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Towards non-surgical therapy for uterine fibroids: catechol-O-methyl transferase inhibitor shrinks uterine fibroid lesions in the Eker rat model

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BACKGROUND: Uterine leiomyomas (fibroids) are the most common pelvic tumors in women. We assessed the potential therapeutic utility of Ro 41-0960, a synthetic catechol-*O*-methyl transferase inhibitor (COMTI), in the Eker rat.

METHODS: We randomized uterine fibroid-bearing Eker rats for treatment with Ro 41-0960 (150 mg/kg/12 h) versus vehicle for 2 and 4 weeks. The fibroids were measured by caliper and subjected to histological evaluation. Urinary levels of 2-hydroxy estrogen (E_2), 16-hydroxy E2 and DPD (osteoporosis marker) and serum liver enzymes were evaluated. Expressions of *Cyclin D1*, proliferating cell nuclear antigen (*PCNA*), Poly [ADP-ribose] polymerase1 (*PARP1*), tumor suppressor gene (*P53*) and transforming growth factor (*TGFβ3*) were assessed in fibroids using immunohistochemical analysis or RT−PCR. Apoptosis was confirmed using terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL).

RESULTS: Ro 41-0960-treated rats exhibited fibroid volumes of $86 \pm 7\%$ and $105 \pm 12\%$ of initial burden, at 2 and 4 weeks post-treatment, respectively, significantly lower than control group ($240 \pm 15\%$ and $300 \pm 18\%$; $P < 0.01$). Ro 41-0960 increased the urinary 2-hydroxy E2/16-hydroxy E_2 ratio, level of *p53* mRNA and TUNEL positivity ($P < 0.05$) and decreased *PARP1*, *PCNA* and *cyclin D1* proteins and *TGFβ3* mRNA ($P < 0.05$). Ro 41-0960 did not change normal tissue histology, liver functions or urinary DPD level.

CONCLUSIONS: Ro 41-0960 (COMTI) arrested growth/shrunk uterine fibroids in Eker rats. This result may be related to modulation of estrogen-dependent genes involved in apoptosis, proliferation and extracellular matrix deposition via accumulation of 2-hydroxy estrogen. The efficacy and safety of Ro 41-0960 in rats suggest its candidacy for treatment of uterine fibroids.

Key words: uterine leiomyomas / catechol-*O*-methyl transferase inhibitor / Eker rats / estradiol / catechol estrogen

Introduction

Uterine leiomyomas, commonly known as fibroids, are the most common pelvic tumors in women, symptomatically affecting at least 25% of women of reproductive age (Healy *et al.*, 1986; Lumsden and Wallace, 1998). Moreover, the prevalence of uterine leiomyomas may be >77% overall (i.e. presence of symptomatic and asymptomatic fibroids in women of reproductive age; Cramer and Patel, 1990). Uterine leiomyomas are the most common indication for hysterectomy in the USA (Wilcox *et al.*, 1994), and they commonly cause severe irregular prolonged menstrual bleeding, infertility and abortion (Hart *et al.*, 2001; Surrey *et al.*, 2001).

The current treatment options for leiomyomas are far from satisfactory, and the gold standard treatment remains surgical removal through either hysterectomy or myomectomy.

Currently, there is no optimal medical treatment option which has been approved by the Federal Drug Agency for uterine leiomyomas. Non-surgical treatment of leiomyomas has been primarily through the use of GnRH agonists that suppress circulating levels of estradiol (E_2) and progesterone (Al-Hendy and Salama, 2006a). Unfortunately current treatment options have serious drawbacks, including the severe—adverse side effects associated with GnRH agonist (GnRHa), such as osteoporosis and menopausal symptoms (Andreyko *et al.*, 1987; Al-Hendy and Salama, 2006a), and loss of procreative

potential as well as the cost and post-operative complications of surgical approaches (VeKaut, 1993; Payne and Haney, 2003; Al-Hendy and Salama, 2006a).

The role of ovarian hormones in the pathogenesis of uterine leiomyoma is well established (Otubu *et al.*, 1982; Maruo *et al.*, 2004). E_2 and its metabolites play a critical role in initiating and promoting leiomyoma growth (Dixon *et al.*, 2002; Yue *et al.*, 2003). Furthermore, there is compelling evidence that endogenous levels of catechol estrogen (CE) metabolites are significantly different between the uterine leiomyoma and adjacent normal myometrium (Reddy *et al.*, 1981). Consequently, estrogen-metabolizing enzymes may be exploited as a potential therapeutic target for the non-surgical treatment of uterine fibroids.

Catechol-*O*-methyl transferase (COMT) metabolizes many endobiotics, including CE (Creveling, 2003). The COMT enzyme catalyzes the conversion of hydroxylated estrogen metabolites (2-hydroxy E2 and 4-hydroxy E2) into their methylated counterparts. We and others have demonstrated that 2-hydroxy E2, a substrate for COMT, exhibits an anti-estrogenic effect in multiple biological systems including human uterine cells (Vandewalle, 1989; Bradlow, 1996; Wentz *et al.*, 2006). We have also demonstrated that the decrease or inhibition of COMT activity is associated with decreased clearance of 2-hydrox E2, leading to a hypoestrogenic milieu and may result in the inhibition of human leiomyoma cell growth *in vitro* (Salama *et al.*, 2006). In addition, 2-methoxy E2, a product of COMT action, may have a mitogenic effect in several cell systems (Banerjee *et al.*, 2003; Liu and Zhu, 2004). Additionally, our previous laboratory studies demonstrated that COMT expression is higher in human leiomyoma tissue compared with adjacent normal myometrium (Salama *et al.*, 2006). Therefore, the inhibition of COMT activity may indirectly modulate the biological effects of E_2 and produce an imbalance between E_2 and its metabolites, which may favor leiomyoma shrinkage.

Female Eker rats, which carry a germline mutation in the tuberous sclerosis-2 tumor suppressor gene, develop uterine leiomyomas with high frequency by 12–16 months of age (Everitt *et al.*, 1995). Eker rats exhibit uterine fibroid lesions that are benign and share many of the same phenotypic, histological and pathological characteristics as the cognate human disease (Everitt *et al.*, 1995; Howe *et al.*, 1995). Therefore, we and others have used the Eker rat experimentally as a model system for human uterine leiomyoma to study the role of hormones in fibroid development and to test the utility of new therapeutic modalities (Everitt *et al.*, 1995; Howe *et al.*, 1995; Hassan *et al.*, 2009, 2010).

The present study was designed to assess the potential therapeutic benefits of Ro 41-0960, a synthetic highly specific COMT inhibitor (COMTI), on uterine leiomyoma lesions in the Eker rat as a preclinical model for developing medical non-invasive treatment of uterine fibroids in women.

Materials and Methods

Drug

The specific, selective COMT inhibitor Ro 41-0960 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ro 41-0960 was suspended in saline using a few drops of Tween 20 just before injection.

Animals and treatment protocol

Female Eker rats from our own breeding colony, which was started with breeders provided by Dr Cheryl L. Walker's closed colony (M.D. Anderson Cancer Center, Smithville, TX, USA) were used. Uterine tumors were initially confirmed and measured by calipers using survival surgery and also at the time of euthanization. Twenty-four, 14–16-month-old rats bearing uterine fibroids were then randomized to receive s.c. injection with either COMTI (Ro 41-0960) at 150 mg/kg twice per day for 28 days (treated group) (Wentz *et al.*, 2007) or vehicle (negative control group). Animals were observed daily for any post-treatment complications. Total 24-h urine samples were collected daily during the treatment course using special metabolic cages. Sample rats (6/group) were selected randomly and euthanized at the following time points: 14 days post-treatment and 28 days post-treatment (end of the experiment). All animals were euthanized by CO_2 asphyxiation. Blood was collected using cardiac puncture, and tissue samples were harvested from the tumor and the following organs: uterus, vagina, cervix, ovary, liver, kidney, lungs, brain and long bones. The care and handling of the rats were in accord with guidelines from the National Institutes of Health and the Association for the Accreditation of Laboratory Animal Care-accredited facilities, and all of the protocols involving the use of these animals were approved by the local Institutional Animal Care and Use Committees.

Histopathological evaluation of tumor tissue

Each tumor tissue was divided into two parts: one part was formalin-fixed and paraffin-embedded and subjected to hematoxylin and eosin (H&E) histological analysis using standard protocols (Bancroft and Stevens, 1996) and was also used for immunohistochemical analysis of proteins that control proliferation and apoptosis; the other part was immediately preserved in liquid nitrogen for later studies. Cell proliferation was assessed in the paraffin-embedded tissue preparations by detection of proliferating cell nuclear antigen (PCNA) and cyclin D1 protein using rat-specific monoclonal antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA) at 1:6000 and 1:3000 dilutions, respectively. Apoptosis was evaluated with the ApopTag Kit (Intergen, Purchase, NY, USA) following the manufacturer's instructions. The death substrate Poly (ADP-ribose) (PAR) polymerase (PARP1) was also evaluated using a rat-specific anti-PARP1 antibody at 1:2000 dilution.

The percentage of apoptotic cells and the protein levels were established by counting the number of ApopTag-labeled nuclei or the number of positively stained cells (brown in color) in three $400\times$ microscopic fields for each tissue, and these numbers were compared with the total number of cells. At least 2000 cells were counted per specimen, and the numbers shown represent the average of three counts.

Determination of urinary levels of 2-hydroxy and 16-hydroxy estrogen metabolites using enzyme immunosorbent assay

The level of estrogen metabolites 2- and 16-hydroxy E2 were estimated using competitive solid-phase enzyme immunoassays (EIA) ESTRAMET 2/16 KIT (Immuna Care Corporation, Sentry Parkway, PA, USA) according to the supplier's protocol. The values of the estrogen metabolites were normalized against the urinary creatinine content measured in our local clinical chemistry laboratory.

RT–PCR analysis

Total RNA was isolated from the tumor tissues preserved in liquid nitrogen using the RNAqueous Kit (Ambion Inc, Austin, TX, USA). The first-strand cDNA was synthesized from 2 μg total RNA using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, USA). PCR

amplification of transforming growth factor (*TGFβ*3) and tumor suppressor gene *P*53 were performed using a rat-specific TaqMan Gene Expression Assay (Applied Biosystem, Foster City, USA) according to the supplier's protocol. Glycerol-3-phosphate dehydrogenase (*G3PDH*) was used as an endogenous control. The potential effects of COMTI on mRNA levels were calculated as described earlier (Pfaffl, 2001; Salama *et al.*, 2007; Othman *et al.*, 2007) and were normalized against the *G3PDH* mRNA level, and changes resulting from COMTI treatment are presented as fold-change compared with control animals.

Evaluation of the safety of COMTI treatment in the Eker rat model

Samples from a wide variety of tissues, including the uterus (myometrium and endometrium), ovary, cervix, vagina, liver and long bones (estrogen-responsive tissues) as well as other organs, such as spleen, lung, kidney and brain, were collected and sent for histopathological assessment by a pathologist (blinded to the assignment of treatment) for signs of tissue damage, inflammation, necrosis or other pathological changes using H&E staining (Bancroft and Stevens, 1996). Blood samples were centrifuged for 10 min at 2000*g* to obtain the serum. Serum samples were carefully placed into clean dry Wassermann tubes for the determination of liver function tests [aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin] using standard techniques (Reitman and Frankel, 1957; Sbrana *et al.*, 2006).

Deoxypyridinoline (DPD) cross-links, a marker of bone resorption, was determined by a sensitive competitive EIA in a microtiter stripwell format utilizing a monoclonal anti-DPD-antibody coated on a strip to capture the DPD using the Metra DPD Kit (Quidal corporation, San Diego, Cal, USA) according to the supplier's instructions and the method described previously (Acil *et al.*, 1996; Seibel *et al.*, 1998). DPD was expressed in nano-mole/millimole creatinine.

Statistics

The data are presented as mean $\pm$ SE. Results were analyzed using two-tailed unpaired *t*-tests (Sigma Stat version 3; SPSS Inc., Chicago, IL, USA); $P < 0.05$ was considered statistically significant.

Results

Ro 41-0960 (COMTI) shrinks Eker rat uterine leiomyoma

As shown in Fig. 1, the Eker rats treated with s.c. injection of Ro 41-0960 (COMTI) at a dose of 150 mg/kg twice/day exhibited significant shrinkage or slowing of growth of the total tumor volume compared with the vehicle-treated group ($P < 0.01$). Animals treated with Ro 41-0960 (COMTI) exhibited a uterine fibroid volume of 86 $\pm$ 7% and 105 $\pm$ 12% of pretreatment volume at 2 and 4 weeks post-treatment, respectively. Conversely, tumor size in the control animals continued to increase and reached 240 $\pm$ 15% and 300 $\pm$ 18% of pretreatment size at the 2- and 4-week time points, respectively.

Ro 41-0960 (COMTI) changes uterine leiomyoma architecture in the Eker rat model

H&E sections of fibroid lesions prepared from control Eker rat groups and stained with H&E showed bundles of smooth muscle cells of
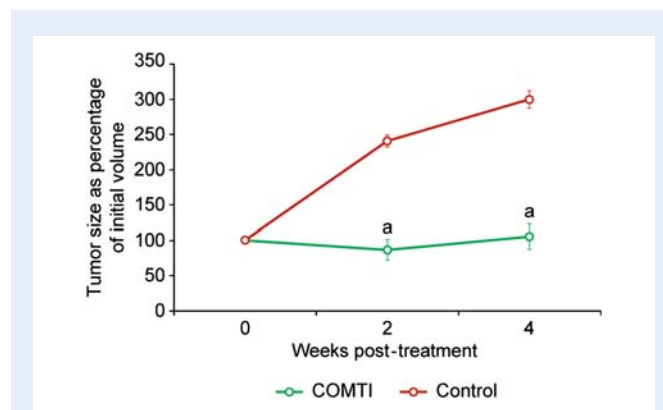

Figure 1 Treatment with Ro 41-0960, a synthetic COMTI, in the Eker rat shrinks total leiomyoma volume compared with vehicle treatment. Tumor volume was calculated as a percentage of the corresponding day zero (pretreatment) volume and presented as mean $\pm$ SE of six animals at each time point. a: indicates a significant difference between the control and treated animals ($P < 0.01$).

various sizes running in different directions. Those muscle cells that were cut longitudinally showed cylindrical nuclei, eosinophilic cytoplasm and ill-defined cell outlines. The nuclei were uniform, and the mitotic figures were absent or sparse. The cells that were transversely cut had nuclei that appeared to be circular with clear surrounding cytoplasm. The muscle fibers were intermixed with fibrous connective tissue (Fig. 2). Conversely, the H&E sections of Ro 41-0960-treated rats showed bundles of edematous smooth muscle cells. The nuclei were smaller and rounded to oval, in shape. No mitotic activity was seen and the cellularity was remarkably decreased (Fig. 2).

Ro 41-0960 (COMTI) treatment induces apoptosis in Eker rat uterine leiomyoma

As shown in Fig. 3A, Ro 41-0960 (COMTI) induced a significant decrease in death substrate PARP1 protein at 2 and 4 weeks compared with control animals at the same time points. At the 2- and 4-week time points, the percentage of PARP1-positive cells were 42 $\pm$ 2.5% and 18 $\pm$ 1% compared with 73 $\pm$ 2% and 69 $\pm$ 2.75% in control tissues, respectively.

We further evaluated apoptosis induction using the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) test. The Ro 41-0960 (COMTI)-treated fibroid lesions exhibited a strong positive TUNEL reaction: 25 $\pm$ 2% and 40 $\pm$ 3% at 2 and 4 weeks, respectively. This positivity was significantly higher ($P < 0.05$) compared with that of the vehicle-treated group at the 2- and 4-week time points (2.5 $\pm$ 0.7% and 2.8 $\pm$ 1.5%, respectively; Fig. 3B).

As shown in Fig. 3C, Ro 41-0960 (COMTI) significantly increased *P53* mRNA levels by 1.22 $\pm$ 0.09 and 1.9 $\pm$ 0.09-fold over the vehicle control ($P < 0.05$) at 2 and 4 weeks after treatment, respectively.

Ro 41-0960 (COMTI) inhibits the expression of proliferation- and extracellular matrix-related genes

Immunohistochemical analysis using rat-specific antibodies against PCNA and cyclin D1 showed that treatment of Eker rat with Ro
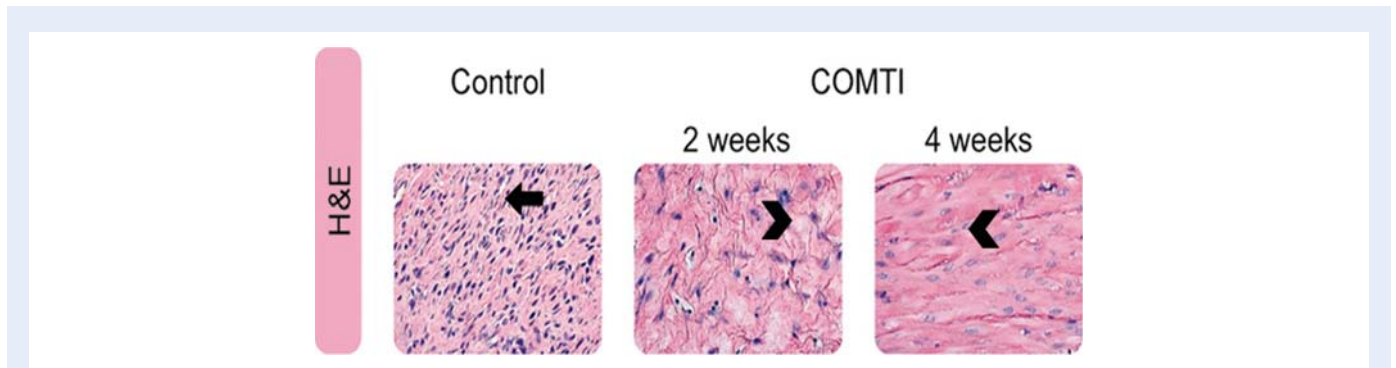
Figure 2 Representative micrographs of fibroid lesions (hematoxylin-eosin stain) from vehicle control and Ro 41-0960 (COMTI)-treated Eker rats at 2 and 4 weeks. Original magnifications 40×. Nuclei in control rats are well defined with clear nuclear membranes (arrow). Tissue generally demonstrates high cellularity. After 2 and 4 weeks of treatment with Ro 41-0960 (COMTI), tissues demonstrate less cellularity and mostly consist of ECM. The few cells remaining are distorted in shape with irregular cellular and nuclear membranes (arrow heads). The control micrograph representing the 4-week time point.
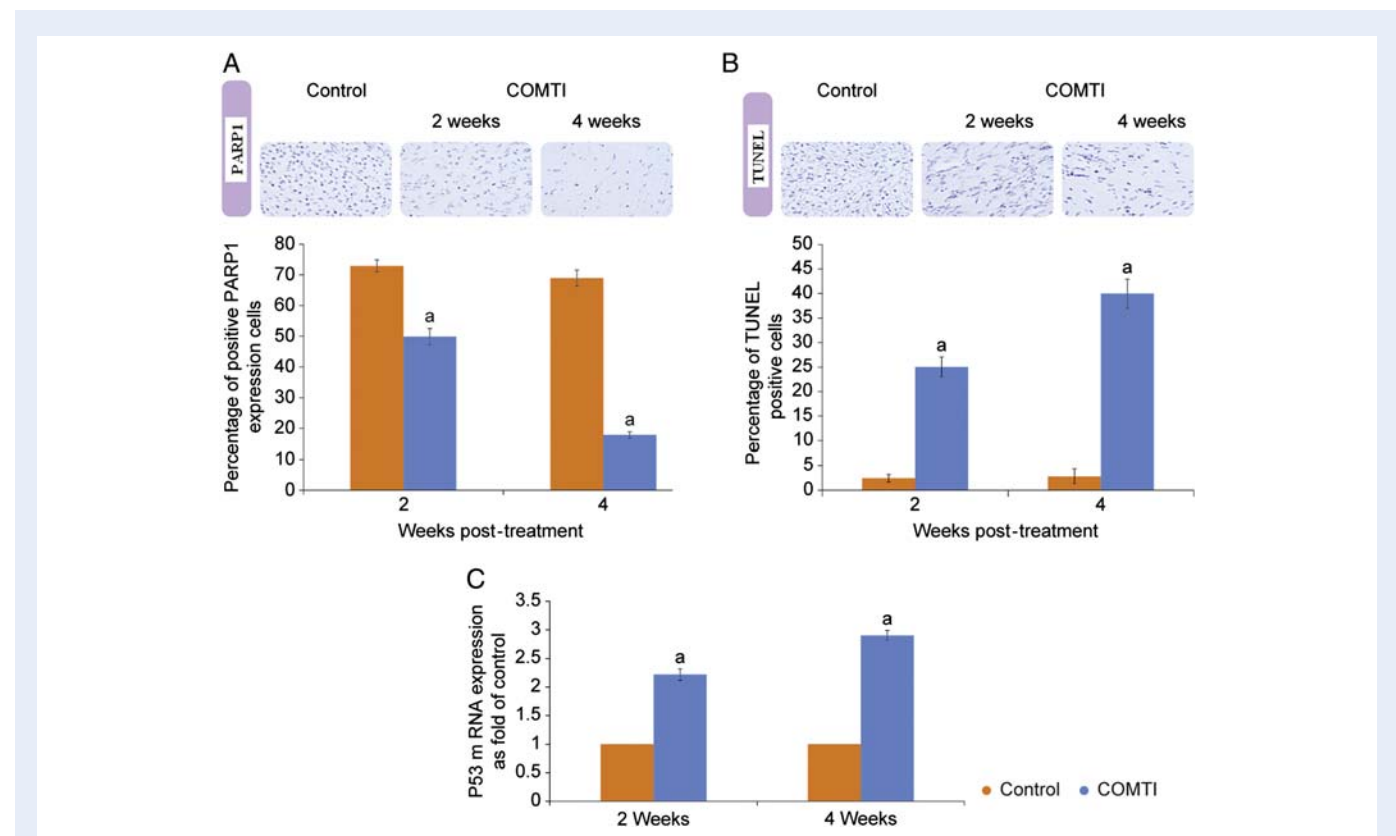

Figure 3 Ro 41-0960 (COMTI) treatment of Eker rats modulates *P53* and *PARP1* apoptotic pathways. Data represent uterine leiomyoma tissues collected at 2 and 4 weeks after Ro 41-0960 treatment compared with those of the vehicle-treated group: (**A**) Ro 41-0960 decreases the death substrate poly (ADP-ribose) (PAR) polymerase (PARP1) protein as assessed by immunohistochemistry; original magnification: 20×; (**B**) Ro 41-0960 increases the percentage of TUNEL-positive cells; original magnification: 20×; and (**C**) Ro 41-0960 increases *P53* mRNA levels. a: indicates a significant difference between the vehicle versus treated rats at $P < 0.05$, using a two-tailed Student's *t*-test. Control data are from the 4-week time point.

41-0960 (COMTI) resulted in a significant ($P < 0.05$) decrease in the expression of proliferation-related genes PCNA and cyclin D1 in sections of uterine leiomyoma tissue collected at 2 and 4 weeks post-treatment, compared with the control. The percentages of PCNA-positive cells in the tumor sections prepared from Ro

41-0960-treated animals euthanized after 2 and 4 weeks post-treatment were $47 \pm 2.4\%$ and $38 \pm 2\%$ compared with $80 \pm 4\%$ and $72 \pm 3.5\%$ in the control animals, respectively ($P < 0.05$; Fig. 4A).

Interestingly, the sections from Ro 41-0960 (COMTI) -treated animals showed $35 \pm 2\%$ and $18 \pm 0.95\%$ cyclin D1-positive cells
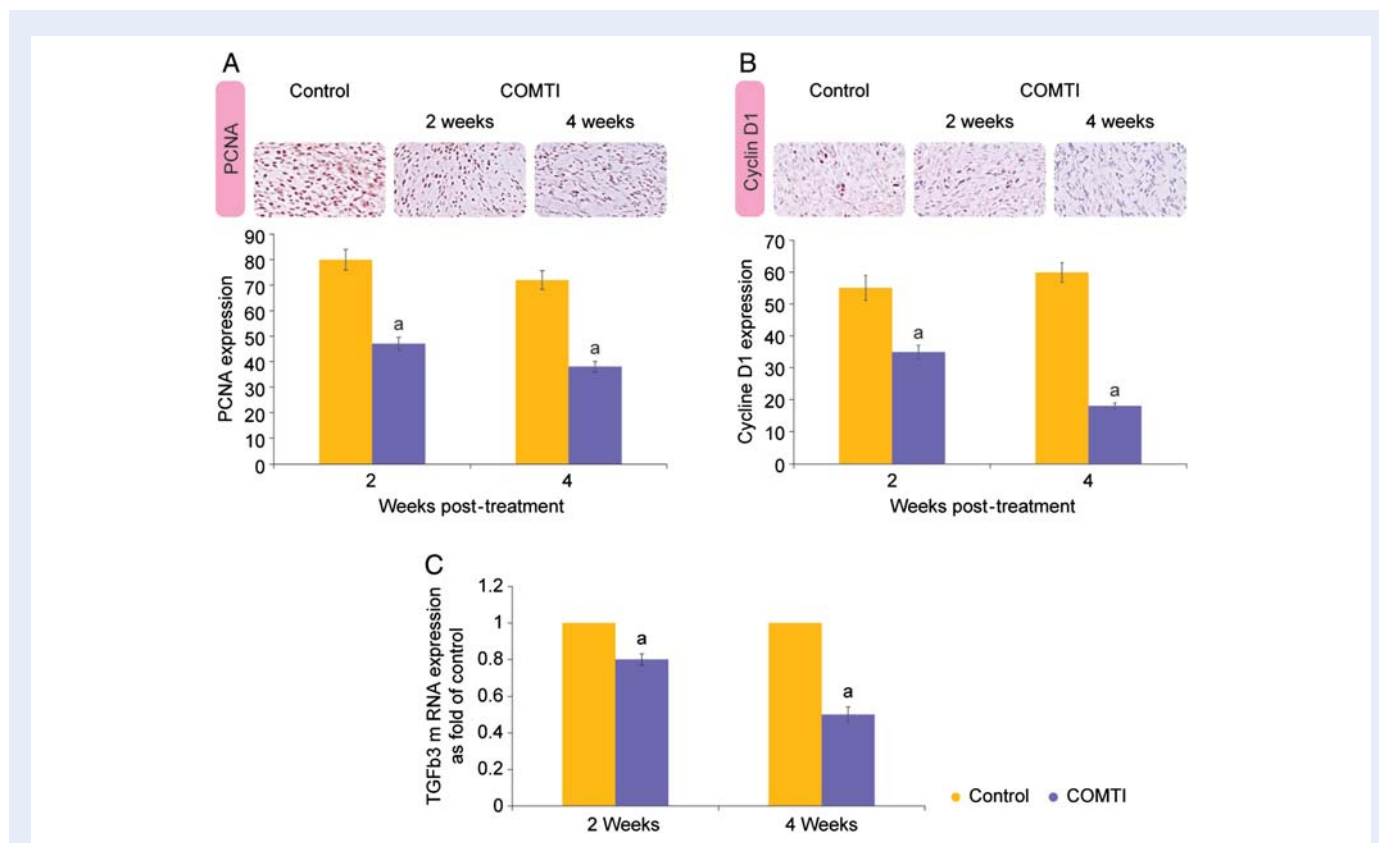
Figure 4 Ro 41-0960 (COMTI) treatment decreases the expression of proliferation- and ECM formation-related genes. Immunohistochemical analysis of proliferation-related (**A**) *PCNA* and (**B**) *Cyclin-D1* in the Eker rat uterine leiomyoma tissue sections collected from animals treated with vehicle only (control) or Ro 41-0960 at 2 and 4 weeks post-treatment. Original magnification: 20×. (**C**) Levels of *TGFβ3* mRNA. a: indicates a significant difference between the Ro 41-0960-treated and vehicle-treated rats, $P < 0.05$. Control data represent the 4-week time point.

compared with $55 \pm 4\%$ and $60 \pm 3\%$ in the vehicle controls at 2 and 4 weeks post-treatment, respectively (Fig. 4B).

Additionally, Ro 41-0960 (COMTI) significantly ($P < 0.05$) decreased the mRNA levels for *TGFβ3* to 0.8 ± 0.03 and 0.5 ± 0.04-fold of that in the control animals at 2 and 4 weeks post-treatment, respectively (Fig. 4C).

Treatment with Ro 41-0960 (COMTI) modulates levels of urinary estrogen metabolites

As shown in Fig. 5A, Eker rats injected with Ro 41-0960 (COMTI) at 150 mg/kg showed a significant ($P < 0.05$) increase in the urinary levels of 2-hydroxy E2 metabolites compared with the vehicle-treated control animals. On the other hand, the urinary level of 16-hydroxy E2, which is not part of the COMT pathway, was not affected by such treatment (data not shown), which in turn resulted in an increase in the ratio of 2-hydroxy E to 16-hydroxy E versus control (Fig. 5B).

Safety of Ro 41-0960 (COMTI) treatment in the Eker rat model

Close monitoring of the animals on a daily basis during the treatment course did not reveal any abnormal observations. All of the animals tolerated the treatment and survived the experiment with no apparent signs of toxicity.

Interestingly, careful histological evaluation using H&E staining of the tissue section obtained from various Eker rat organs at different time points (2 and 4 weeks) exhibited no signs of tissue damage or necrosis (some examples are shown in Supplementary data, Fig. S1). Moreover, Ro 41-0960 did not change the urinary levels of the bone decalcification marker DPD during the treatment period compared with the control (Fig. 6).

Most importantly, Ro 41-0960 treatment had no significant effect on the liver function tests (AST, ALT and total bilirubin) in Eker rats at 2 and 4 weeks post-treatment compared with vehicle-treated controls (Fig. 7A and B).

Discussion

Estrogen plays a pivotal role in leiomyoma pathogenesis and growth (Murphy and Ghahary, 1990; Rein et al., 1995; Chegini et al., 2003; Yue et al., 2003; Al-Hendy et al., 2004; Maruo et al., 2004). However, results published earlier, which have recently been confirmed in our laboratory, suggest that in addition to the parent molecule the oxidative metabolites of estrogen, including the CEs 2-hydroxy-E2, 4-hydroxy E2 and 16-hydroxy E2, may also affect estrogen-induced human uterine leiomyoma growth (Reddy et al.,
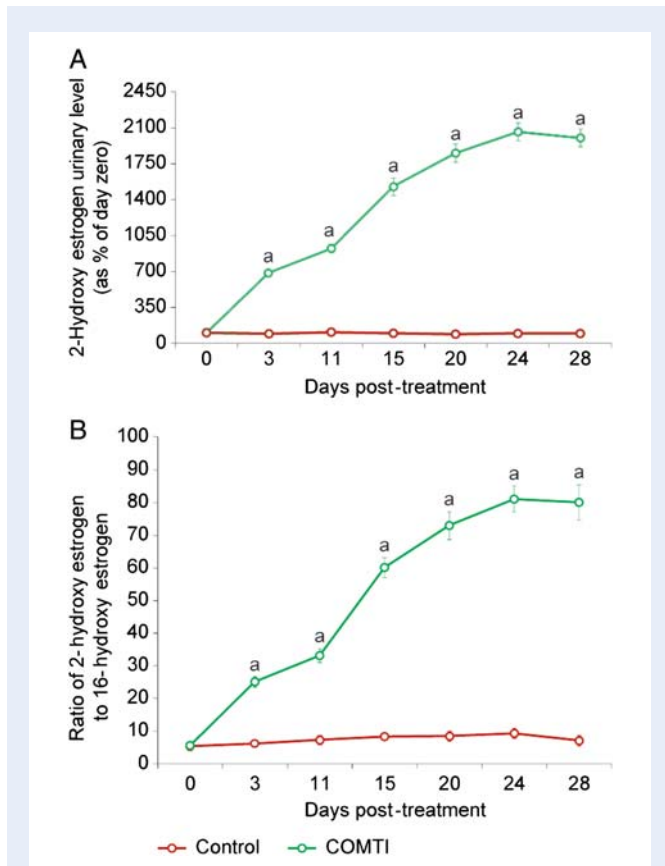
Figure 5 Ro 41-0960 (COMTI) increases the urinary level of 2-hydroxy estrogen metabolite (**A**) and increases the ratio of 2-hydroxy estrogen to 16-hydroxy estrogen metabolites (**B**). Data are calculated as mean and SE for six animals at each time point and are represented as (A) a percentage of the data at day zero; and (B) a ratio of absolute values. A indicates a significant difference between the Ro 41-0960-treated rats and the vehicle-treated rats ($P < 0.05$).
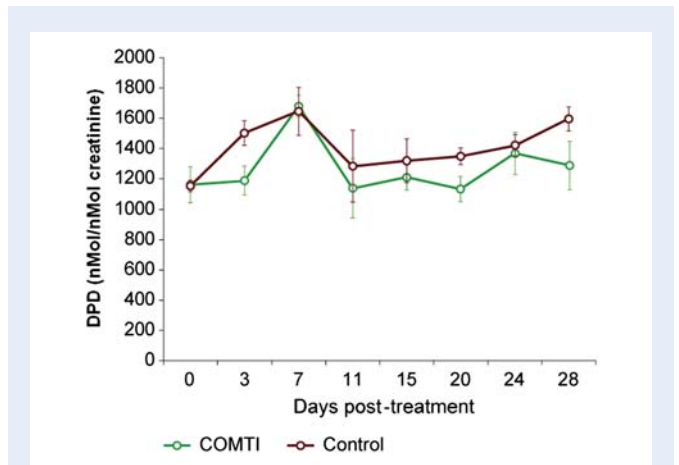

Figure 6 Effect of Ro 41-0960 (COMTI) injection at 150 mg/kg bid on Eker rat urinary levels of the bone decalcification marker Deoxy Pyridinoline (DPD). DPD was evaluated using a competitive enzyme immunoassay and normalized against creatinine. Data are mean ± SE of six animals for each time point. There is no significant difference between the Ro 41-0960-treated rats and the vehicle-treated rats.
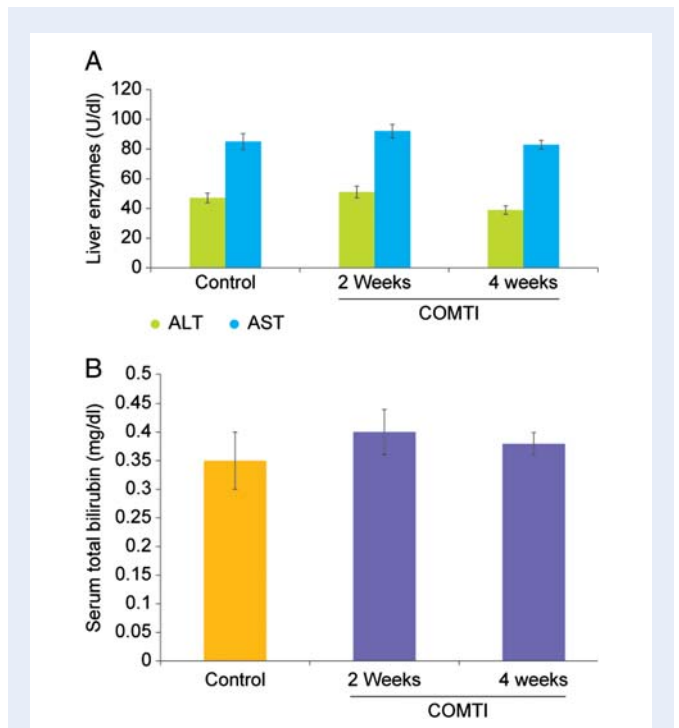

Figure 7 Effect of Ro 41-0960 (COMTI) on liver function tests in Eker rats. Eker rats were treated with Ro 41-0960 at a dose of 150 mg/kg bid or vehicle. Ro 41-0960 produce no significant changes in liver function tests: (**A**) AST, ALT and (**B**) total bilirubin. Liver function was evaluated 2 and 4 weeks after Ro 41-0960 injection and compared with vehicle-treated control animals using Student's two-tailed *t*-test.

1981; Al-Hendy and Salama, 2006b). Recent reports from our lab demonstrated that the development of uterine leiomyoma is associated with high *COMT* expression, and that specific *COMT* genotypes are associated with higher enzymatic activity (Al-Hendy and Salama, 2006b; Salama *et al*., 2006). Therefore, interrupting estrogen metabolism or the estrogen receptor (ER) signaling pathway may be a viable strategy for many therapeutic modalities. One of these approaches exploits COMTI to interfere with estrogen metabolism in many estrogen-dependent neoplasms, such as breast cancer (Lavigne *et al*., 2001).

In the present report, we aimed to develop an alternative localized non-surgical conservative treatment approach for uterine leiomyoma utilizing this novel COMTI approach. To that end, we evaluated the therapeutic modality of COMTI in the Eker rat, the only authentic immunocompetent animal model that spontaneously develops uterine leimyoma lesions and shares many of the same characteristics as human leiomyoma (Everitt *et al*., 1995). Our goal was to explore the treatment efficiency and safety of COMTI in the Eker rat model

as a first preclinical step in preparation for a possible future clinical trial.

Our studies revealed that *Ro 41-0960* (COMTI) treatment was able to significantly shrink pre-existing Eker rat uterine leiomyoma lesions compared with a vehicle-treated group (Fig. 1). The shrinkage/growth arrest reached a maximum level after 2 weeks of treatment and appeared to be sustained for the full treatment period (28 days). Furthermore, *Ro 41-0960* (COMTI) modulated the expression of several estrogen-regulated genes that control various cellular functions, such as apoptosis (*P53*, *PARP1* and TUNEL assay; Fig. 3), proliferation (*PCNA* and *cyclin-D1*; Figs. 4A and B) and extracellular matrix (ECM) formation (*TGF-β3*; Fig. 4C; Imai *et al.*, 1998; Dixon *et al.*, 2002; Gao *et al.*, 2002; Sozen and Arici, 2002; Chegini *et al.*, 2003). In addition, *Ro 41-0960* (COMTI) treatment changed the ratio of 2-hydroxy E2 to 16-hydroxy E2 in favor of a systemic hypoestrogenic status (high 2-hydroxy E2/16-hydroxy E2), which provides a potential mechanism of action for this COMTI-mediated therapeutic approach and possibly explains the rapid and impressive tumor shrinkage in the uterine fibroid lesions (Fig. 5B). Notably, this treatment approach appeared to be safe and well tolerated in rats; for example, it did not induce any macroscopic or microscopic tissue damage in the organs tested. COMTI treatment also did not cause any significant change in the liver function tests or in urinary levels of the bone decalcification marker DPD, suggesting no major effects of COMTI on liver and bone in the 28-day study period (Figs. 6 and 7).

Accumulating evidence indicates that uterine leiomyoma growth is, in part, regulated by the balance between the genes controlling proliferation and apoptosis (Dixon *et al.*, 2002; Loy *et al.*, 2005). *PCNA* and *cyclin D1* have been reported as markers of cellular proliferation (Kurki *et al.*, 1986; Baldin *et al.*, 1993; Gao *et al.*, 2002) and are expressed at a higher level in leiomyoma compared with adjacent normal myometrium (Dixon *et al.*, 2002). As shown in Fig. 4A and B, in the COMTI-treated rats, tumor lesions exhibited significantly decreased PCNA and cyclin-D1 expression, which might reflect a cell cycle arrest (Kurki *et al.*, 1986; Baldin *et al.*, 1993; Dixon *et al.*, 2002) mediated by active p53 overexpression (Fig. 3C).

The tumor suppressor *P53* has been categorized as both a caretaker and a gatekeeper for the cell cycle (Lane, 1992). Additionally, *P53* controls multiple downstream targets that regulate variable cellular pathways, such as cell cycle, apoptosis, DNA repair and replication (Bargonetti and Manfredi, 2002; Haupt *et al.*, 2003). Interestingly, estrogen stimulates leiomyoma growth in part by decreasing p53 protein levels in the nucleus and suppressing normal p53 function. Conversely, GnRH agonist treatment, one of the treatment modalities currently available for uterine leiomyoma, increases leiomyoma p53 tumor suppressor content (Gao *et al.*, 2002). *P53*-mediated apoptosis has been documented by two separate pathways (Haupt *et al.*, 2003): the extrinsic death-receptor pathway and the intrinsic mitochondrial pathway, which shift the balance in the Bcl-2 family toward the proapoptotic members, such as Bax. Both pathways lead to activation of caspases as the common final pathway, which degrades cellular components (Haupt *et al.*, 2003). In accordance with this mechanism, our results indicated that the COMTI-treated animals had a significantly lower death substrate PARP1 protein levels at 2 and 4 weeks compared with the control animals, suggesting an activation of the caspase cascade (Shanmugathasan and Jothy, 2000; Chiarugi, 2002; Soldani and Scovassi, 2002). Apoptosis was also confirmed in our

study by the TUNEL assay, and all of the tissue sections prepared from *Ro 41-0960* (COMTI)-treated animals exhibited a significant increase in the percentage of apoptotic cells compared with those from the control group at both time points (Fig. 3B). On the other hand, Ro 41-0960 (COMTI) treatment lowered the protein level of proliferation-related genes, namely *PCNA*, an endogenous marker of proliferation (Kurki *et al.*, 1986; Dixon *et al.*, 2002) and *cyclin D1*, a cell cycle regulating gene (Baldin *et al.*, 1993), which may indicate an overall growth arrest in uterine leiomyoma after treatment with Ro 41-0960.

The TGF-β family includes pleiotrophic cytokines that play key roles in tissue morphogenesis and growth (Ingman and Robertson, 2002). TGF-β increases the expression of ECM proteins (Ignotz and Massague, 1986; Ignotz *et al.*, 1987). Leiomyomas contain abundant quantities of ECM and contain increased levels of estrogen-induced TGF-β3 relative to adjacent normal myometrium (Sozen and Arici, 2002). In the present study, we demonstrated that the interference of estrogen metabolism by Ro 41-0960 (COMTI) resulted in a marked decrease in the level of TGF-β3 mRNA (Fig. 4C), which possibly led to a decrease in leiomyoma ECM formation and ultimately contributed to a decrease in leiomyoma volume.

Microsomes from human uterine leiomyoma tumor tissue have been shown to metabolize E_2 to catechol metabolites, including 2-hydroxy E2 and 4-hydroxy E2, and non-catechol metabolites, such as 16-hydroxy E2 (Vandewalle, 1989; Bradlow, 1996; Creveling, 2003; Wentz *et al.*, 2006). Ro 41-0960 (COMT) converts the CEs 2- and 4-hydroxyE to 2- and 4-methoxyestrogen, respectively (Creveling, 2003). The CE 2-hydroxy E2 can act as an estrogen antagonist (Vandewalle and Lefebvre, 1989; Bradlow, 1996; Devanesan *et al.*, 2001; Al-Hendy and Salama, 2006b), while the methylated counterparts (2- or 4-methoxy E_2) can act as estrogen agonists in multiple biologic assays (Banerjee *et al.*, 2003; Lippert *et al.*, 2003; Liu and Zhu, 2004; Sutherland *et al.*, 2005). Both rat and human COMT have a higher catalytic activity toward 2-hydroxy E2 compared with 4-hydroxy E2 (Zhu and Liehr, 1993; Goodman *et al.*, 2001). Thus we elected to focus on the more abundant 2- and 16-derivatives as possible ER modulators (Vandewalle and Lefebvre, 1989; Martucci and Fishman, 1993; Zhu and Liehr, 1993; Bradlow, 1996; Cavalieri *et al.*, 2000; Devanesan *et al.*, 2001; Goodman *et al.*, 2001; Lord *et al.*, 2002; Banerjee *et al.*, 2003, Lippert *et al.*, 2003, Liu and Zhu, 2004; Sutherland *et al.*, 2005)

Eker rats injected with 150 mg/kg Ro 41-0960 (COMT) showed a remarkable increase in urinary levels of 2-hydroxy estrogen metabolites compared with the vehicle-treated animals. On the other hand, the urinary level of 16-hydroxy E2, a potent estrogenic metabolite associated with increased cancer risk (Martucci and Fishman, 1993; Muti *et al.*, 2000), was not affected by such treatment. This, in turn, resulted in an increase in the 2/16-hydroxy E2 metabolite ratio, which leads to a low estrogenic environment and may eventually lead to the shrinkage of uterine leiomyoma lesions.

Several prior reports have shown that the ratio of 2-hydroxy E2 to 16-hydroxy E2 can be used as a biomarker that correlates with carcinogenicity as well as tumor follow-up, and the ratio was described as more representative of the intrinsic tissue estrogen milieu than each metabolite separately (Fishman *et al.*, 1995; Kabat *et al.*, 1997; Meilahn *et al.*, 1998; Muti *et al.*, 2000; Lord *et al.*, 2002). This was described in several types of cancer in various anatomical sites, such

as breast cancer, mammary tumor, head and neck cancer as well as endometrial cancer (Persson, 1985; Taioli *et al.*, 1996; Wong et al., 1997; Auborn *et al.*, 1998; Ursin *et al.*, 1999; Yoo *et al.*, 2001; Lord *et al.*, 2002).

From a clinical point of view, COMTI therapy for uterine leiomyoma must be safe and well tolerated and have no or minimal toxic side effects on other normal organs, especially the liver, a common target for systemically injected drugs (Thirunavukkarasu and Sakthisekaran, 2001). Interestingly, careful histologic and pathologic examination of the various Eker rat organs after treatment with Ro 41-0960 (COMTI), especially the liver and estrogen-dependent organs such as the uterus, revealed no evidence of adverse effects or organ damage. Furthermore, the liver function tests were unaffected after 4 weeks of daily Ro 41-0960 (COMTI) treatment (Fig. 7). Because of the deleterious effects of GnRHa on bone mineral density, which is secondary to hypoestrogenemia, we wanted to evaluate the effects of Ro 41-0960 (COMTI) on bone health. As shown in Fig. 6, the urinary level of DPD, a sensitive osteolytic bone marker that cross-links collagen molecules in bones (Colwell *et al.*, 1993; Eastell *et al.*, 1997), showed no significant change in the Ro 41-0960 (COMTI)-treated rats compared with the vehicle-treated control animals, which supported the favorable safety profile of this Ro 41-0960 (COMTI) treatment regimen on bone health (Fig. 6).

The role of COMT in mammary tissue neoplasia is controversial however, with some studies showing a higher COMT activity associated with breast cancer risk (Thompson *et al.*, 1998; Mitrunen *et al.*, 2001), others describing a possible protective effect (Davis *et al.*, 1997; Lavigne *et al.*, 2001; Salama *et al.*, 2007) and many reporting no association between COMT and increased cancer risk (Millikan *et al.*, 1998; Hamajima *et al.*, 2001; Kocabas *et al.*, 2002; Wedren *et al.*, 2003; Ahsan *et al.*, 2004; Wen *et al.*, 2005). Thus we are currently planning a detailed toxicity evaluation following a long period of treatment with COMTI, including the effect on the mammary gland.

It is noteworthy to mention that most of the currently available medical treatment options for uterine fibroids, such as selective progesterone receptor modulators and oral GnRH antagonists, are being developed for chronic therapy of this condition (Kettle *et al.*, 1998; Hara *et al.*, 2003; Armer and Smelt, 2004; Sankaran and Manyonda, 2008; Engman *et al.*, 2009). It is mechanistically inconceivable that a medication would totally eliminate all fibroid lesions in a short period of time, after which the drug could be stopped. A developing theme is gradually being accepted in the reproductive medicine community that uterine fibroids could, and should, be considered as a chronic disease, just as hypertension or diabetes mellitus is, in which a medication will be used chronically. As COMT expression is higher in leiomyoma tissues than adjacent normal myometrium (Reddy *et al.*, 1981; Salama *et al.*, 2006), a low dose of COMTI is expected to affect human fibroids while sparing normal myometrium and other normal organs (as supported by data in the current study), which might provide a safe therapeutic window of favorable anti-fibroid effects. In addition COMTIs are relatively inexpensive compared with GnRHa, and are available as an oral formula, which would encourage patient compliance. All of these advantages would suggest COMTI as a potential candidate for further evaluation in future clinical trials in women with symptomatic uterine fibroids.

In conclusion, Ro 41-0960, a highly specific and selective synthetic COMT inhibitor, produced a dramatic reduction in uterine leiomyoma volumes in Eker rats over 4 weeks of treatment with 150 mg/kg/12 h. The Ro 41-0960-mediated tumor shrinkage may be in part related to the modulation of certain estrogen-dependent genes regulating leiomyoma apoptosis (*P53* and *PARP1*), proliferation (*PCNA* and *cyclin D1*) and ECM formation (*TGFβ3*). These mechanisms are conceivably affected by a decrease in the estrogenic milieu, by increasing the anti-estrogenic metabolite 2-hydroxy E2. Daily administration of Ro 41-0960 was well tolerated by all of the animals and had a favorable safety profile for target and non-target tissues.

This study has provided some essential preclinical data to support the development of an alternative safe non-surgical, non-invasive treatment option for uterine leiomyoma.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

M.H.H. made Substantial contributions to the conception and design, acquisition of data, analysis and interpretation of data. Participated in drafting the article and revising it critically for important intellectual content. He made substantial contribution in the physical work. He was involved in the final approval of the version to be published. H.F. made substantial contributions to acquisition of data, analysis and interpretation of data. Was involved with revising the article critically for important intellectual content, examining the histopathological finding and the final approval of the version to be published. S.B. made substantial contributions to acquisition of data, analysis and interpretation of data. Was involved in revising the article critically for important intellectual content and the final approval of the version to be published. A.A.-H. made Substantial contributions to conception and design, acquisition of data, analysis and interpretation of data. Was involved in drafting the article and revising it critically for important intellectual content and supplying the laboratory space and facilities. Made substantial contribution in physical work and financial support. Participated in the final approval of the version to be published.

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