Modulation of oestrogenic effects by progesterone antagonists in the rat uterus

Kristof Chwalisz[1], Klaus Stöckemann, Karl-Heinz Fritzemeier and Ulrike Fuhrmann

Research Laboratories of Schering AG, Müllerstrasse 170–178, 13342 Berlin, Germany

TABLE OF CONTENTS

Antiprogestins can modulate oestrogenic effects in various oestrogen-dependent tissues, dependent on species, tissue, dose and duration of treatment. Enhanced oestrogenic responses to mifepristone and onapristone occur in vitro and in vivo. However, the antiprogestins mifepristone, onapristone, and ZK 137 316 can block the ability of oestradiol to increase endometrial growth in non-human primates. Our purposes were firstly, to decide whether mifepristone and onapristone had direct oestrogenic activity in vitro and in the uterus of spayed and immature rats, and secondly, to discover whether antiprogestins exhibit inhibitory effects on oestrogen action in the uterus in spayed, oestrogen-substituted rats. In transactivation assays, mifepristone induced oestrogenic response, whereas onapristone had only marginal effects on reporter gene transcription. In immature rats, onapristone and mifepristone markedly increased uterine weights, and onapristone, but not mifepristone significantly enhanced endometrial luminal epithelial height, a sensitive oestrogen parameter. Conversely, in spayed and adrenalectomized rats, neither onapristone nor mifepristone changed uterine weights or endometrial morphology, indicating that their effects in immature rats were indirect. In spayed, oestrogen-substituted rats, antiprogestins did not block oestradiol-stimulated endometrial growth and luminal and glandular epithelium were stimulated more after antiprogestin plus oestrogen, than after oestradiol alone. All compounds induced compaction of the uterine stroma. In spayed rats, onapristone and some other 13α-configured (type 1) antagonists (ZK 135 569, ZK 131 535) reduced oestradiol-stimulated myometrial proliferation and induced an overall uterine weight reduction in animals treated with oestrogen and antiprogestins, in comparison with oestradiol-treated controls. 13β- configured (type II) antagonists, including mifepristone, lilopristone and ZK 112 993, were not effective. In the uteri of spayed rats, onapristone was also found to enhance the oestradiol-stimulatory effect on expression of the oestrogen-dependent proto-oncogene, c-fos. In conclusion, antiprogestins do not inhibit, but rather enhance, oestrogen-induced uterine glandular and luminal epithelium in spayed rats, contrary to their effects in primates. The rat model is unsuitable to study endometrial antiproliferative effects of antiprogestins in primate uteri.

Key words: oestrogen action/progesterone antagonists/proliferation/uterus

Introduction

11β-aryl-substituted steroidal progesterone antagonists (antiprogestins), e.g. mifepristone (RU 486), onapristone (ZK 98 299), lilopristone (ZK 98 734), ZK 112 993, and other structurally-related compounds bind with high-affinity to progesterone receptors (PR) and block progestagenic effects both in vitro and in vivo (Neef et al., 1984; Phillibert et al., 1985). Mifepristone and onapristone are the most widely-studied antiprogestins. Both also bind to glucocorticoid (GR) and androgen (AR) receptors and exhibit

[1]To whom correspondence should be addressed

antiglucocorticoid and anti-androgenic activity *in vitro* (U.Fuhrmann, unpublished results) and *in vivo*; onapristone being less antiglucocorticoid than mifepristone. These two antiprogestins show a very weak binding to human and rat oestrogen receptors (ER) (Chwalisz *et al*., 1995).

Overall control of growth and functions in the reproductive tract is regulated by oestrogen and progesterone. In the uterus, oestrogens stimulate endometrial epithelial proliferation, control many metabolic events, and are necessary for normal uterine growth. Progesterone generally inhibits oestrogen-dependent uterine epithelial proliferation and involves endometrial differentiation to the secretory type. Specific mechanisms proposed for the antiproliferative action of progesterone include: (i) a down-regulation of ER in target tissues (Hsueh *et al*., 1975; Katzenellenbogen 1980); (ii) uterine enzyme induction catalysing oestradiol conversion to less active metabolites (Tseng and Gurpide, 1975); (iii) a decrease in oestrogen-induced specific protein expression (Bhakoo *et al*., 1977); and (iv) an inhibition of oestrogen-induced proto-oncogenes (Kirkland *et al*., 1992; Fuhrmann and Stöckemann, 1993).

Antiprogestins and oestrogen responses in the uterus

Progesterone is a major sex steroid controlling oestrogen action in reproductive tracts and other oestrogen-dependent tissues. Therefore, it is not surprising that antiprogestins also interfere with various oestrogenic responses. The major concern of chronic antiprogestins administration in women is endometrial hyperplasia due to unopposed oestrogen effects. Paradoxically, antiprogestins, including mifepristone (Wolf *et al*., 1989; Slayden *et al*., 1993; Slayden and Brenner, 1994; Heikinheimo *et al*., 1996), onapristone (Chwalisz *et al*., 1994) and the new antiprogestin ZK 137 316 (Slayden *et al*., 1997), inhibit endometrial proliferation in both ovariectomized and intact monkeys. Antiprogestins administered chronically at relatively low doses inhibit mitotic activity in endometrial epithelium and induce a dose-dependent stromal compaction in spayed and intact monkeys at high oestradiol concentrations. Similar endometrial antiproliferative effects arise in ovariectomized rabbits where onapristone selectively inhibits oestrogen-induced endometrial gland formation (Chwalisz *et al*., 1991). Recently, Gemzell-Danielsson *et al*. (1998) found that low-dose mifepristone (0.5 mg daily for 3 months) delayed endometrial maturation and significantly reduced the glandular diameter in premenopausal women. These effects were accompanied by a reduction in endometrial glycodelin expression and *Dolichus biflorus* agglutinin (DBA)–lectin binding. Stromal compaction and an absence of mitoses arose with 2 mg mifepristone daily for 30 days (Cameron *et al*., 1996). In monkeys and rabbits antiproliferative effects were endometrium-specific and oestrogenic effects in the oviduct and vagina were not inhibited by antiprogestins (Chwalisz *et al*, 1991; Slayden and Brenner, 1994). The endometrial antiproliferative effects of antiprogestins in the primate endometrium is a most important property of antiprogestins offering a unique opportunity to selectively inhibit oestrogenic effects in the uterus without affecting oestrogenic response in other tissues. The mechanism underlying this endometrial antiproliferative effect is still unknown, yet it differs from the inhibitory effect of a progesterone on the endometrium.

However, uterine oestrogenic responses occur in monkeys and women after treatment with chronic, high-dose antiprogestins. Cystic endometrial hyperplasia occurred after chronic, high dose oral onapristone (50 mg/kg, Schering toxicological study, unpublished data) of intact cynomolgus monkeys. Atypical cystic changes also arose after chronic treatment of endometriotic women with a relatively high dose (50 mg daily) of mifepristone (Murphy *et al*., 1995). Recently, Croxatto *et al*. (1998) reported the occurrence of endometrial gland dilation in 34% of women treated with 1 mg/day mifepristone for 150 days. The significance of this finding is unclear, since no signs of endometrial hyperplasia were found in this study. A similar dilation of endometrial glands was frequently observed in monkeys after chronic treatment with different antiprogestins (K.Chwalisz, unpublished data). Interestingly, this effect could be observed even within an atrophic endometrium accompanied by a drastic reduction of mitotic activity in the glandular epithelium strongly suggesting that antiprogestin-induced gland dilation is due to altered glandular fluid outflow rather than to endometrial hyperplasia in non-human primates. Moreover, there are experimental studies in rats and mice which suggest that both mifepristone (Dibbs *et al*., 1995) and onapristone (Bigsby and Young, 1994) may exhibit some oestrogenic-like activities by directly interacting with ER.

These conflicting results indicate that the precise mechanism of the divergent antiprogestin effects on the endometrium are still poorly understood. These studies also show that the modulatory impact of oestrogen by antiprogestins is quite complex, since it may depend on species, tissue, antiprogestin dose and type, and duration of treatment. We describe the modulatory effect of various antiprogestins in the non-pregnant rat uterus, since rats are widely used to study oestrogenic and anti-oestrogenic activities. We specifically address the question of whether antiprogestins exert oestrogenic or antiproliferative (anti-oestrogenic) effects in the uteri of castrated rats, and whether type I (onapristone-type) and type II (mifepristone-type) antiprogestins act differently in the rat uterus. The

oestrogen-like effects of antiprogestins are described using in-vitro and in-vivo models. In addition, the modulatory effects of antiprogestins on various parameters of oestrogen action, including c-fos expression, uterine growth, morphology, and morphometry are discussed. Experiments were performed on spayed and immature rats, both commonly used to study oestrogenic effects. We also wished to find out whether rat models are of use in predicting antiproliferative endometrial effects of antiprogestins in primates. We also examined onapristone and mifepristone effects on uterine ER protein concentrations in ovariectomized rats, in the presence and absence of oestradiol.

Immature and adult Wistar rats (Schering, Berlin, Germany) were kept in Makrolon cages (type III) in an air-conditioned room at a temperature of 22 + 2°C and relative humidity of 50 + 5%, under a regime of 14 h light: 10 h dark cycle (light 6:30–20:30). The animals had free access to the standard pellet diet Altromin® (Altromin Ltd, Lage, Germany) and to tap water containing 0.9% saline.

Figure 1 shows the progesterone antagonists used in this study. The 13β-methyl-substituted (type II) antiprogestins used were: mifepristone (RU 486; ZK 95 890: 11β-[4-(dimethylamino)-phenyl]-17β-hydroxy-17β-(prop-1-ynyl)estra-4, 9-dien-3-one); lilopristone (ZK 98 734; 11β-[4-(dimethylamino)-phenyl]-17β-hydroxy-17α-(3-hydroxyprop-1-ynyl)estra-4, 9-dien-3-one). The 13α-methyl-substituted (type I) antiprogestins used were: onapristine (ZK98 299; 11β-[4-(dimethylamino)-phenyl]-17α-hydroxy-17β-(3-hydroxypropyl)-11β-13α-estra-4, 9-dien-3-one); ZK 131 535 (17α-hydroxy-17β-(3-hydroxypropl) (11β-[4-1-methylethenyl)phenyl]-13α-estra-4, 9-dien-3-one); and ZK 135 695 (11β-[4-(3-furanyl)phenyl]-17α-hydroxy-17β (3-hydroxypropyl)-13α-estra-4, 9-dien-3-one). All compounds were from Schering AG. For oral administration, the antiprogestins were formulated in 0.5 ml Myrj® 53 (ICI, Essen, Germany) saline (85 mg Myrj® 53 in 100 ml 0.9% saline). For s.c. administration, the compounds were formulated in 0.2 ml benzylbenzoate + castor oil (1:4 v/v). 17β-oestradiol and the pure anti-oestrogen ICI 182 780 (ZK 156 901) were formulated in 0.2 ml benzyl benzoate plus castor oil (1:4 v/v).

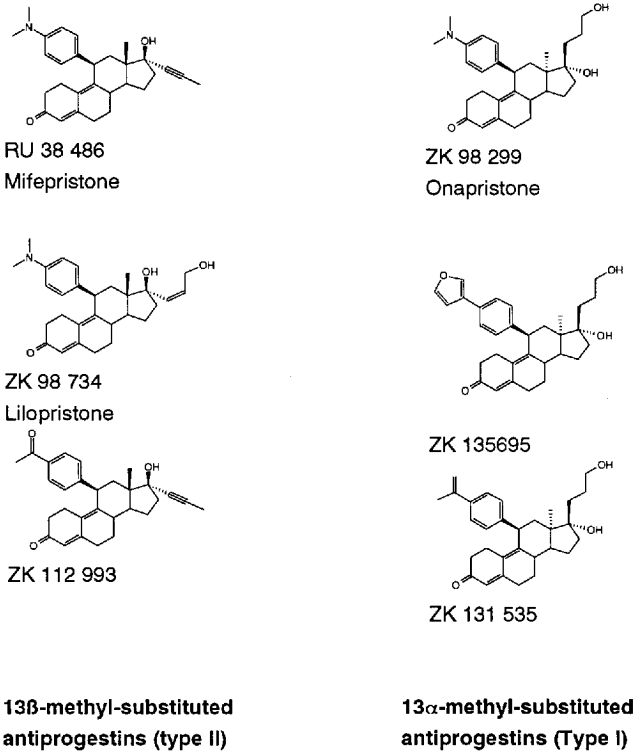

Figure 1. Structure of 13α- (type I) and 13β-methyl substituted (type II) antiprogestins used in the experiments.

Oestrogenic effects of anti-progestins *in vitro* and *in vivo*

Oestrogen-like activities of onapristone and mifepristone

Effects of onapristone and mifepristone on the oestrogen-responsive reporter gene VITtk-LUC in MVLN cells expressing human endogenous ER

MVLN cells (MCF-7 cells stably transfected with Vitellogenin-Luciferase-reporter gene and Neomycin resistance gene) were cultured in Dulbecco's modified Eagle's medium (DMEM) without Phenol Red, supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine, penicillin, and streptomycin. To study hormonal effects, MVLN cells were trypsinized, pooled and replated onto 96-well dishes at a density of 1.2 ψ 10^4 cells/well. Cells were cultured in medium supplemented with 3% charcoal-stripped FCS in the presence of 10^{-9} M of the anti-oestrogen ICI 182 780 to reduce high background and the appropriate compound. Cells cultured in 1% ethanol were used as negative controls for reporter gene induction. Transactivation assays were carried out at least three times. The Luc Assay was performed using the Promega kit.

Oestrogenic-like effects of onapristone and mifepristone in immature rats

Rats aged 21 days (body weight ~50 g) were randomly allocated to nine groups (n = 5/group) and treated s.c. for 3 consecutive days as follows: group 1, vehicle; group 2, oestradiol 0.1 μg/rat; group 3, onapristone 500 μg/rat; group 4: ICI 182 780 (ZK 156 901, 500 μg/rat); group 5, oestradiol plus ICI 182 780 (0.1 μg/rat and 500 μg/rat respectively); group 6, onapristone plus ICI 182 780 (500 μg/rat each); group 7, onapristone plus oestradiol (500 μg/rat and 0.1 μg/rat respectively); group 8,

mifepristone (RU 486) at 500 μg/rate. Oestradiol doses induced a submaximal (50–60%) stimulation of uterine growth. During autopsy, the uteri were carefully excised from surrounding tissue, weighed and placed in Bouin's solution for histology, morphometric analysis, and proliferating cell nuclear antigen (PCNA) staining.

Effects of onapristone and mifepristone on selected parameters of oestrogen action in ovariectomized and adrenalectomized adult rats

Adult female rats (n = 40; body weight 170–180 g) were ovariectomized and adrenalectomized under ether anaesthesia. An additional group of 10 rats remained intact until the start of experiment (group 1, intact controls). At 12 days after surgery the ovariectomized and adrenalectomized animals were randomly allocated to four groups and treated s.c. for 15 consecutive days with either vehicle (group 2, ovariectomized plus adrenalectomized controls), oestradiol (0.3 μg/rat; group 3), onapristone, 10 mg/rat (group 4), and mifepristone 10 mg/rat (group 5). During autopsy, which took place 1 day after cessation of treatment, the uteri and vaginae were carefully excised from the surrounding tissue, weighed and placed in Bouin's solution for histological and morphometric analysis.

Modulation of oestrogenic effects by onapristone and mifepristone

Effects of onapristone on the oestrogen-induced expression of c-fos in the rat uterus

Adult female Wistar rats (225–250 g body weight) were ovariectomized and randomly allocated to seven experimental groups (six rats per group). At 10 days after ovariectomy the animals were treated s.c. as follows: group 1, vehicle, 0.2 ml; group 2, oestradiol, 3 μg/rat; group 3, oestradiol plus progesterone, 3 μg/rat and 3 mg/rat respectively; group 4, oestradiol plus onapristone, 3 μg/rat and 10 mg/rat respectively; group 5, oestradiol plus onapristone plus progesterone, 3 μg/rat, 10 mg/rat, and 3 mg/rat respectively; group 6, progesterone, 3 mg/rat, group 7, onapristone, 10 mg/rat. At 2 h after the treatment the animals were decapitated and the uteri were removed for RNA preparation.

Effects of onapristone and mifepristone on oestrogen receptor (ER) protein in the uterus of ovariectomized rats

With the exception of group 1 (intact controls, n = 10), all other rats were ovariectomized under ether anaesthesia 14 days prior to the experiment, randomly allocated to nine groups and treated s.c. for 3 days as follows: group 2, vehicle (n = 25); group 3, oestradiol (0.3 μg/rat/day) plus vehicle (n = 5); group 4, onapristone (10 mg/rat/day) plus vehicle (n = 15); group 5, mifepristone (10 mg/rat/day) plus vehicle (n = 15); group 6, oestradiol plus onapristone (1 mg/rat, n = 10); group 7, oestradiol plus onapristone (3 mg/rat/day; n = 10); group 8, oestradiol plus onapristone (10 mg/rat/day; n = 10); group 9, oestradiol plus mifepristone (10 mg/rat/day; n = 15). The animals were killed 24 h after the last treatment and the whole uteri were removed for ER measurements.

Effects of various type I and type II antiprogestins on selected parameters of oestrogenic action in ovariectomized, oestradiol-substituted rats

Adult Wistar rats were ovariectomized under ether anaesthesia at least 11 days before the experiment. The rats were then randomly allocated to treatment and control groups and treated for 3 consecutive days with a substitution dose of oestradiol (0.3 μg/rat s.c.) in combination with oral treatment of various antiprogestins including onapristone, mifepristone (RU 486), lilopristone (ZK 95 734) and ZK 122 993 (1, 3, and 10 mg/rat each). During autopsy, which was performed ~24 h after the last treatment, the uterine wet weights were measured and the uteri were fixed in Bouin's solution for histological and morphometric analysis. In a separate experiment the effects of two additional 13α-configurated compounds (ZK 131 535, ZK 135 695) were studied. (see Figure 7 for details).

Molecular and morphometric analyses

RNA preparation and Northern blot analysis

Total RNA was prepared according to Maniatis et al. (1982). Briefly, RNA was extracted from the uteri by immediate homogenization in a buffer containing 4 M guanidinium thiocyanate using a Polytron homogenizer (Kinematic AG, Littau, Switzerland). Uteri from each group were pooled for the preparation of each RNA sample. RNA was purified by ultracentrifugation through 5.7 M CsCl dissolved in SET buffer (10 mM Tris–HCl, pH 7.4) containing 5 mM EDTA, and 0.1% sodium dodecyl sulphate) and precipitated with ethanol. Poly $(A)^+$ RNA was separated from total RNA by affinity chromatography on oligo(dT)-cellulose columns (Pharmacia, Freiburg, Germany). Northern blot analysis was carried out according to Maniatis et al. (1982), applying 5 μg poly$(A)^+$ RNA per lane. Blots were hybridized with [^{32}P]-labelled cDNA probes for c-fos (Dianova, Hamburg, Germany). To assure that a constant amount of RNA was loaded, the blots were re-hybridized with the cDNA probe for 1A (subunit of cytochrome C

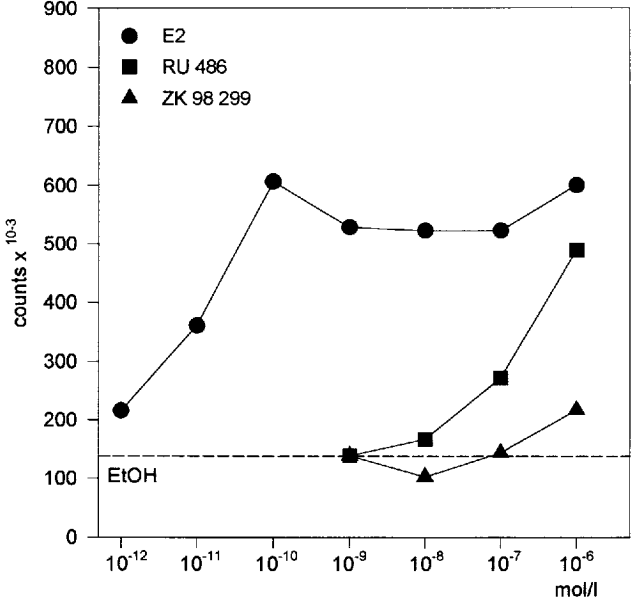

Figure 2. Effects of onapristone and mifepristone (RU 486) in the transactivation assay. MVLN cells expressing endogenous human ER and stably transfected with VITtk-LUC were cultured in the absence (EtOH control) and presence of increasing amounts of oestradiol (E2), mifepristone (RU 486) and onapristone (ZK 98 299). Note the weak oestrogenic activity of mifepristone.

oxidase; M.Lessl, unpublished data) a ubiquitous mRNA in the rat uterus whose expression is not regulated by steroid hormones.

Assay for total (cytosolic and nuclear) ER

After excision, rat uteri were deep frozen in liquid nitrogen and stored at –805C until use. Receptor determination was performed essentially as described earlier (Chwalisz et al., 1991). Briefly, 1 g frozen tissue was broken up in a microdismembrator (Braun, Melsungen, Germany). The powder was suspended (5 ml/g) in homogenization buffer (10 mM Tris–HCl, pH 7.4, 1.5 mM EDTA, 0.4 mM KCl, 5 mM $NaMoO_4$, 10% glycerol) and was homogenized with a Polytron homogenizer. The homogenate was incubated at 05C for 60 min. During incubation the slurry was gently stirred. After that the homogenate was centrifuged for 90 min at 100 000 g. Aliquots of the supernatant were diluted to a protein concentration of 1 mg/ml and were analysed by an ER enzyme immunoassay kit from Abbott Laboratories, (Chicago, IL, USA) in accordance with the manufacturer's instructions.

Immunohistochemistry

Tissue sections were prepared from Bouin-fixed paraffin-embedded uterine samples. Proliferation was studied by using a monoclonal antibody against PCNA and by applying the avidin–biotin–peroxidase (ABC) technique. After deparaffinization and washing in methanol, the slides were incubated in 3% hydrogen peroxide and diluted in methanol for 15 min. Non-specific binding of avidin/biotin reagents was prevented by using a blocking kit (Vector Laboratories, Burlingame, CA, USA) for 20 min. Thereafter the slides were incubated with the specific mouse monoclonal antibody against PCNA (DAKO–PCNA; PC Dako, Glostrup, Denmark), diluted 1:100, for 60 min at room temperature. The sections were then incubated with a biotinylated sheep anti-mouse immunoglobulin (Ig)G (RPN 1061; Amersham Life Science, Braunschweig, Germany, diluted 1:300) for 60 min at room temperature, and followed by incubation with avidin DH–biotinylated horseradish peroxidase H complex (Vectastain Elite ABC Kit; Vector Laboratories) for 60 min at room temperature in accordance with the manufacturer's instructions. Finally, the sections were developed in a substrate solution of 0.05% diaminobenzidine tetrachloride and 0.01% hydrogen peroxide. Slides were then washed in tap water, dehydrated in ethanol, cleared in xylene and mounted in DPX. Control sections were prepared by substituting the primary antibody with unspecific mouse IgG.

Morphometric evaluation

Measurements of the luminal epithelial height and luminal perimeter were performed on haematoxylin/eosin-stained paraffin sections, which were sectioned vertically to the longitudinal axis using Axioplan II microscope (Carl Zeiss GmbH, Jena, Germany) and Vidas 2.0 (Kontron Electronics, Eching, Germany) software. A double-sided t-test ($\alpha = 0.5$) was used for the statistical comparison of the treatment and the corresponding control groups with respect to various parameters.

Oestrogen-like activities of onapristone and mifepristone

Effects of onapristone and mifepristone in transactivation assays in vitro

To determine the oestrogenic activity of mifepristone and onapristone, MVLN cells expressing endogenous ER and stably transfected with VITtk-LUC were used. ER-mediated activity was investigated by treating the cells

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.