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The evaluation of the immunomodulating properties of ERA-63 a pharmaceutical with estrogenic activity

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ABSTRACT

This paper describes studies performed with ERA-63 a low molecular weight pharmaceutical with intended immunomodulatory effects. Since this compound was also known to have estrogenic activity a non-conventional approach was taken in order to differentiate between estrogenic and non-estrogenic-induced immunomodulatory effects. EE was included not only for qualitative comparison (hazard identification) between immunomodulatory effects but also, in case of similar effects, to facilitate the extrapolation of the findings in the rat to anticipated effects in humans.

After 28 days of treatment with dosages ranging from pharmacological up to clearly toxic levels for both compounds the immunotoxic potential was assessed by performing a T cell-dependent antibody response and a host resistance assay in rats. Selected ERA-63 dose levels (0.167–0.2, 1.67–2 and 16.7–20 mg/kg) were expected to have comparable estrogenic activity to respective EE dose levels (0.05, 0.5 and 5 mg/kg).

General toxicity parameters reflecting estrogenic activity (i.e. decreased body- and organ weights of thymus and testis, and increased bilirubin and GGT levels) confirmed the comparable estrogenic activity for both compounds at the dose levels tested. Together with the comparable estrogen-related immune suppression (i.e. decreases in specific antibody responses and an increased susceptibility for *Listeria monocytogenes* infects) for both compounds, this indicates that available clinical data for EE facilitates the human risk assessment of ERA-63.

1. Introduction

The pharmaceutical under investigation, ERA-63 (Fig. 1), showed efficacy in inflammatory models and was taken under development for possible treatment of rheumatic diseases (Dulos et al., 2006). However, the immunomodulatory effects of ERA-63 in the tested inflammatory models could potentially be due (in part) to its estrogenic activity (ER-α agonist). Immunomodulating effects as induced by estrogens are pleiotropic and may lead to both enhancement and suppression of many aspects of the immune response. Sex steroid hormones are important factors that contribute to the noted strong sexual dimorphism in the mammalian immune response. Males generally exhibit lower immune responses than females (Cernetich et al., 2006; Klein, 2000; Schuurs and Verheul, 1990). Conversely, females from many species have been shown to be more prone to producing immune responses against self-tissues and thus exhibit an increased tendency to develop autoimmune diseases in comparison to male individuals. In addition, an elevated humoral immune response (i.e. antibody production by B-cells) has been demonstrated in females. In male rats physiological levels of estrogen enhances immune response by increasing the synthesis of IgM antibodies (Myers and Petersen, 1985). The cell-mediated immune response also differs between males and females with females exhibiting a more pronounced Th2 response than males (Myers and Petersen, 1985).

Although physiological levels of estrogens are immunostimulatory, supraphysiological levels appear to be immunosuppressive (Kovacs et al., 2002). Levels of estrogen during pregnancy may be 100–1000 times that of cycling females. This suppresses maternal Th1 responses in order to protect the conceptus and it attenuates the severity of certain disorders which are mediated by aberrant immune function, such as autoimmune diseases (Kovacs et al., 2002). Likewise, estrogen treatment protects female mice from experimental autoimmune encephalomyelitis by inhibiting the recruitment of T cells and macrophages into the CNS (Subramanian et al., 2003). In pregnant mice B cell lymphopoiesis appeared to

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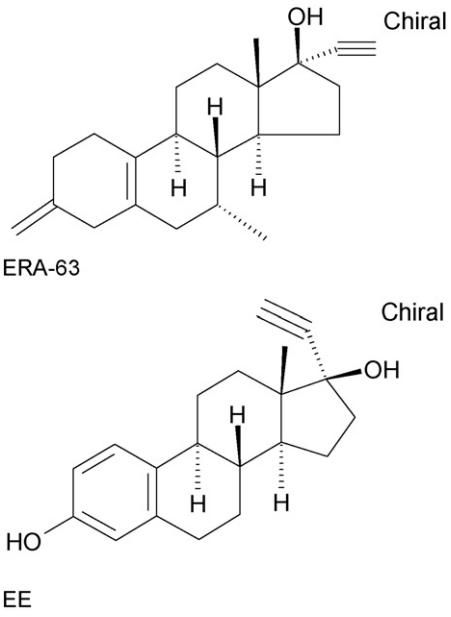

Fig. 1. Structures of ERA-63 and EE.

be selectively suppressed due to the increased levels of estrogen (Medina and Kincade, 1994). In rats estrogens enhance antibody production in the physiological range and suppress antibody production in the pharmacological range (Trawick and Bahr, 1986). Female mice treated with either physiological or pharmacological doses of estrogens are more susceptible to *Listeria monocytogenes*, *Salmonella typhimurium* and *Toxoplasma gondii* than non-treated female or males (Kita et al., 1985, 1989; Pung et al., 1985).

Functional estrogen receptor expression is required for both the humoral (Erlandsson et al., 2003; Smithson et al., 1998; Thurmond et al., 2000) and the cell-mediated immune response (Huber et al., 1999; Liu et al., 2003; Maret et al., 2003). The two nuclear estrogen receptor isoforms (ERα and ERß) are differentially expressed according to immune cell type (Pillet et al., 2006). Mechanisms by which estrogen affect the functionality of the immune system may be (i) regulation of cytokine expression, (ii) affecting the elimination of autoreactive cells (negative and positive selection) in development organs (e.g. thymus and bone marrow) through an effect on the morphology and (iii) altering the patterns of apoptosis of T and B cells (Ahmed, 2000).

In summary, the above indicates that interactions of estrogenic compounds are complex and due to the induced pleiotropic effects not easily predictable. The overall aim of the performed studies (the PFC assay and the host resistance model using *L. monocytogenes*) was to investigate whether ERA-63, a compound with intended immunomodulatory effects and having estrogenic activity, showed immunomodulating properties that could not be attributed to it's estrogenic activity. Both the PFC assay (testing the T cell-dependent antibody response to SRBC) and the host resistance assay (testing the non-specific immunity towards a *L. monocytogenes* infection) are mentioned in the ICH S8 guideline for immunotoxicity testing of human pharmaceuticals as recommended functional immune tests to screen for potential immunotoxicity of compounds, and are expected to give sufficient data for risk assessment and risk management. Effects of test compounds in the PFC assay alone are considered to be about 80% predictive for immunotoxicity (Luster et al., 1992).

Estrogenic potency was assessed mainly by the indirect parameter body weight as in toxicological experiments this parameter appeared to be a very sensitive parameter for estrogenic activity in the rat (Andrews et al., 2002b). Moreover, in previous in-house performed sub-acute toxicity experiments (data not shown) a dose level of 0.05 mg/kg EE (pharmacological dose) produced a slightly less decreased body weight in female Sprague–Dawley rats than a dose of 0.2 mg/kg ERA-63 in Wistar rats. Hence these dosages and 10 times multiples were selected for the present studies. Besides a qualitative comparison (hazard identification) of immunomodulatory effects the parallel testing with EE and the evaluation at estrogenic equipotent dose levels allowed also for a quantitative risk assessment in case similar results would be obtained.

2. Materials and methods

2.1. Test chemical

Ethinyl estradiol (19-nor-17alpha-pregna-1,3,5[10]-trien-20-yne-3, 17beta-diol, CAS no 57-63-6, purity 100%) was originally obtained from N.V. Organon, Oss, The Netherlands. It was formulated by the Investigational Products Supply Department, Organon S.A., Riom, France, as a freeze dried preparation. After reconstitution with water for injection a suspension containing 5 mg ethinyl estradiol/mL in gelatin/mannitol 0.5%/5% (w/v) was obtained. Final dosing preparations were prepared by dilution with gelatin/mannitol 0.5%/5% (w/v).

ERA-63 ((7-alpha,17-alpha)-7-methyl-3-methylene-19-norpregn-5[10]-en-20-yn-17-ol, solution in ethanol) was originally obtained from N.V. Organon, Oss, The Netherlands. It was formulated by the Investigational Products Supply Department, Organon S.A., Riom, France, as a suspension containing 50 mg ERA-63/mL, gelatin/mannitol 0.5%/5% (w/v) and ethanol <2%. Final dosing preparations were prepared by dilution with gelatin/mannitol 0.5%/5% (w/v).

Cyclophosphamide, i.v. lyophilisate (CPS, Product code: RVG 08058) was obtained from Asta Medica, Frankfurt/Main, Germany. A solution of 25 mg/mL (males) or 12.5 mg/mL (females) was prepared in water for injection shortly before administration.

2.2. Animals

Male and female rats HSD/CpB: WU (spf-bred) obtained from Charles River Deutschland, Sulzfeld, Germany, were kept in macrolon cages with a soft wood bedding (four animals of one sex per cage, except for the control group of the plaque-forming cell assay in which five animals of the same sex were housed per cage). The rats were fed ad libitum using a standard pelleted rodent diet (RM3 (E) SQC, obtained from SDS Special Diets Services, Whitham, England). Animals were 6–8 weeks old upon arrival and 8–10 weeks at the start of dosing. Body weight range at the start of dosing was 158–201 g for females and 219–287 g for males in the plaque-forming cell assay and 135–188 g for females and 184–253 g for males in the host resistance assay. The animal room had a 12-h light/dark regime, a temperature of 21 ± 2 °C, a relative humidity of 30–71%, and a rate of air exchange of approximately 10 times per hour. All procedures used in this experiment were compliant with the regional ethics regulations and approved by a local ethics committee.

2.3. Experimental design

Two studies were performed each consisting of eight groups of male and female rats dosed orally with the vehicle (control group), ethinyl estradiol at a low dose of 0.05 mg/kg, a mid dose of 0.5 mg/kg and a high dose of 5 mg/kg (comprising a dose range starting at approximately the pharmacological dose up to clearly toxic levels), ERA-63 at a low dose of 0.2 mg/kg, a mid dose of 2 mg/kg and a high dose of 20 mg/kg (comprising a dose range starting at approximately the pharmacological dose up to clearly toxic levels), and cyclophosphamide at a dose level of 50 mg/kg (males in the host resistance assay received 100 mg cyclophosphamide/kg). Due to human error the dose levels ERA-63 in "Study II: host resistance assay with *Listeria monocytogenes*" were from the second day of dosing onwards 0.167, 1.67 and 16.7 mg/kg for the low, mid and high dose, respectively, instead of the above-mentioned dosages.

The control group, the ethinyl estradiol treated groups and the ERA-63 treated groups were treated daily by gavage (1 mL/kg) for 29 days (first day of treatment is day 0). Cyclophosphamide was administered once intravenously (4 mL/kg) on day 27 in the host resistance assay and on day 24 in the plaque-forming cell assay (on both occasions the day before challenge with antigen or pathogen).

2.3.1. Study I: T cell-dependent antibody response in the plaque-forming cell (PFC) assay

In the plaque-forming cell (PFC) assay eight animals/(group sex) (ten animals/sex for the control group) were dosed as indicated above. On day 24, all groups received an i.v. injection of 0.5 mL with $5 \pm 1 \times 10^8$ sheep red blood cells (SRBC, Biotrading Benelux b.v., Mijdrecht, The Netherlands).

Autopsy was performed on day 29. The animals were sacrificed using CO_2/O_2 and spleen was collected aseptically, weighed and further processed to determine the number of plaque-forming cells, in addition, the thymus was weighed.