of RU 56187 and its metabolites administered at 1 mg/kg, with respect to prostate and seminal vesical weights

	Prostate weight (mg)	Seminal vesicle weight (mg)
Castrated animals	19.9 ± 1.6	13.8 ± 1.8
Castrated + TP	160.1 ± 7.3	178.7 ± 9.6
Castrated + TP + RU 56187	74.0 ± 9.0*	49.3 ± 9.5*
Castrated + TP + RU 56191	52.3 ± 3.4*	19.6 ± 3.1*
Castrated + TP + RU 56279	80.7 ± 6.1*	33.0 ± 2.2*
Castrated + TP + RU 58336	83.6 ± 6.9*	31.1 ± 4.2*

Each value represents the mean of 5 animals ± SEM. *$P < 0.01$, using Dunnett's test, indicates a significant difference between the groups.

Antiandrogenic activity of RU 58841 and its metabolites by subcutaneous route

The antiandrogenic activity of RU 58841 was compared with that of its metabolites RU 56279 and 59416 identified after i.v. administration. After chronic treatment at a dose of 1 mg/kg, RU 58841 and 59416 did not induce any significant decreases in prostate or seminal vesicle weights. On the contrary, RU 56279 caused a strong decrease in these parameters, with reductions in prostate and seminal vesicle weights of 76 and 94%, respectively (Table 2).

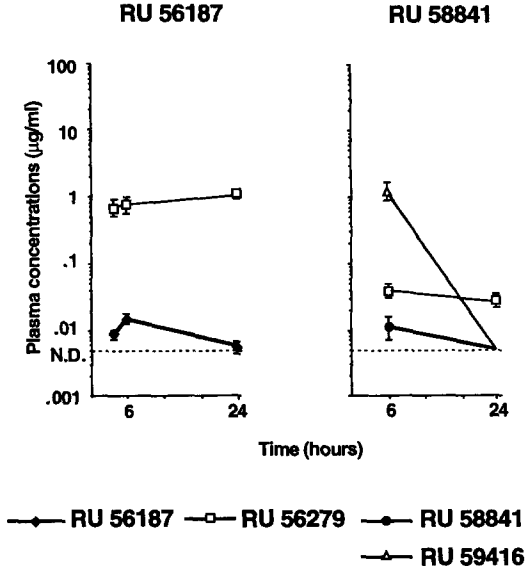


Fig. 7. Plasma concentration–time curves of parent compound and metabolites after cutaneous administration of 10 mg/kg of RU 56187 (left) or RU 58841 (right). Each point represents the mean ± SEM of 4 animals.

DISCUSSION

Antiandrogens are compounds of potential therapeutic interest for the treatment of either systemic androgen-dependent disorders such as prostate cancer or topical ones such as acne, alopecia or hirsutism [5]. Several non-steroid products (flutamide, Anandron) are available for the treatment of prostate cancer [6, 7]

Table 2. Subcutaneous antiandrogenic activity of RU 58841 and its metabolites administered at 1 mg/kg, with respect to prostate and seminal vesicle weights

	Prostate weight (mg)	Seminal vesicle weight (mg)
Castrated animals	17.7 ± 1.2	11.0 ± 0.6
Castrated + TP	169.5 ± 10.0	156.8 ± 7.1
Castrated + TP + RU 58841	146.0 ± 8.9	118.9 ± 8.4
Castrated + TP + RU 56279	47.8 ± 6.0*	19.6 ± 2.1*
Castrated + TP + RU 59416	143.3 ± 10.3	134.2 ± 11.4

Each value represents the mean of 5 animals ± SEM. *$P < 0.01$, using Dunnett's test, indicates a significant difference between the groups.

or in development (Casodex) [8] but at the moment there is no topically acting compound which is devoid of systemic effects. As a result of structure–activity studies, new molecules with dissociated effects have been developed [2].

RU 56187 which shows strong systemic inhibitory activity on accessory sex organ weights (prostate and seminal vesicles) could be used for the treatment of prostate cancer [2]. However, despite an affinity for the androgen receptor *in vitro* about 100 times higher than that of the compounds cited above, it appears to be only 3–10 times more active *in vivo* [2]. RU 58841 on the other hand, which is a powerful topical antiandrogen with respect to the hamster flank organ but with very weak systemic effects, is of interest for the treatment of acne [1]. Its affinity is in good agreement with its pharmacological profile [1].

The metabolism of this series of compounds has not yet been described. That of Anandron, a hydantoin which differs from RU 56187 by the presence of a nitro group on the benzene ring, has been described in man and in various animal species [9, 10], but its metabolites contribute very little to its activity [11]. It therefore seemed of interest to make an initial study of the principal metabolites of RU 56187 and RU 58841, in an attempt to explain their dissociated effects and the difference between the *in vivo* and *in vitro* results observed for RU 56187. Since these compounds have a very short elimination half-life (about 1 h), the difference in their activity on accessory sex organs would not seem to be related to their rate of elimination and could not explain the systemic activity of RU 56187. It could however, be explained by a metabolism product.

After i.v. administration of RU 56187, three metabolites were identified in the plasma: RU 58336 which is formed by oxidation of the thio function, RU 56191 arising from an *N*-demethylation and RU 56279 resulting from both these biotransformations. RU 56279 is the principal metabolite: about 80% of RU 56187 administered is transformed into RU 56279 which is found at relatively high concentrations. Its elimination is slow, the half-life being about 20 h. RU 58336 is present at concentrations of the order of those of RU 56187 but it is rapidly eliminated. RU 56191 was detected but not quantified. Preliminary results (not yet published) have shown that these three compounds display a low affinity for the androgen receptor. Thus, in order to investigate their activities, they were administered orally to castrated rats supplemented with testosterone propionate, an experimental model very sensitive to the systemic activities of antiandrogens [12]. After 2 weeks of treatment, they cause an important reduction in prostate and seminal vesicle weights, equivalent to that induced by RU 56187 itself. Thus the systemic activity of this compound which is not well correlated with its affinity, could be mediated by its metabolites and specially by the most important of them: the *N*-desmethyl hydantoin derivative (RU 56279). This metabolite is found at high concentration in the plasma and it remains there for a long time so, despite a low affinity, it displays a long acting antiandrogenic effect both on prostate and seminal vesicle weights.

As far as RU 58841 is concerned, two principal metabolites have been identified: RU 56279, resulting from *N*-dealkylation and RU 59416, the acid formed by oxidation of the butyl alcohol function. RU 56279 is formed in a very low proportion, representing about 1% of RU 58841 administered, compared with RU 59416, which represents 93%. RU 58841, a topically active compound has little systemic activity. Its metabolites were therefore administered s.c. so as to mimic the complete passage of the compounds through the skin and to measure their effects at the level of the prostate and seminal vesicles. In these conditions, RU 56279 causes an important reduction in prostate and seminal vesicle weights (as after oral administration). RU 59416 on the other hand does not significantly modify these parameters. Furthermore, it displays a very low affinity for the androgen receptor (preliminary results, not yet published). Thus the lack of systemic effects of RU 58841 could be explained by the fact that it is metabolized principally to a compound which is inactive on accessory sex organ weight in the rat.

Thus, the dissociation of the effects RU 56187 and 58841 would therefore seem to be related to the extent of their metabolism and to the proportion of the common *N*-desalkyl metabolite formed.

RU 58841 being at present in clinical development for the local treatment of acne, alopecia and hirsutism, its pharmacokinetics have also been studied after cutaneous administration. The same metabolites were found in the plasma but at concentrations much lower than after i.v. administration. These results showing that RU 58841 is transformed primarily into an inactive metabolite suggest that, provided that its metabolism in man is the same as in the rat, it could be used topically without the risk of affecting accessory sex organ weight.

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