

# Inhibition of leiomyoma cell proliferation in vitro by genistein and the protein tyrosine kinase inhibitor TKS050

Asher Shushan, M.D.,<sup>a</sup> Hannah Ben-Bassat, Ph.D.,<sup>b</sup> Eyal Mishani, Ph.D.,<sup>c</sup> Neri Laufer, M.D.,<sup>a</sup> Benjamin Y. Klein, M.D.,<sup>b</sup> and Nathan Rojansky, M.D.<sup>a</sup>

<sup>a</sup>Department of Obstetrics and Gynecology, <sup>b</sup>Laboratory of Experimental Surgery, and <sup>c</sup>Department of Nuclear Medicine, Hadassah University Hospital, Jerusalem, Israel

**Objective:** To determine the potency of TKS050, a new epidermal growth factor receptor (EGFR) inhibitor and genistein, a naturally occurring protein tyrosine kinase inhibitor, to inhibit leiomyoma cell proliferation in vitro.

**Design:** Establishment of paired cultures of leiomyoma and normal myometrial samples.

**Setting:** University clinical research laboratory.

**Patient(s):** Hysterectomy specimens from premenopausal women affected by uterine leiomyomas.

**Intervention(s):** The suppressive effect of TKS050 and genistein on the cells, before and after steroidal hormone treatment, was examined.

**Main Outcome Measure(s):** Cell proliferation, recovery after treatment, cell cycle analysis, and immunochemical analysis of relevant proteins were performed.

**Result(s):** TKS050 (2  $\mu\text{mol/L}$ ) and genistein (50  $\mu\text{mol/L}$ ) completely suppressed leiomyoma cell proliferation, and the cells did not recover after cessation of treatment. TKS050 induced cell cycle arrest and apoptosis in a dose- and time-dependent manner. Cells accumulated in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle at the expense of the S and G<sub>2</sub>+M phases. Treatment of cells with TKS050 resulted in a dose-dependent inhibition of EGFR autophosphorylation and of phosphorylated signal transducer and activator of transcription 3 (Stat3). Genistein inhibited the phosphorylated Stat3 but did not affect EGFR autophosphorylation. The inhibitory effects of TKS050 or genistein were unaffected by the presence of physiologic concentrations of estradiol-17 $\beta$ .

**Conclusion(s):** Leiomyoma cell growth is effectively blocked by TKS050 and genistein. The inhibitory action of newly developed and natural inhibitors derived from diet may be useful as a possible alternative therapy for leiomyomas. (Fertil Steril® 2006;xx:xxx. ©2006 by American Society for Reproductive Medicine.)

**Key Words:** Leiomyoma, genistein, signal transduction therapy, protein tyrosine kinase (PTK) inhibitors

Uterine leiomyomas, or fibroids, are a major health concern. Although the leiomyoma is a benign tumor, which arises from smooth muscle cells of the myometrium, the disease can severely impair women's quality of life. Leiomyomas can cause infertility, miscarriage, menorrhagia, and pain (1). Fibroids, which are the most common pelvic tumors in women, also contribute to the high hysterectomy rate in the western world (2). Recently it has been reported that the estimated cumulative incidence of uterine leiomyomas by age 50 years was >80% for black women and nearly 70% for white women. These authors conclude that a very significant number of women in the United States have uterine fibroid tumors before menopause (3).

The precise pathophysiology of leiomyoma growth is still unresolved. Chromosomal abnormalities, hormonal deregulation, and growth and angiogenic factors have all been implicated in the etiology of these clonal smooth muscle cell

proliferations (4–7). Because no medical therapeutic strategy currently exists to adequately manage uterine leiomyoma, treatment is primarily surgical. Development of new therapeutic agents for the treatment of leiomyoma would therefore have important health benefits for women.

Accumulating data indicate that the effects of steroid hormones on leiomyoma proliferation are mediated through the local production of epidermal growth factor (EGF) (8, 9). Therefore, selective inhibitors of the EGF receptor (EGFR) might be useful in the treatment of uterine fibroids. Indeed, we have previously shown that leiomyoma cell growth was effectively blocked by AG1478, a selective EGFR blocker, that was unaffected by the presence of physiologic concentrations of P and estradiol-17 $\beta$  (E<sub>2</sub>) separately or in combination. At a concentration of  $\geq 10$   $\mu\text{mol/L}$ , AG1478 completely suppressed proliferation and the cells did not recover after cessation of treatment (10).

In the present study, we evaluated the potential of TKS050 (also known as MLO5), a new selective irreversible EGFR blocker, and genistein, an abundant dietary constituent, regarded as a natural protein tyrosine kinase (PTK) inhibitor, as candidates for "signal transduction therapeutics" for alternative treatment of leiomyomas.

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## MATERIALS AND METHODS

TKS050 (also known as MLO5) (N-{4-[(3,4-dichloro-6-fluorophenyl)amino]-quinazoline-6-yl}-2-chloroacetamide) was designed and synthesized at the Cyclotron/Radiochemistry unit of the Hadassah University Hospital (under the project of TK-Signal on May 2002). The compound and its affinity toward the EGFR were presented in 2003 at the Fifteenth International Symposium on Radiopharmaceutical Chemistry, Sydney, Australia. Genistein (molecular weight 270.23; catalog no. G-6055, CH-144) was obtained from LC Laboratories, Woburn, Massachusetts.

The antibodies used to monitor the level and state of various signaling proteins are the anti-EGFR external domain (clone F4) monoclonal antibody (catalog no. 1 428 551; Boehringer, Mannheim, Germany) and the following antibodies, all from Upstate Biotechnology UBI, Lake Placid, New York: anti-phosphotyrosine—4G10 mouse monoclonal antibody (catalog no. 05321); Bcl-2—mouse monoclonal anti-human antibody (catalog no. 05-341); signal transducers and activators of transcription 3 (Stat3)—rabbit polyclonal IgG, anti-human; and for phosphorylated Stat3 (pStat3)—mouse anti-phosphoStat3-Tyr 705 synthetic peptide (Y704 catalog no. 06-596).

Fluorescein isothiocyanate conjugated goat anti-mouse antibody (catalog no. F2653; Sigma Chemical Company, St. Louis, MO) was used for immunofluorescence staining. Fetal bovine serum (United States origin) was from GIBCO-BRL (Life Technologies, Inc., Grand Island, NY). Tissue culture media, trypsin-ethylenediaminetetraacetic acid (EDTA) solution, and antibiotics were from Biological Industries (Beit Haemek, Israel). Tissue culture reagents, growth supplements, and the ovarian steroid hormones E<sub>2</sub> and P were from Sigma Chemical Company (St. Louis, MO).

### Cell Cultures

Paired cell cultures of leiomyoma and adjacent normal myometrial tissue samples were established from premenopausal women following hysterectomy conducted for benign disease. Helsinki approval and informed consent were obtained. Primary cell cultures were initiated in Ham's/F12 Dulbecco's modified essential medium (DMEM) 1:1 with 20% fetal bovine serum and antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL). Thereafter the cell cultures were propagated in phenol red-free DMEM and 10% charcoal-treated fetal bovine serum, specifically for the experiments with ovarian steroid hormones. The experiments were performed on secondary and/or tertiary cultures. Cells were maintained at 37°C in a humidified incubator, containing 5%–8% CO<sub>2</sub>. Logarithmically growing cells were used in the experiments. Immunocytochemical staining with α-actin antibody was routinely performed to verify the smooth muscle cell origin of the cells in culture.

### Protein Tyrosine Kinase Inhibitors and Treatment of Cells

Stock solutions of 10 mmol/L TKS050 or genistein in dimethyl sulfoxide (DMSO) were stored at –70°C (11). For the experiments the stock solution was diluted with DMEM containing 10% fetal bovine serum. The highest concentration of DMSO was 0.1%.

### Experimental Design

Cells were seeded  $1 \times 10^4$ /well in 96-well microplates and grown for 2 to 3 days. Thereafter, the medium was replaced every 2 days with medium containing TKS050 or genistein. Two days after the second treatment (4 days treatment) medium was removed, new medium without the compound was added, and the cells were grown for another 4 days (rescue/recovery after treatment) followed by determination of growth with the colorimetric methylene blue assay. Control cells were grown in medium and in medium with the appropriate concentration of DMSO.

### Calculation of Growth Inhibition

For each concentration of TKS050 or genistein used, medium containing only DMSO was used for the control. Thus, for each concentration the control was taken as 100% growth. Cell growth was determined by the automated microculture methylene blue assay (10).

### Fluorescence-activated Cell Sorter Analysis of DNA Content and Determination of Apoptotic Cells

Fluorescence-activated cell sorter (FACS) analysis was performed to determine DNA content on selected samples of cell cultures. Samples of cells treated with TKS050 or genistein for predetermined periods were dispersed with Trypsin 0.25% 1:1 EDTA 0.05% and stained with propidium iodide in suspension. Cell cycle analysis and determination of the apoptotic cell fraction of the cell populations were carried out with FACS FPAR PLUS (Becton-Dickinson, Inc., Mountain View, CA).

### Biochemical Activities: Western Blot Analysis and Phosphotyrosine

Western blot analysis for relevant proteins and phosphotyrosine was performed as previously described and according to the manufacturer's recommendation (10, 12).

Cell cultures were seeded  $5 \times 10^5$  cells/35 mm plate in DMEM with 10% fetal calf serum (FCS) medium and grown for 2 days. Thereafter the cultures were washed, fed with medium without serum, and starved for 48 hours. TKS050 or genistein at the appropriate concentration in medium without serum (= starvation medium) were added for 4 hours. Cells were then stimulated with 30 ng/mL EGF for 10 minutes. The reaction was terminated by placing the cultures on ice and washing the cells with ice-cold phosphate-buffered saline solution. Whole cells were lysed with hot buffer,

scraped, boiled for 5 minutes, and then run on 7% SDS for 4 hours. They were then transferred to nitrocellulose paper and incubated overnight at 4°C with a monoclonal anti-phosphotyrosine antibody 4G10, according to the manufacturer's recommendations. Goat anti-mouse fluorescent antibody was added for a 2-hour incubation at room temperature (1 mL/20 mL). After drying, the gels were exposed to x-ray film in cassettes. Densitometric analysis was performed with use of the ImageJ program. Our method to determine the effects of E<sub>2</sub> and P on cell growth of paired leiomyoma and normal myometrial cell cultures was previously described (10).

The same protocol was used to determine the effects of E<sub>2</sub> and P on the biochemical activities of TKS050 and genistein, but on the third day the cultures were washed, fed with medium containing the steroid: 10 ng/mL E<sub>2</sub> or 100 ng/mL P without serum, and starved for 48 hours. Thereafter TKS050 or genistein at the appropriate concentration in medium + E<sub>2</sub> or medium + P without serum (= starvation medium) was added for 4 hours. Immunoblot analysis of relevant proteins was performed on whole cell lysates.

### Effects of E<sub>2</sub> and P on the Inhibitory Capacity of TKS050 or Genistein

Myometrial and leiomyoma paired cells were seeded 5 × 10<sup>5</sup> cells/35 mm plates in DMEM with 10% FCS medium and grown for 2 days. Thereafter the cultures were washed, fed with medium containing the steroid: 10 ng/mL E<sub>2</sub> or 100 ng/mL P without serum and TKS050 at 5 μmol/L and 10 μmol/L in medium without serum + E<sub>2</sub> or medium without serum + P. The cultures were grown for an additional 5 days, followed by determination of growth with the colorimetric methylene blue assay.

### Statistics

Data obtained from determination of growth, FACS analysis, and densitometry were expressed as mean ± SD. Statistical analysis was conducted with use of Student's *t*-test, ANOVA (Figs. 1 and 2), and multivariate ANOVA (Fig. 3), and verified also with the Kruskal-Wallis test. Statistical significance was established at *P* values of <.05.

## RESULTS

### Inhibition of Growth by TKS050 and Genistein

TKS050 effectively inhibited the growth of leiomyoma and myometrial cell cultures in a dose- and time-dependent manner (Fig. 1), with an inhibitory concentration of 50% (IC<sub>50</sub>) = 0.7 μmol/L for leiomyoma and 1.1 μmol/L for myometrial cell cultures, respectively (IC<sub>50</sub>s are depicted from Fig. 1). TKS050 was already effective at ≥1.0 μmol/L, and the cells remained arrested after withdrawal of the compound on day 4 as monitored on day 7. The inhibitory effect of 0.5 μmol/L TKS050 was already evident and highly significant on day 1 of treatment (*P*≤.001). Generally, a similar pattern

of growth inhibition was obtained for normal myometrial cell cultures. It is of interest that in some myometrial complete growth inhibition was obtained only with ≥2 μmol/L TKS050, suggesting that some myometrial cells are less sensitive to the suppressive effect of TKS050 (Fig. 1).

The inhibitory effect of genistein was significantly weaker (IC<sub>50</sub> = 9.0 μmol/L for leiomyoma and 12.5 μmol/L for myometrial cell cultures). Genistein was effective at ≥10.0 μmol/L, but only approximately 30% of the cells remained in growth arrest after withdrawal of the compound on day 4 as monitored on day 7 (*P*≤.001). Complete growth suppression was obtained with ≥50 μmol/L genistein, and the cells remained in growth arrest after withdrawal of the compound (*P*≤.01 and .001, respectively) (Fig. 2A).

### Recovery After Treatment

TKS050 at ≥1.0 μmol/L completely inhibited growth with no recovery of the leiomyoma cells after cessation of treatment (*P*≤.001). After treatment with ≥0.1 μmol/L TKS050 the cells resumed growth and approximately 60% survival was obtained. Generally the self-renewal capacity of leiomyoma and normal myometrium cell cultures after treatment with TKS050 is similar (Fig. 1A). After treatment with ≥1.0 μmol/L genistein the cells resumed growth and an almost complete survival was obtained. After treatment with ≥10 μmol/L genistein the cells resumed growth and approximately 30% survival was obtained (*P*≤.001). The self-renewal capacity of leiomyoma and normal myometrium cell culture after treatment with genistein is similar (Fig. 1A). Complete growth suppression was obtained with ≥50 μmol/L genistein, and the cells remained arrested after withdrawal of the compound on day 4 as monitored on day 7 (*P*≤.001) (Fig. 2C).

### Ovarian Steroids Do Not Affect Genistein Inhibitory Activities

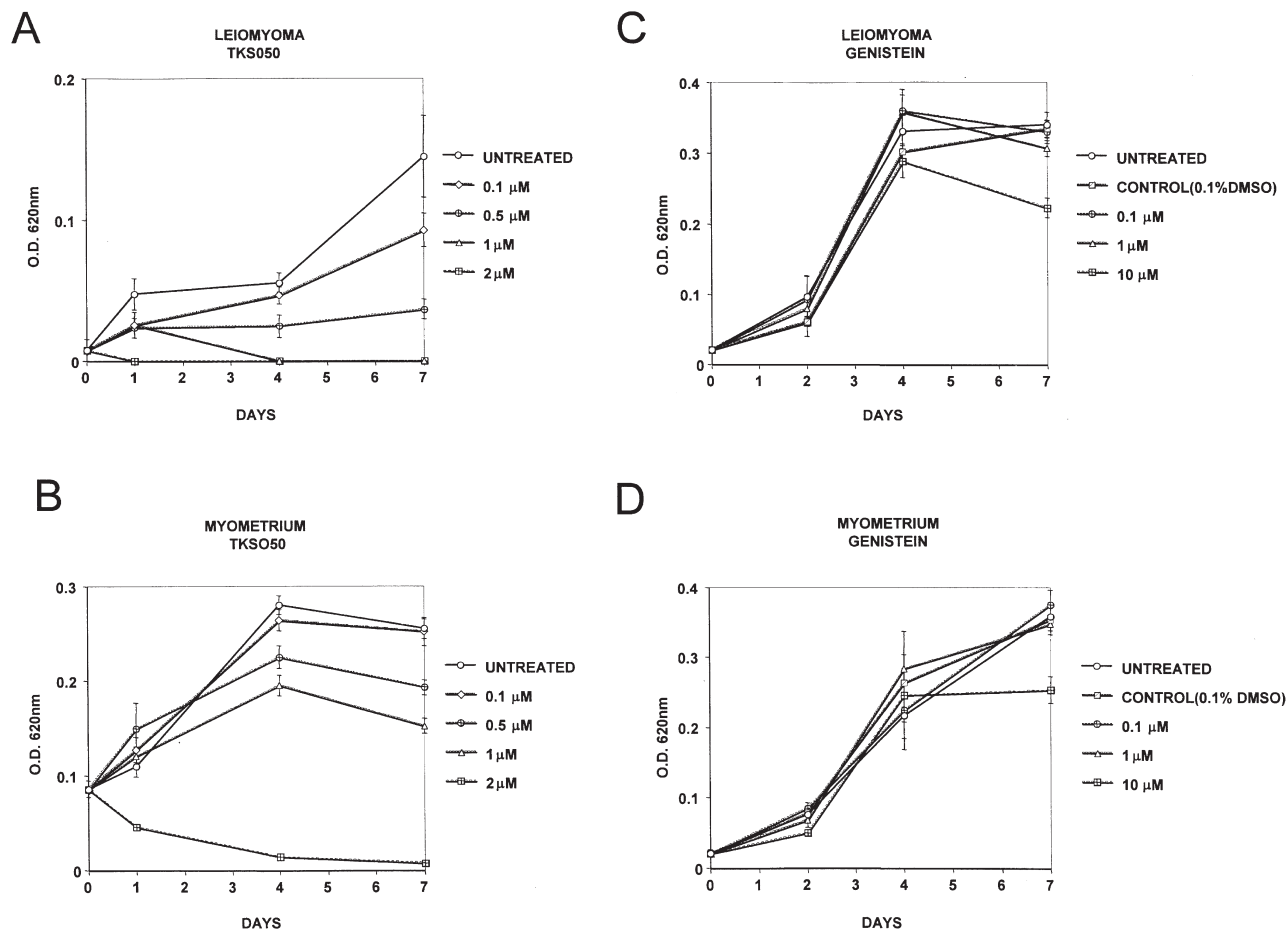
We examined the effect of E<sub>2</sub> alone and in combination with genistein on the growth of leiomyoma and myometrial cell cultures. The concentrations we chose, 10 ng/mL E<sub>2</sub> or 100 ng/mL P, are within the physiologic tissue concentrations found in leiomyomas and myometrium (13). The results indicated that E<sub>2</sub> alone had no effect on the growth of leiomyoma or myometrial cell cultures, and the combination of E<sub>2</sub> with genistein did not affect the growth suppressive effect of genistein (*P*>.05) (Fig. 2). Similar results were obtained with P (data not shown).

### Points of Growth Arrest in the Cell Cycle and Apoptosis

Cells exposed to TKS050 and genistein were assessed by FACS analysis on day 2 after initiating treatment. The results indicate that TKS050 alters the cell cycle distribution (Fig. 3A and B). After one treatment with ≥5 μmol/L TKS050 for 2 days, there was a concentration-dependent decrease in G<sub>0</sub>/G<sub>1</sub> (*P*<.05) and increase in G<sub>2</sub>+M (*P*<.05), with a

## FIGURE 1

Inhibitory effect of TKS050 and genistein on the proliferation and rescue after treatment of leiomyoma (A, C) and myometrial (B, D) cell cultures. The cells were treated with TKS050 or genistein and medium was changed after 2 days with new agent. Two days after the second treatment (4 days treatment) medium was removed, new medium without the agent added, and the cells were grown for another 3 days to determine recovery after treatment, followed by determination of growth with the automated microculture methylene blue assay. Greater than or equal to 1  $\mu\text{mol/L}$  TKS050 completely suppressed growth without recovery of the cells after cessation of treatment. Genistein is effective at  $\geq 10.0 \mu\text{mol/L}$ ; approximately 30% of the cells remained arrested after withdrawal of the compound on day 4 as monitored on day 7 (detailed in Materials and Methods).



Shushan. *EGF receptor inhibitors to treat leiomyoma. Fertil Steril* 2006.

significant increase in the apoptotic cell fractions  $\geq 33\%$  ( $P < .05$ ) (Fig. 3A and B). Genistein alters the cell cycle distribution in a dose- and time-dependent manner (Fig. 3C and D). After one treatment with  $\geq 25 \mu\text{mol/L}$  genistein for 2 days the proportion of cells in  $G_0/G_1$  was slightly decreased ( $P \leq .05$ ) and the proportion of cells in S and  $G_2 + M$  increased ( $P \leq .05$ ), with no effect on the apoptotic cell fraction (Fig. 3C and D).

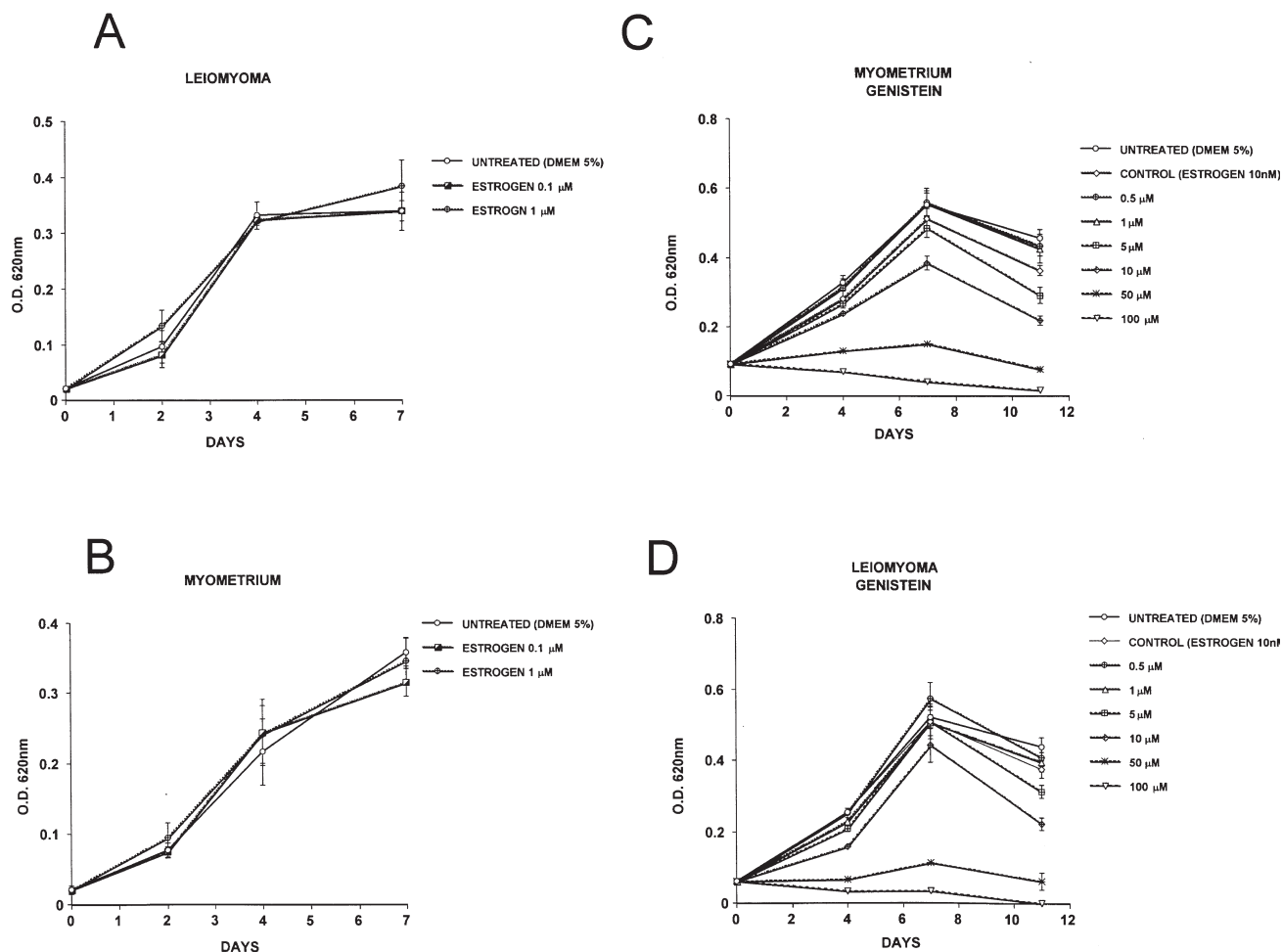
#### Biochemical Activities of TKS050 and Genistein

TKS050 induced a dose-dependent inhibition of EGFR autophosphorylation that was enhanced greatly in the presence

of the ligand. TKS050 was already effective at  $\geq 0.1 \mu\text{mol/L}$  ( $\geq 51\%$  inhibition in the presence of the ligand of leiomyoma and myometrial cultures; Fig. 4A and B, respectively). With  $\geq 1.0 \mu\text{mol/L}$  TKS050 the autophosphorylation inhibition was slightly increased ( $\geq 59\%$  inhibition in the presence of the ligand of leiomyoma cultures and  $\geq 61\%$  inhibition of myometrial cultures; Fig. 4A and B, respectively). The results also indicate that TKS050 did not alter the level of EGFR in both cell types. These findings are in accordance with conclusive evidence that TKS050 specifically and potently inhibits ligand-induced autophosphorylation of the EGFR (14).

## FIGURE 2

Effect of E<sub>2</sub> alone and with genistein on the proliferation and rescue after treatment of leiomyoma (A, D) and myometrial (B, C) cell cultures. The cells were treated with 0.1 μmol/L or 1.0 μmol/L of E<sub>2</sub> and with 0.01 μmol/L E<sub>2</sub> and various concentrations of genistein and harvested on day 7 or 12 (A and C, respectively), followed by determination of growth with the automated microculture methylene blue assay. Greater than or equal to 50 μmol/L genistein suppressed ≥80% growth and no recovery of the cells after cessation of treatment, whereas ≥100 μmol/L genistein completely suppressed growth with no recovery of the cells after cessation of treatment (detailed in Materials and Methods).



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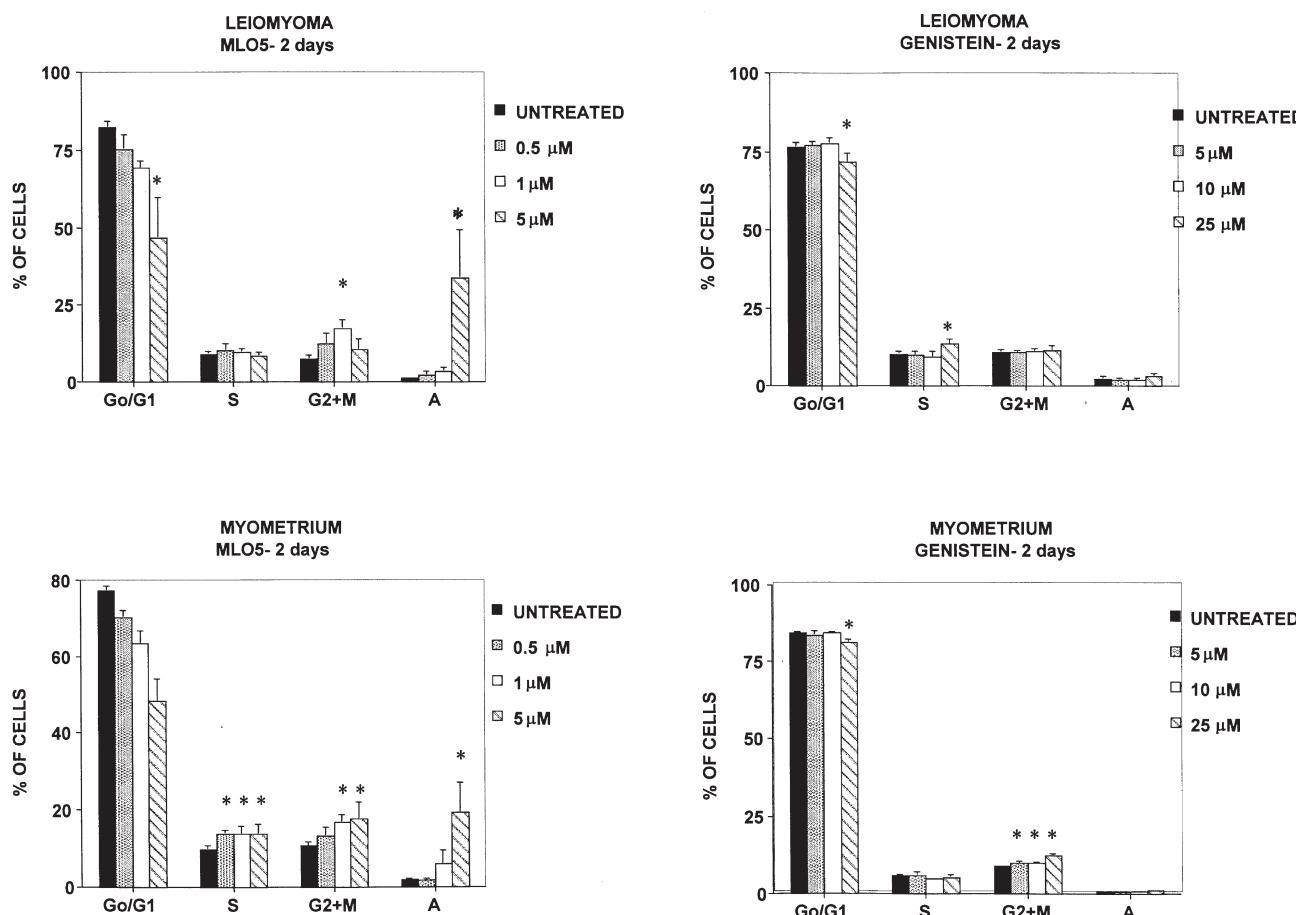
Thereafter, the effect of TKS050 on protein expression of two major regulatory proteins of apoptosis Bcl2 (antiapoptotic) and Bax (proapoptotic) was examined. TKS050 had no significant effect on the expression of the Bcl2 or Bax proteins in both cell culture types. Greater than or equal to 5 μmol/L TKS050 did not alter the level of pStat3 and Stat3 of the leiomyoma cell cultures, but a slight inhibition of pStat3 was detected in the myometrial cell cultures (≥15.5 % without EGF stimulation and ≥26% inhibition after EGF stimulation; Fig. 4A and B, respectively).

Genistein (0.1–50.0 μmol/L) did not induce inhibition of EGFR autophosphorylation nor did it alter the level of EGFR in the cells that was enhanced in the presence of the ligand

(Fig. 5). Genistein had no effect on the expression of the Bcl2 or Bax proteins, in accordance with the FACS analysis showing no detectable changes in the apoptotic cell fraction (Fig. 3). The leiomyoma cell cultures of this biopsy sample did not show a higher level of the antiapoptotic Bcl2 protein compared with normal myometrial cell cultures as reported by Matsuo et al. (15). Western blot analysis of the BCL2 and Bax, as well as of the EGFR proteins, in additional matched leiomyoma and myometrial cell cultures did not demonstrate consistent and/or clearly defined differential level of these proteins' expression in the cultures (10). Interestingly, in the leiomyoma cell cultures (but not in the myometrium), after EGF stimulation, ≥1.0 μmol/L genistein suppressed Bcl2

## FIGURE 3

Effect of TKS050 and genistein on the cell cycle phase distribution and apoptosis of leiomyoma (A, C) and myometrial (B, D) cell cultures. The cells were treated with various concentrations of TKS050 or genistein and harvested on day 2. Cell cycle distribution and apoptosis were determined by FACS analysis. TKS050 alters the cell cycle distribution in a dose-dependent manner. The proportion of cells in  $G_1$  is decreased, the proportion of cells in  $G_2/M$  increased, with a significant increase on the apoptotic cell fraction. Greater than or equal to  $25 \mu\text{mol/L}$  genistein decreased the fraction of cells in  $G_1$  and increased the fraction of cells in S or  $G_2+M$  with no effect on the apoptotic cell fraction of the leiomyoma and myometrial cell cultures (\*significant difference).



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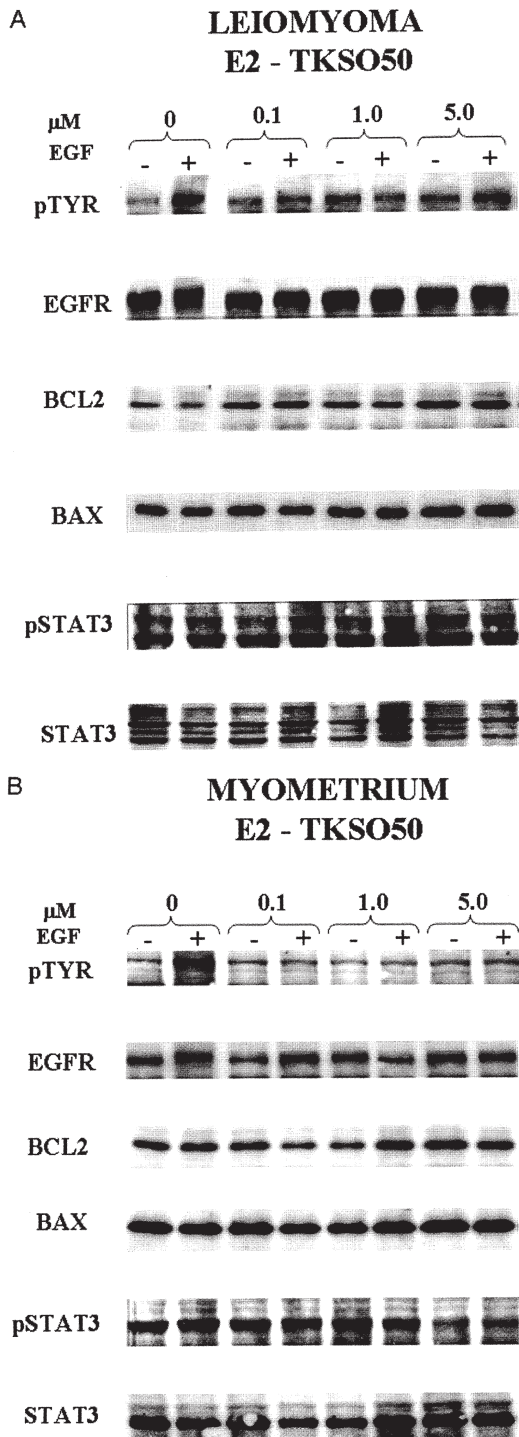
protein level by 50%. Greater than or equal to  $50.0 \mu\text{mol/L}$  genistein also suppressed significantly the level of pStat3 protein (55.2% and 57.7% with or without EGF stimulation, respectively).

## DISCUSSION

We have shown that  $2 \mu\text{mol/L}$  TKS050 and  $50 \mu\text{mol/L}$  of genistein completely suppressed leiomyoma cell proliferation and the cells did not recover after cessation of treatment. TKS050 induced cell cycle arrest and apoptosis in a dose- and time-dependent manner. Rescue experiments in which we measured the self-renewal capacity of the cells after removal of the agent on day 4 confirmed the growth inhibi-

tion experiments. Cells accumulated in  $G_0/G_1$  phase of the cell cycle at the expense of S and  $G_2+M$ . TKS050 blocked the growth of leiomyoma cell culture, mainly by decreasing the fraction of cells in  $G_0/G_1$  (with a concomitant increase in the fraction of apoptotic cells). Genistein induced cell cycle alteration in a dose- and time-dependent manner, but without apoptosis. On the molecular level, treatment of cells with TKS050 or genistein resulted in a slight dose-dependent inhibition of pStat3 in the myometrial cultures. TKS050, but not genistein, caused a dose-dependent inhibition of EGFR autophosphorylation. The inhibitory effect of TKS050 and genistein was unaffected by the presence of physiologic concentrations of  $E_2$  or P.

FIGURE 4



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FIGURE 4 CONTINUED

Biochemical activities of TKS050 (immunoblot analysis). Leiomyoma (**A**) and myometrial (**B**) paired cell cultures were grown for 2 days. Thereafter the cultures were washed, fed with medium without serum, and starved for 48 hours. TKS050 at the appropriate concentration in starvation medium was added for 4 hours. The cells were then stimulated with 30 ng/mL EGF for 10 minutes and analyzed. TKS050 inhibited EGFR autophosphorylation in a dose-dependent manner that is enhanced greatly in the presence of the ligand. TKS050 did not alter the level of EGFR or the expression of the apoptotic proteins Bcl2 and Bax. Greater than or equal to 5 μmol/L TKS050 did not alter the level of Stat3, pStat3 of the leiomyoma cell cultures, but a slight inhibition was detected in the myometrial cell cultures (≥15.5% without EGF stimulation and ≥26% inhibition after EGF stimulation (densitometric analysis was performed with the ImageJ program).

inhibition of EGFR by micromolar concentrations of TKS050 might contribute to the design of medical therapies based on ligand-mediated growth suppression and apoptosis. Previous biochemical analyses of AG1478, another EGFR-specific inhibitor, have shown that this tyrosinase inhibitor effectively inhibits EGFR autophosphorylation in a dose-dependent manner, without an effect on the level of EGFR expression (10–12, 17, 18). However, AG1478 suppressed leiomyoma cell growth but did not induce apoptosis, whereas TKS050 increased the fraction of apoptotic cells. TKS050 did not alter the expression of the apoptosis-related proteins Bcl2 (antiapoptotic) and Bax (proapoptotic) in the leiomyoma and myometrial cultures. Further, TKS050 had no effect on the expression of the Bcl2 and Bax proteins in leiomyoma and myometrial cells cultured with E<sub>2</sub> or P. Our previous and present results indicate that Western blot analysis of the Bcl2 and Bax proteins in matched leiomyoma and myometrial cell cultures did not demonstrate consistent and/or clearly defined differential expression level of these proteins between the two types of cells (10). Matsuo et al. have reported that Bcl2 protein was overexpressed in leiomyoma cell cultures compared with normal myometrium (15). Matsuo et al. have also suggested that Bcl2 protein expression was up-regulated by P, but down-regulated by E<sub>2</sub>. Thus, P might be involved in leiomyoma development through induction of Bcl2 (13). One possible explanation for the discrepancy between our findings and the report of Matsuo et al. could be the monoclonal origin of individual fibroids leading to differences between fibroids even from the same individual uterus.

The possibility of using synthetic inhibitors of EGFR for the nonsurgical treatment of uterine leiomyoma requires

Wei et al. have recently investigated tissue microarray data and found at least two broad groups of leiomyomata presented by the dysregulation of different groups of gene products (16). Importantly, one of these groups was characterized by up-regulation of the EGFR. Thus, the effective

FIGURE 5

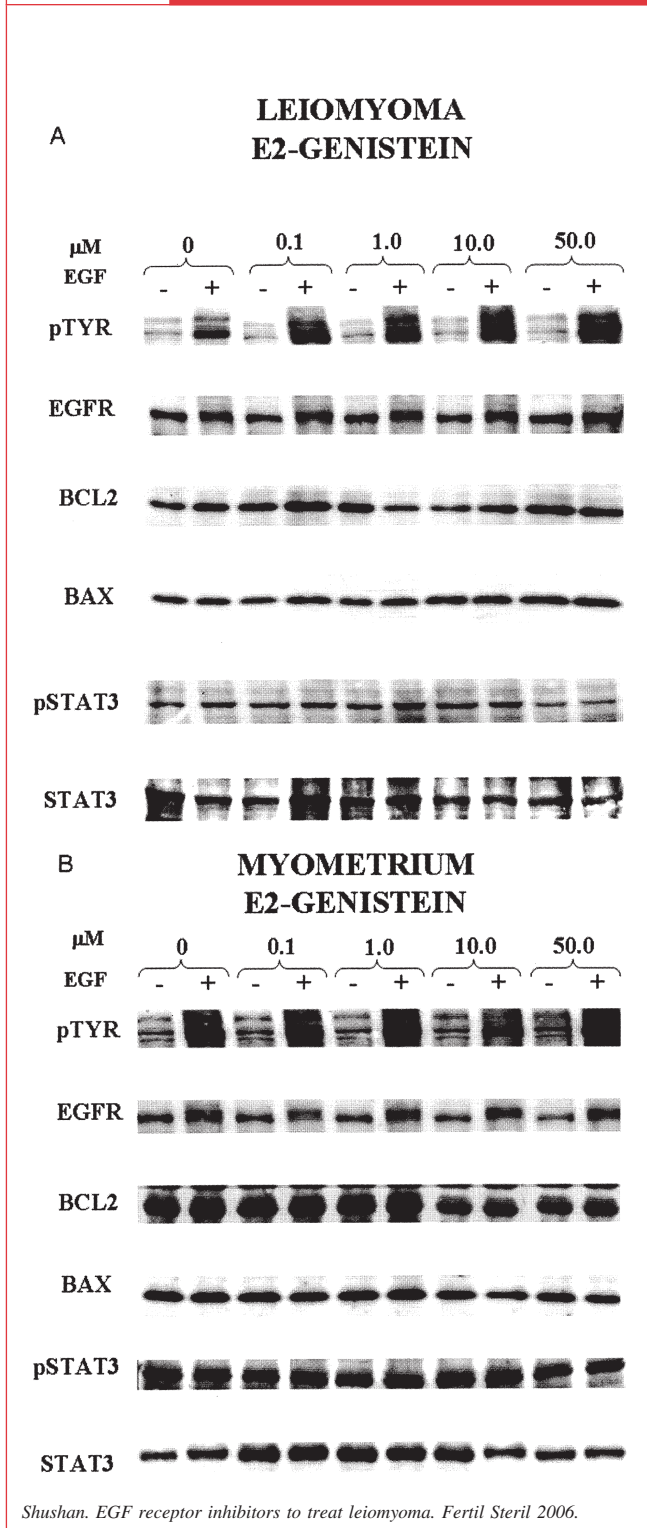


FIGURE 5 CONTINUED

Biochemical activities of genistein (immunoblot analysis). Leiomyoma (A) and myometrial (B) paired cell cultures were grown for 2 days. Thereafter the cultures were washed, fed with medium without serum, and starved for 48 hours. Genistein at the appropriate concentration in starvation medium was added for 4 hours. The cells were then stimulated with 30 ng/mL EGF for 10 minutes and analyzed. Genistein did not induce inhibition of EGFR autophosphorylation that is enhanced in the presence of the ligand. Genistein was not effective at 0.1–50.0 μmol/L on the leiomyoma and myometrial cell cultures. The results also indicate that genistein did not alter the level of EGFR in the cells that is enhanced in the presence of the ligand. Greater than or equal to 1.0 μmol/L genistein suppressed Bcl2 protein level by 50% after EGF stimulation of leiomyoma cell cultures not detected in the myometrial cells. Greater than or equal to 50.0 μmol/L genistein significantly suppressed the level of pStat3 protein (55.2% and 57.7% with or without EGF stimulation, respectively) (densitometric analysis was performed with the ImageJ program).

Of special interest is the suggestion that use of genistein, an isoflavonoid phytoestrogen, considered a naturally occurring PTK, may be an effective inhibitor of leiomyoma cell growth. If indeed genistein would prove beneficial it might be considered an ideal drug to treat leiomyoma as it is a natural product present in diet, safe with minimal side effects, and inexpensive. Our hypothesis was that if the synthetic inhibitors of PTK act as effective inhibitors of leiomyoma cells growth, genistein might have a similar beneficial effect. Furthermore, soy-derived phytoestrogens have been shown to exert several health beneficial effects. Genistein and a panel of phytoestrogens were shown to exert a potent inhibitory effect on cell invasion of MDA-MB-231 breast cancer cells in vitro without affecting cell viability (24). Soy isoflavones have differential intestinal metabolism likely to influence their ability to prevent prostate cancer. Indeed, high consumption of these compounds in Asian diets has been correlated with a lower incidence of clinically important cases of prostate cancer (25). It has also been demonstrated that genistein exhibited dose-dependent (0–50 μmol/L) growth inhibition of many murine and human bladder cancer cell lines, providing evidence of cell growth arrest at the G<sub>2</sub>/M phase followed by apoptosis in some cell lines (26, 27). In the present study, we found that genistein significantly suppressed the level of pStat3 protein. Importantly, recent studies with non-small cell lung cancers indicated a strong positive correlation between phosphorylated EGFR expression and pStat3 expression and an inverse correlation between pStat3 and apoptosis, consistent with less apoptosis in tumors ex-

further examination and evaluation. To this point, two quinoxaline derivatives of AG1478, AG1517/SU5271, have been shown as effective suppressors of psoriatic keratinocyte growth (18, 19). More recently, AG1478 has been selected for clinical development as treatment for glioma multiformis and several other cancer cells (20–23).

pressing high amounts of pStat3. Cell lines with mutant EGFR have increased levels of pStat3 compared with cell lines without mutant EGFR, and this correlates with their sensitivity to gefitinib (a specific inhibitor of autophosphorylation of EGFR) (28). Thus suppression of EGFR-pStat3 might contribute to growth arrest of leiomyomas.

In summary, we have identified TKS050 as a potent inhibitor of leiomyoma and myometrial cell growth. It selectively inhibits autophosphorylation of EGFR and downstream signal transduction events, including cell proliferation and cell cycle progression at micromolar concentrations. A number of PTK inhibitors, especially gefitinib-Iressa-ZD1839 (inhibitor of autophosphorylation of EGFR) are progressing through clinical development to provide new treatment options for a range of malignancies (29). Our results suggest that specific EGFR inhibitors as well as phytoestrogens—broad PTK inhibitors—may be considered as a future therapeutic approach for uterine fibroids. PTK inhibitors—tyrphostins are the first signal transduction agents to be used in clinical practice.

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**000 Inhibition of leiomyoma cell proliferation in vitro by genistein and the protein tyrosine kinase inhibitor TKS050**

A. Shushan, H. Ben-Bassat, E. Mishani, N. Laufer,  
B. Y. Klein, and N. Rojansky  
*Jerusalem, Israel*

Growth of leiomyoma cells is effectively blocked by TKS050 and genistein. The inhibitory action of newly developed and natural inhibitors derived from an individual's diet may be useful as a future therapy for leiomyomas.