Inactivation of estrogen receptor by *Schistosoma haematobium* total antigen in bladder urothelial cells

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Abstract. We recently reported the expression of an estradiol-like molecule by a trematode parasite *Schistosoma haematobium*. We further established that this estradiol-like molecule is an antagonist of estradiol, repressing the transcriptional activity of the estrogen receptor (ER) in estrogen-responsive MCF7 cells and also that *S. haematobium* total antigen (Sh) contains estrogenic molecules detected by mass spectrometry. In the present study, we used HCV29 cells, a cell line derived from normal urothelial cells, as well as an *in vivo* model to evaluate the expression of ER in the bladders of Sh-instilled animals. We show that, similarly to MCF7 cells, Sh down-regulates the transcriptional activity of ER in HCV29 cells and also in the bladders of Sh-treated mice. The antiestrogenic activity of the *S. haematobium* extract and its repressive role in ER could have implications in the carcinogenic process in bladders with *S. haematobium* infection.

Introduction

Schistosomiasis is the first in prevalence among all water-borne infectious diseases and one of the main occupational risks encountered in the rural endemic areas of the world (1). It is a common parasitic disease that ranks second only to malaria as a global cause of morbidity and mortality. It occurs in 76 tropical and subtropical countries and affects about 10% of the world's population. The prevalence of this infection remains high despite the availability of efficacious anti-schistosomal drugs. Individuals become infected with the parasite at an early age after exposure to the free-swimming larval stage (termed cercariae) in contaminated water (2). Despite affecting 200 million people, schistosomiasis remains a neglected disease. There are two main reasons for this: the availability of an effective and cheap drug, praziquantel, has contributed to the wrong perception that chemotherapy would represent the final solution for the control of schistosomiasis, and the disability adjusted life years as a measure of the impact of disease has been underevaluated because of rebound morbidity following chemotherapeutic intervention (3).

Estrogen receptors (ERs) are known to mediate important physiological functions, including reproduction, metabolism, maintenance of bone density, and growth of estrogen-responsive tumors, including breast and endometrial cancers. They are members of a superfamily of nuclear receptors that are able to transduce extracellular signals into a transcriptional response. There are 2 nuclear receptors, ERα and ERβ, which are known to mediate the physiological response to estrogens. They share several functionally conserved structural domains, such as the regions responsible for DNA binding, dimerization, ligand binding and ligand-dependent transactivation of gene expression (4). ERα and ERβ have different transcriptional activities in certain ligand, cell-type, and promoter contexts and also exhibit distinct tissue- and cell-type-specific expression patterns. ERα is found predominantly in the normal pituitary, breast, uterus, vagina, testis, liver, and kidney, whereas ERβ is mainly expressed in thyroid, ovary, prostate, skin, bladder, lungs, gastrointestinal tract, bone and cartilage (4).

We recently reported the expression of an estradiol-like molecule by a trematode parasite *Schistosoma haematobium* (5). We further established that this estradiol-like molecule is an antagonist of estradiol, repressing the transcriptional activity of the estrogen receptor (ER) in estrogen responsive MCF7 cells and also that *S. haematobium* total antigen (Sh) contains estrogenic molecules detected by mass spectrometry (6).

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In the present study, we used HCV29 cells, a cell line of normal urothelial cells, as well as an *in vivo* model to evaluate the expression of ER in the bladders of Sh-instilled animals. We show that, similarly to MCF7 cells, Sh is able to repress the transcriptional activity of ER in HCV29 cells and also in the bladders of Sh-treated mice.

The antiestrogenic activity of *S. haematobium* extract and its repressive role in the ER could have implications in the carcinogenic process in bladders with *S. haematobium* infection.

Materials and methods

Animals. Eight-week-old female golden hamsters (LVG/SYR) and CD-1 mice were provided by Charles River (Barcelona, Spain). Animals were allowed to acclimate for one week under routine laboratory conditions before starting the experiments. They did not receive any treatment prior to the study. Hamsters were kept in separated cages and mice were kept in 6-littermate cages. They were fed standard balanced food and water *ad libitum*. All the animals were maintained at the National Institute of Health (Porto, Portugal) in rooms with controlled temperature (22±2˚C) and humidity (55±10%) and continuous air renovation. Animals were housed in a 12-h light/12-h dark cycle (from 8 am to 8 pm). All animal experiments were performed in accordance with the National (DL 129/92; DL 197/96; P 1131/97) and European Convention for the Protection of Animals used for Experimental and Other Scientific Purposes and related European Legislation (OJ L 222, 24.8.1999).

Experimental infections. Urine specimens were collected from *S. haematobium*-infected individuals living in Angola, an endemic area for schistosomiasis. All individuals were given instructions for the collection of midstream urine specimens. *S. haematobium* infection was detected by the microscopical observation of the eggs in the sediment of the centrifuged urine. Informed consent was obtained from all patients. The eggs were hatched and the resulting miracidia, were used to infect snails from susceptible species.

Golden hamsters were experimentally infected with 100 cercariae by tail immersion. The control animals consisted of littermates. The cercariae were obtained by shedding of snails infected with miracidia.

S. haematobium total antigen (Sh) production. *S. haematobium* adult worms (Angolan strain) were collected by perfusion of the hepatic portal system of golden hamsters at seven weeks after infection with 100 cercarie. The worms were suspended in PBS and then sonicated. The protein extract was then ultracentrifuged and the protein concentration was estimated using a micro BCA protein assay reagent kit.

Experimental design. Forty mice were randomly divided into six experimental groups. Groups 1 and 3 (n=10) were Sh-instilled animals. Groups 2 (n=5) and 4 (n=5) were control animals with their bladders instilled with saline only. Groups 1 and 2 were sacrificed 19 weeks after treatment; groups 3 and 4 were sacrificed 32 weeks after treatment. For the duration of the study, the state of health of the mice was monitored daily.

For intravesical instillation of the parasite total antigen, 50 μg/ml of Sh was introduced directly into the urinary bladders of female mice via the urethra according to a previously described procedure (7). Briefly, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). The bladder was catheterized via the urethra with a 24-gauge plastic intravenous cannula and the bladder mucosa was then traumatized to remove the umbrella cells of the urothelium by instilling 0.1 ml of 0.1 N HCl solution for 15 sec, neutralized with 0.1 ml of 0.1 N KOH and flushed with sterile saline.

A total of 150 μl of Sh with a concentration of 50 μg/ml was instilled via the cannula. The dwell time after instillation was approximately 1 h. The mice were turned 90 degrees every 15 min to facilitate whole bladder exposure to the intravesically-instilled Sh. One hour after the instillation, micturition of the mice was induced by light abdominal massage so that the duration of exposure was constant. Body temperature was maintained with a homeothermic bandage. All the experiments described herein were carried out under aseptic conditions and performed once during this study.

All animals were sacrificed by intraperitoneal administration of sodium pentobarbital (130 mg/kg). Complete necropsies were carefully conducted. All organs were examined macroscopically for any changes. The urinary bladders were inflated *in situ* by injection of 10% phosphate-buffered formalin (150 μl), ligated around the neck to maintain proper distension and then immersed in the same solution for 12 h. After fixation, the formalin was removed; the urinary bladder was cut into two strips, and routinely processed. Tissue sections (2 μm) from urinary bladder, spleen, liver and lung were stained with hematoxylin and eosin (H&E) to characterize changes and inflammatory infiltrate.

Chemicals and materials. HCV29 cells were cultured in phenol red-free RPMI-1640 medium (Sigma-Aldrich, Portugal), with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, purchased from Invitrogen Life Technologies (Paisley, Scotland, UK). Estradiol (E2) was obtained from Sigma-Aldrich (Portugal) and the antiestrogen ICI 182,780 (ICI) was kindly provided by AstraZeneca (Portugal), both chemicals dissolved in 100% ethanol and added to cell culture medium.

Cell culture. HCV29 cells were maintained at 37˚C in a 5% CO_2 humidified atmosphere. Cells were passaged every 5 days. Before treatments cells were serum-starved for 16 h. The cells were treated with Sh antigen for 24 h in serum-free conditions.

Lactoferrin assay. HCV29 cells were cultured in 0.3 ml RPMI-1640 medium in 24-well flat-bottom plates. Cultures were seeded at $5x10^4$ cells/well and allowed to attach overnight. Cells were then treated with 50 μg/ml of Sh for 24 h. Lactoferrin in the supernatant of HCV29 cells was determined using ELISA with an antibody specific for lactoferrin (Hycult, Netherlands) according to the manufacturer's instructions.

Isolation of RNA. At defined time intervals HCV29 cultured cells were harvested in TriPure reagent (Roche, Portugal).

Total RNA was isolated from the cell material according to the manufacturer's instructions. RNA recovery and quality were ascertained by measuring the optical density ratio (260/280 nm) and RNA quantification was also determined from the optical density (Nanodrop).

Real-Time PCR. Real-Time PCR was performed in HCV29 cultures. For this purpose cDNA was obtained using the Promega cDNA kit. For each sample, 1.0 μg of RNA was reverse transcribed in a reaction volume of 20 μl in the presence of 10 mM deoxynucleotide triphosphate and 2 μl RT-PCR enzyme mix. Taqman GeneExpression assays (Applied Biosystems, USA) were used for gene-specific primers and probes for ERα (*ESR1*, Applied Biosystems, USA) and ERβ (*ESR2*, Applied Biosystems, USA). Both target gene transcripts were normalized to the reference gene GAPDH mRNA (Applied Biosystems, USA) content and to the control sample, through the $-2^{\Delta\Delta Ct}$ semi-quantitative method. Quantifications were performed in triplicate in a 7500 Real-Time PCR System (Applied Biosystems, USA).

ER immunohistochemistry. Immunohistochemistry was performed in 5 μm sections attached to silane-coated slides that were dried for 48-72 h at 37˚C before use. After dewaxing in xylene and rehydration through graded alcohol to water, antigens were retrieved using microwaving at 850 W in 10 nM citrate buffer, pH 6.0 for 10 min and allowed to cool. After a brief wash in distilled water endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide for 10 min. Non-specific binding sites were blocked with 10% normal rabbit serum (Dako, Glostrup, Denmark) diluted in phosphate-buffered saline (PBS). Sections were incubated with primary mouse monoclonal antibody against ER (Zymed, San Francisco, CA, USA) in a humidified container for 18 h at 4˚C. After washing with the appropriate PBS for 20 min the sections were incubated with biotinylated rabbit anti-mouse IgG (Dako; 1:400) for 30 min at room temperature. After another wash with buffer for 20 min the speci mens were incubated with Vectastain ABC Elite avidin-biotin complex-linked horseradish peroxidase reagent for 30 min at room temperature in a humidified chamber. After a further wash with buffer for 30 min the immunoreactivity was visualized with exposure to the 3,3'-diaminobenzidine substrate (Vector Laboratories, Peterborough, UK) for 5 min. Sections were briefly counterstained with Mayer's hematoxylin (Sigma).

Immunohistochemistry analysis. The distribution of positively stained cells in the urothelium, urethral epithelium and smooth muscle cells was determined as the percentage of positive cell nuclei. The intensity of the reaction was recorded as follows: negative (-), weak (1+), moderate (2+), and strong (3+).

Statistical analysis. For *in vitro* experiments data were expressed as mean ± SD. T-test was used to assess the statistical significance of differences between groups.

For animal experiments, statistical analysis was performed using Microsoft Excel analysis ToolPak for Windows. The differences in estrogen receptor intensity of expression between groups were assessed by the χ^2 test. For all analyses a $P<0.05$ was accepted as statistically significant.

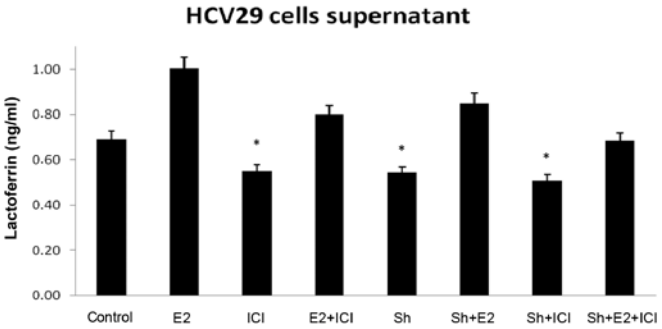

Figure 1. Lactoferrin production in supernatant of HCV29 cells. Cells were treated with 50 μg/ml of Sh. Data represent the means ± SEM from 3 independent experiments (*P<0.05 Sh vs. control; ICI vs. control; and Sh+ICI vs. control).

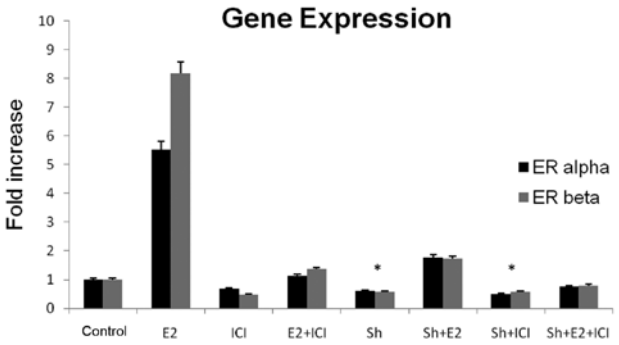

Figure 2. Real-time PCR for ERα and ERβ in HCV29 cells. Bars correspond to the fold-increase of ERα and ERβ gene expression after normalization to the endogenous control gene GAPDH and control sample. Results are representative of three independent experiments. Statistically significant differences in ERα and ERβ gene expression were found between Sh-treated and vehicle-treated HCV29 cells (*P<0.05 vs. control).

Results

S. haematobium total antigen reduces the production of lactoferrin in the supernatant of HCV29 cells. Lactoferrin is a target gene of the ER and can be a useful marker of estrogen action on estrogen responsive cells. Therefore, HCV29 cells were seeded on 96-well plates, serum-starved, treated with 50 μg/ml of Sh for 24 h, cultured for 24 or 48 h, and the supernatants were analyzed by ELISA for lactoferrin (Fig. 1). Bars show that Sh-treated cells produced less lactoferrin than control cells. In addition, incubation with ICI or Sh+ICI also led to an identical decrease in lactoferrin secretion ($P<0.05$ vs. controls). These results suggest that Sh treatment represses estrogen receptor activity in HCV29 cells.

S. haematobium total antigen down-regulates ERα and ERβ in HCV29 cells. To confirm the involvement of the ER, the expression of the *ESR1* and *ESR2* genes were analyzed in HCV29 cells after E2, ICI and Sh treatment. Incubation with E2 clearly increased both *ERS1* and *ERS2* expression (Fig. 2). Furthermore, both ER isoforms were down-regulated by Sh treatment in this cell culture, either alone or in the presence of

Table I. Expression of ER in Sh-instilled bladders and control groups.

Group	N	Evaluated structures	No. of samples showing the stated ER immunoreactivity (%)			
			Negative	Weak (+)	Moderate (++)	Strong (+++)
1	10	Urothelium	10 (100)		-	-
		Urethra	6 (60)	4 (40)	-	-[a]
		Smooth muscle	6 (60)	4 (40)		-[b]
2	5	Urothelium	5 (100)	-	-	-
		Urethra	1 (20)	-	3 (60)	1 (20)
		Smooth muscle	1 (20)	-	3 (60)	1 (20)
3	10	Urothelium	10 (100)	-	-	-
		Urethra	5 (50)	5 (50)	-	-[c]
		Smooth muscle	5 (50)	5 (50)	-	-[d]
4	5	Urothelium	5 (100)			
		Urethra	2 (40)	-	2 (40)	1 (20)
		Smooth muscle	2 (40)	-	2 (40)	1 (20)

[a]P=0.01 for the urethra group 1 vs. group 2; [b]P=0.01 for the smooth muscle group 1 vs. group 2; [c]P=0.03 for the urethra group 3 vs. group 4; [d]P=0.03 for the smooth muscle group 3 vs. group 4.

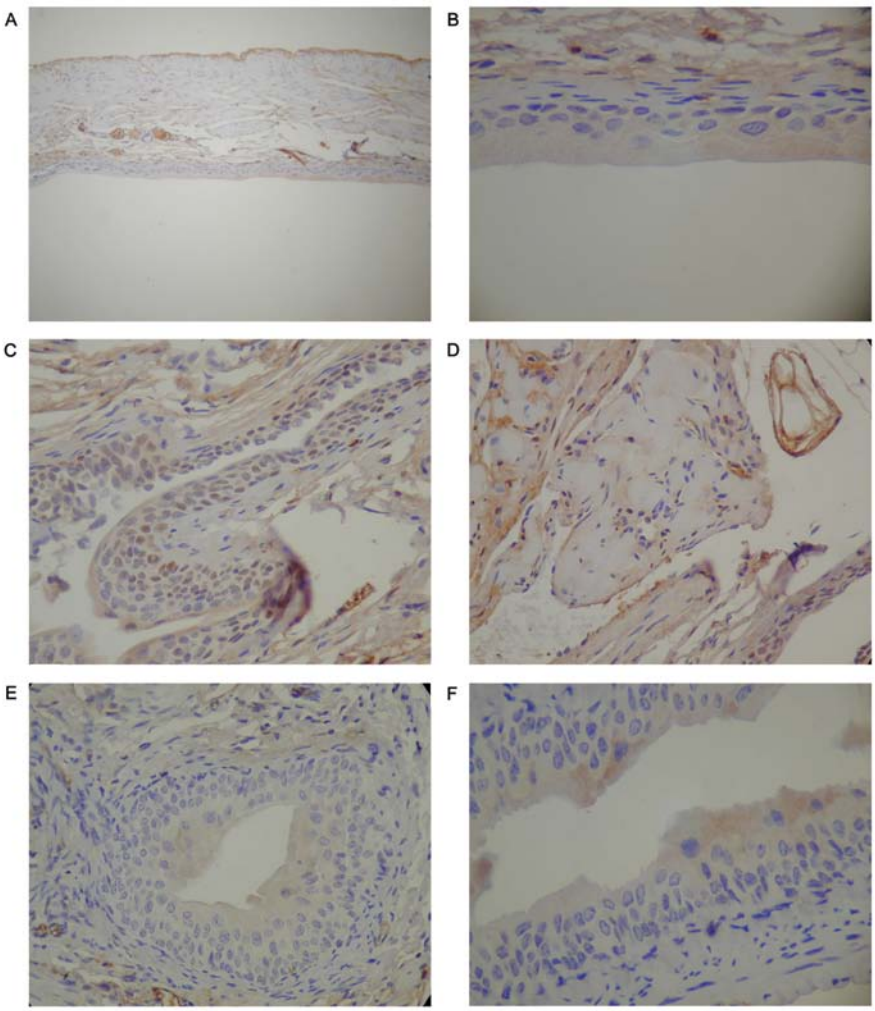

Figure 3. Immunohistochemical staining for ER in the bladders of mice. (A and B) Urothelium of a control animal showing negative staining for ER; (C) urethral epithelium of a control animal showing stained nuclei; (D) smooth muscle cells (SMC) of a control animal with positive staining; (E) urethral epithelium of Sh-treated mouse with negative expression; (F) SMC lining of urethral epithelium of a Sh-treated mouse with negative staining. Magnification, x400.

ICI in comparison to the control (Fig. 2). Interestingly, Sh incubation reduced *ESR1* and *ESR2* expression, even in the presence of E2 ($P<0.05$ Sh+E2 vs. E2).

S. haematobium total antigen down-regulates ER expression in the bladders of CD-1 mice. A total of 30 bladders, 10 from controls and 20 from treated animals, were analyzed for ER expression. The urothelium was always negative for ER protein expression in all animals of the 4 groups. The controls of group 2, sacrificed after 20 weeks, showed positive staining in the urethral epithelium and in the smooth muscle cells (SMC) in 80% (4 out of 5) of the bladders (Table I, Fig. 3A-D). The treated bladders were negative for ER expression in the urethral epithelium (Table I, Fig. 3E) and SMC (Table I, Fig. 3F) in 60% (6 out of 10) of the bladders of mice belonging to Group 1 which were sacrificed after 20 weeks. The bladders of the animals in this group that were considered positive for ER expression (40%) exhibited week immunostaining. The treated animals of Group 3, sacrificed after 40 weeks, were ER-negative for urethral epithelium and SMC in 50% (5/10) of the bladders. The other half showed a pattern of staining similar to the positive bladders of Group 1 (Table I). Group 4, controls sacrificed after 40 weeks, showed positive ER expression in the urethral epithelium and in the SMC in 60% (3 out of 5) of the bladders.

Discussion

This study addresses the suppression of ER transcriptional activity in urothelial cells and the ER expression in the bladders of mice in response to *S. haematobium*. Data presented here add new observations to this area by demonstrating that: i) *S. haematobium* extracts reduce the production of lactoferrin by urothelial cells; ii) urothelial cells produce both transcripts of ER which can be down-regulated by *S. haematobium* extracts; and iii) bladders of mice instilled with *S. haematobium* extracts exhibit down-regulation of ER in urethral epithelium and smooth muscle cells in contrast to controls.

We have previously shown that extracts of this parasite had an antagonistic effect to E2 *in vitro*. We observed decreased production of lactoferrin, an estradiol target gene, in the supernatants of the Sh-treated estrogen responsive cell line MCF-7 and down-regulation of both ERα and β in these cells (6). We now demonstrate that this effect also occurs in urothelial cells and in the bladders of Sh-treated mice. The bladders of mice exhibit down-regulation of ER in the urethral epithelium and the SMC in contrast with controls, but we did not find expression of ER in the bladder urothelium of controls and Sh-treated mice. In accordance, ER was found to localize to the urethral epithelium but has been reported to be absent from the transitional epithelium (8).

The present study addresses the regulation of the anti-estrogenic activity *S. haematobium* as an important mechanism used by the parasite to influence ER transcription. The benefit that this ER repression may provide to the parasite could be explained by the following hypothesis: E2 treatment has been shown to increase smooth muscle density in the bladder of female rats (9). In our study we observed down-regulation of ER expression in the smooth muscle of Sh-treated bladders. During infection with *S. haematobium* the parasite eggs must cross the bladder mucosa to reach the lumen of the bladder to be excreted, in order to survive and continue their life cycle. A thinner smooth muscle layer in the bladder mucosa of infected individuals could possibly increase the number of eggs released.

On another level, ER repression during *S. haematobium* infection could be a collateral damage of the infection. Nevertheless, ER repression could have implications in the long-term sequelae of bladder cancer associated with this infection. We have previously identified by mass spectometry in *S. haematobium* extracts and in the serum of infected individuals, four new estrogenic molecules that are capable, according to their molecular structure, to bind ER and exert the repression effect observed (6). The molecules detected by the LC-MS analysis of both the schistosoma extract and the plasma of a schistosoma-infected human were formed by reactions of estrogen-quinones with DNA (Fig. 4).

The quinone forms generated by metabolic activation of catechol estrogens are highly electrophilic species. Some studies have reported that these species are able to covalently bind DNA bases, forming stable adducts (10-13). These adducts can be considered as estrogen-bound metabolites generated *in vivo* that are produced by nucleophilic attack of the DNA base on different positions of the steroid ring (11-14). Catechol estrogen-3,4-quinone adducts of DNA have been proposed as endogenous initiators of breast, prostate and other human cancers (15). In such cases, oxidation of the catechol estrogen metabolites 4- and 3-hydroxyestradiol would lead to formation of, respectively, estradiol-3,4-quinone and estradiol-2,3-quinone, whose reactions with DNA have shown to be much more reactive and correlated with the carcinogenicity and mutagenicity of their precursors (15). The apparently higher reactivity of estradiol-3,4-quinone with DNA may be possibly due to its longer half-life, providing extended reaction time with DNA (16). Although evidence from cell-culture and animal studies support the estrogen adduct-mediated pathway for the referred cancers, data from humans are scarce. Some authors have reported the presence of 4-OH-E1-1-N3Ade at 8.4 pmol/g in breast tissue from a woman with carcinoma (17), and a non-depurinating hydroxyestradiol guanosine found upon hydrolysis of DNA from breast tissues (18). Interestingly, urine levels of 4-OH-E1-1-N3Ade are low in men with prostate cancer or other urological problems (17), whereas a study involving 76 women showed high urine levels of this estrogen adduct in women at high risk of or with breast cancer, as compared to controls (19). Convert *et al* (20) developed an LC-MS/MS method for assessment of the reactivity of estradiol-2,3-quinone towards deoxyribonucleosides in crude reaction mixtures and proved that LC-ESI-MSn is a very powerful analytical tool for the detection of DNA-estrogen adducts generated *in vivo*. Also, Zhang *et al* (21) have recently described an extraction procedure and a LC-MS/MS assay to detect an estrone-metabolite-modified adenine in 100-200 mg samples of human breast tissue. The carcinogenic effect of this estrogen adduct-mediated pathway of the estrogenic molecules present in *S. haematobium* extracts could explain the link between this parasite and squamous cell carcinoma of the bladder. In accordance, we have previously demonstrated that normal cells treated in the laboratory with *S. haematobium* total antigen, acquire cancer-like characteristics. In fact, these

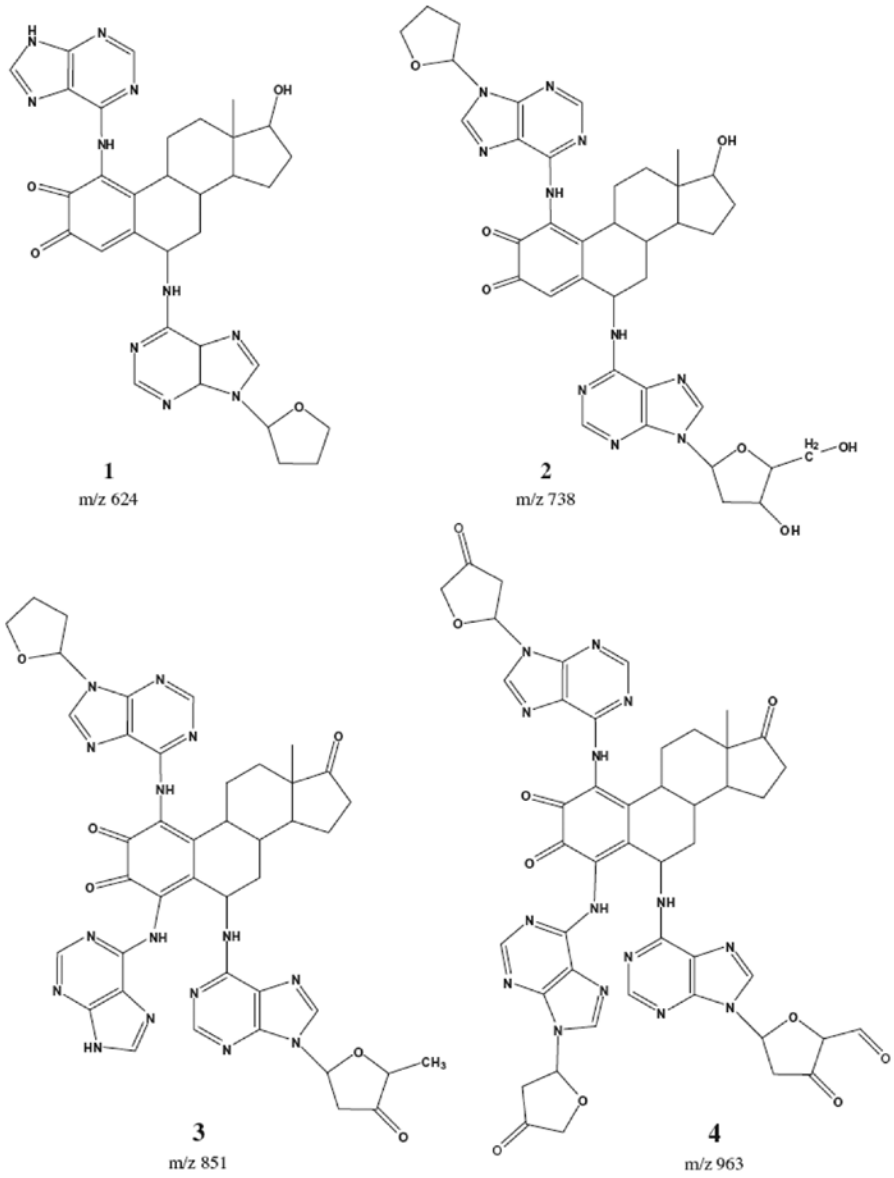

Figure 4. Estrogenic molecules identified in *S. haematobium* extracts. These new estrogenic molecules were detected on the LC-MS/MS analysis and formed by reactions of estrogen-quinones with DNA (6).

cells present rapid uncontrolled division, high resistance to death and an abnormal capability to migrate (22) and, when injected into mice with no immune system, lead to the formation of tumours. Crucially, animals injected with non-exposed cells have shown no growth (23). We also demonstrated that *S. haematobium* total antigen in CD-1 mice normal bladders after intravesical administration of the parasite antigens induced the development of urothelial dysplasia and inflammation (24). The increased migration and invasion of CHO cells induced by Sh previously observed (22) could be explained by the estrogens found in Sh. Corroborating this hypothesis, Maynadier and collaborators (25) demonstrated that E2 could inhibit the invasion in the ERα-negative MDA-MB-231 cell line transiently transfected with ERα, and that this inhibitory effect was reversed by antiestrogens.

In summary, this study reports a decrease of ER expression in urothelial cells after treatment with *S. haematobium* extract. Both ERα and ERβ are down-regulated in these cells by the parasite. Moreover, we also demonstrate that instillation of the *S. haematobium* extract in the bladders of mice down-regulates the expression of ER in the urethral epithelium and in smooth muscle cells. These findings may have relevance to the development of bladder cancer associated with infection with *S. haematobium*. Future studies should assess the specific effects of the estrogenic molecules previously identified in the parasite extract.

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