Micronucleus induction in the bone marrow of rats by pharmacological mechanisms. I: glucocorticoid receptor agonism

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A novel selective glucocorticoid receptor (GR) agonist, AZD2906, was found to increase the incidence of micronucleated immature erythrocytes (MIE) in the bone marrow of rats given two oral doses at the maximum tolerated level. Because GR agonists as a class are considered not to be genotoxic and AZD2906 showed no activity in the standard in vitro tests or in vivo in a rat liver comet assay, investigative studies were performed to compare AZD2906 with a reference traditional GR agonist, prednisolone. Emphasis was placed on blood and bone marrow parameters in these studies because GR activation has been reported to induce erythropoiesis which, in turn, is known to increase MIE in the bone marrow. Both compounds induced almost identical, small increases in micronucleus frequency at all doses tested. Directly comparable changes in haematological and bone marrow parameters were also seen with significant decreases in lymphoid cells in both compartments and significant increases in numbers of circulating neutrophils. Although no evidence of increased erythropoiesis was seen as increased immature erythrocyte numbers either in the blood or in the bone marrow, histopathological examination showed focal areas in the bone marrow where the erythropoietic tissue was enriched in association with an atrophic myeloid lineage. This could have been due to direct stimulation of the erythroid lineage or a secondary effect of myelosuppression inducing a rebound increase in erythropoiesis into the vacant haematopoietic cell compartment. It was concluded that the increased MIE frequencies induced by both AZD2906 and prednisolone are a consequence of their pharmacological effects on the bone marrow, either by directly inducing erythropoiesis or by some other unknown effect on cellular function, and do not indicate potential genotoxicity. This conclusion is supported by the lack of carcinogenic risk in man demonstrated by decades of clinical use of prednisolone and other GR agonists.

Introduction

Corticosteroid drugs such as prednisolone and dexamethasone have been in clinical use for a considerable time and there has been no suggestion that, as a class, they present any genotoxic or carcinogenic risk. Steroids in general are considered not to be genotoxic and even the genotoxic potential of oestradiol, oestrone and ciproterone acetate is believed to be irrelevant under normal physiological and therapeutic conditions (1). There appear to be very few studies on the genotoxicity of corticosteroids in the open literature, probably because they are generally considered to be inactive, and a relatively recent review (1) found only a single paper which reported that dexamethasone induced chromosome aberrations in human lymphocytes in vitro and increased micronuclei in the bone marrow of mice (2). It was surprising, therefore, when a novel selective glucocorticoid receptor (GR) agonist AZD2906, discovered jointly by AstraZeneca and Bayer HealthCare Pharmaceuticals, which had given negative results in a bacterial mutation test and mouse lymphoma T2 assay, was found to increase micronucleus frequency in the bone marrow of rats. Unpublished observations within the pharmaceutical industry indicated that this may be a class effect and a subsequent review of the US product listings for traditional GR agonists found that the warning labels for a number, including betamethasone valerate (Lunix; 2002), clobetasol propionate (Olux-E; 2006), fluocinonide (Vanos; 2006) and triamcinolone acetonide (Trivaris; 2008), stated that positive results had been seen in rodent bone-marrow micronucleus tests. The CDER Pharmacology review (3) for ciclesonide contains data showing weak, but clear, increases in micronuclei in the bone marrow of mice given high oral doses and comparable increases were also seen with dexamethasone and budesonide included as reference agents. Ciclesonide was subsequently shown not to increase tumour incidence in mouse oral and rat inhalation carcinogenicity studies and from these data it was concluded that its effect on the mouse bone marrow was a consequence of GR agonism and did not predict carcinogenic potential.

The purpose of the present study was to examine the effect of a reference traditional GR agonist, prednisolone, on bone marrow micronucleus frequency and a range of haematology and bone marrow parameters in the rat for comparison with AZD2906 and to make publicly available definitive data to show them to be a pharmacological class effect.

Materials and methods

All chemicals and reagents were purchased from Sigma (Dorset, UK) unless specified. The chemical structures of AZD2906 and prednisolone, together with their IC50s at the GR receptor, are shown in Figure 1.

Animal husbandry

Male Wistar Han rats (substrain HsdHan) were obtained from Harlan UK and housed three or four per cage. All animals were ~10 weeks old at dosing. Environmental controls were set to maintain conditions of 19–23°C and 40–70% relative humidity, with a 12-h light/dark cycle. All animals were treated in accordance with approved UK Home Office licence requirements.

Bone marrow micronucleus tests

For both prednisolone and AZD2906, rat bone marrow micronucleus tests were performed according to current Organisation for Economic Cooperation and Development (OECD) guidelines, except that concurrent positive control groups were not included, in the standard protocol in use at AstraZeneca. Groups of seven male rats were given two doses of the vehicle control or test compound 24 h apart and killed 24 h after the second dose by halothane administration and cervical dislocation. A femur was removed from each rat, the ends clipped to expose the bone marrow and smears prepared and stained essentially as described by Tinwell and Ashby (4). The femoral cells were
flushed out using foetal bovine serum containing 25 mmol/l EDTA, filtered through a 150 μm bolting cloth and centrifuged at 200g for 5 min. The supernatant was removed, the cell pellet carefully mixed by repeated aspiration with a Pasteur pipette and a small drop spread onto a clean microscope slide, air dried and then fixed with methanol. After drying again, slides were dipped in fresh phosphate buffer pH 6.4, stained with 12.5% acridine orange and then rinsed in two changes of fresh phosphate buffer and air dried. Prior to analysis, slides were coded, wet mounted with phosphate buffer and examined blind by fluorescence microscopy at ×400 magnification. Identification of immature erythrocytes (IE), mature erythrocytes (E) and micronucleated erythrocytes (MIE) was described by Hayashi et al. (5).

Initially, for each animal, 2000 IE were scored for the incidence of micronuclei and the ratio of immature to mature erythrocytes (IE:E) was determined in 1000 cells. With AZD2906, the analysis was extended by scoring an additional 4000 IE to give a total of 6000 IE for each animal (6). In order to act as a quality control procedure, six bone marrow slides from rats treated with cyclophosphamide at 20 mg/kg (72 μmol/kg) in a previous study were included prior to staining and coding all the slides (7).

### AZD2906

AZD2906 (purity 96.5%) was synthesised at AstraZeneca R&D. It was suspended in 0.5% w/v hydroxypropyl methylcellulose and 0.1% Tween 80 in 50 mM citrate buffer of pH 4.0 and groups of seven rats were given two oral doses of the vehicle, 5, 25 or 50 mg/kg. A previous study had shown 50 mg/kg to be the maximum tolerated dose. After increases in bone marrow micronucleus frequency were seen, a subsequent study was performed to investigate possible haematological and bone marrow changes. Groups of eight male rats were again be the maximum tolerated dose. After increases in bone marrow micronucleus frequency were seen, a subsequent study was performed to investigate possible haematological and bone marrow changes. Groups of eight male rats were given two oral doses of the vehicle, 500, 1000 or 1500 mg/kg and then killed 24 h after the second dose. Blood samples (0.5 ml into EDTA) were taken for analysis of the following haematological parameters: erythrocytes, platelets, leukocytes, neutrophils, lymphocytes, monocytes, basophils, eosinophils, large unstained cells.

One femur was removed for flow cytometric analysis of the following bone marrow parameters: erythroid cells per femur, percent erythroid cells, lymphoid cell per femur, percent lymphoid cells, myeloid/erythroid ratio, myeloid cells per femur, percent myeloid cells, percent red blood cells, total nucleated cells per femur, percent total nucleated cells.

The second femur was prepared for microscopic pathological assessment as follows. The femur, attached to the tibia, was removed from each rat, dissected free of as much skin and muscle as possible and the distal ends of both bones were cut with bone forceps to expose the inner marrow cavity for fixation. The entire femorotibial joint was then immersed in approximately 10× its own volume of 10% phosphate-buffered formalin at pH 7.4. The joint was allowed to fix for approximately 10 days before being washed and then immersed in a decalcifying fluid composed of equal parts of 40% formic acid and 10% acetic acid. The decalcifying fluid was changed every 2 days until, by inspection, the bone was soft enough to allow slicing with a scalpel without the obvious presence of hard bone. This occurred after approximately 8–10 days of immersion in decalcifying fluid. The femorotibial joint was then trimmed to fit an embedding cassette and washed in running tap water, dehydrated in graded ethanol solutions and embedded whole in paraffin wax following clearing in xylene.

The dehydration and embedding schedule was standard for this tissue. Slices of liver and thymus, 3–4 μm thick, were sampled and fixed in the same fixative as described above for the femorotibial joint, for a minimum of 7 days. The samples were then dehydrated and embedded in paraffin wax using standard processing schedules. About 4–5 μm thick longitudinal wax sections from femorotibial joints and transverse sections of liver and thymus were prepared on a microtome, dewaxed and stained with haematoxylin and eosin stain before being examined by light microscopy.

#### Prednisolone

Prednisolone (purity 99%) was suspended in 0.5% hydroxypropyl methylcellulose/0.1% w/v polysorbate 80, groups of seven rats were given two oral doses of the vehicle, 500, 1000 or 1500 mg/kg and then killed 24 h after the second dose. A previous study had shown 1500 mg/kg to be the maximum tolerated dose. The incidence of MIE in the bone marrow and the haematological and flow cytometry bone marrow parameters were analysed as for AZD2906. Because the dose-setting study had shown some evidence of gastrointestinal ulceration, at necropsy the thoracic and abdominal cavities of all animals were examined macroscopically.

#### Statistical analysis

Prior to analysis of the micronucleus tests, the MIE counts were subjected to an average square-root transformation and transformed counts were analysed in all cases. Analysis of variance, with appropriate linear contrasts, was used to test for dose-related trends in MIE and was determined in the following way. If a significant increase was observed across all test doses, then the trend test was reapplied in a cascade—first excluding the top dose, then the intermediate dose(s) and finally the control and the lowest dose. This closed testing procedure stops when either a non-significant effect is detected or all dose levels have been tested. Positive controls were excluded from this analysis. Positive controls were compared with the concurrent vehicle controls using Fisher’s exact test and was one sided at the 5% level.

Haematology and bone marrow data were analysed pairwise using Shirley’s test with significance at the 5% level.

### Results

#### Bone-marrow micronucleus frequency

AZD2906 was found to give biologically significant increases in MIE at all doses after analysis of the standard 2000 IE. The increases exceeded the concurrent and historical control values but, because there was inter-animal variability in the groups given 5 or 25 mg/kg, and also because the results were unexpected at that time, the analysis was extended to score a total of 6000 IE. This confirmed that statistically significant (p < 0.001) increases greater than 3-fold the concurrent control value were seen at all three dose levels (Table 1; supplementary Appendices 1 and 2, available at Mutagenesis Online).
Histopathology investigation was only conducted on rats given AZD2906 and doses of 5 or 25 mg/kg/day induced histological changes in the liver, thymus and bone marrow of the femorotibial joint.

Discussion

The results from this study clearly show that both AZD2906 and prednisolone have comparable effects on the bone marrow of rats and the maximum increases in micronucleus frequency over the concurrent control values, 4.0 per 2000 IE, were exactly the same for both compounds. No-effect levels (NOEL) were not established and similar increases were seen at all
doses of both AZD2906 and prednisolone, presumably because the blood levels are so far in excess of their IC50s at the GR. For AZD2906, two doses of 5 and 25 mg/kg gave C max values of 643 and 2520 nmol/l and area under the curve (AUC) of 7270 and 36,400 nmol.h/l, respectively, in comparison with the IC50 of 7.5 nmol/l determined in whole rat blood. Similar plateau responses have also been reported in the mouse with doses of ciclesonide from 150 to 2000 mg/kg and dexamethasone from 25 to 1000 mg/kg and the maximum increases, 4.1 and 3.3 per 2000 IE, respectively (3), were also remarkably similar to those in the rat in the present study. It appears that the propensity to increase micronucleus frequency in rodents is common to several GR agonists with differing chemical structures.

In terms of the mechanism of action, it appears unlikely that DNA reactivity is responsible for the effects in the bone marrow because there are no reliable reports in the literature of any of the GR agonists considered here giving positive results in standard in vitro tests for genotoxicity. Prednisolone has been reported not to be a bacterial mutagen in a limited study (8) and also in a test using a standard set of tester strains (9). Although it has been reported to be weakly active in the mouse lymphoma assay (10), the increases in mutant frequency do not satisfy current criteria for a positive result (11). Dexamethasone has also been shown to give negative results in the Ames test but to induce chromosome damage and sister chromatid exchanges in human lymphocytes (2). Ciclesonide has given negative results in the Ames test, an assay for mutation at the hprt locus in Chinese hamster ovary cells and an assay for chromosome aberrations in human lymphocytes (3). For AZD2906, no evidence of genotoxicity was seen in vitro in Good Laboratory Practice-compliant bacterial mutation and mouse lymphoma Tk assays performed to current International Conference on Harmonisation (ICH) and OECD guidelines. The pattern of metabolites of AZD2906 in the rat has been determined and none (nor AZD2906 itself) has any structural alert for bacterial mutagenicity. Further, a quantitative whole-body autoradiography study in rats showed no evidence of tissue retention of radioactivity, with the exception of the choroid and retinal pigment, indicating a lack of formation of reactive metabolites and negative results were also obtained in a rat liver comet assay (supplementary Appendix 5, available at Mutagenesis Online) using the same doses as those increasing bone-marrow micronucleus frequency. For both ciclesonide and AZD2906, potential aneugenicity can be dismissed from negative results in in vitro micronucleus tests using Chinese hamster V79 (3) and mouse lymphoma cells (supplementary Appendix 6, available at Mutagenesis Online), respectively.

![Fig. 2. Marrow atrophy in the sub-growth plate region of the tibia induced by AZD2906. (a) Control marrow: microscope magnification ×10. (b) Control marrow: microscope magnification ×40. (c) Marrow from AZD2906-treated rat: microscope magnification ×10. (d) Marrow from AZD2906-treated rat showing retention/hyperplasia of erythroid cells: microscope magnification ×40.](https://academic.oup.com/mutage/article-abstract/28/2/227/1084975)
Because of the lack of evidence of genotoxicity with AZD2906 and other GR agonists, it seems likely that the increased incidence of micronuclei in the bone marrow is a consequence of pharmacology-related effects and this is supported by the similarity in the haematology and bone marrow changes with both AZD2906 and prednisolone (Table II). There was evidence for retention of the erythroid colonies amongst a general pattern of myeloid atrophy in AZD2906-treated animals. This may represent an early hyperplasia of the erythroid series and/or retention of mature, nucleated erythroid precursors within the marrow, but it was not possible to arrive at this interpretation unequivocally in the histopathological sections presented. The effects of glucocorticoids on haematopoietic cells are well documented in the published literature and have recently been reviewed (12). Activation of the GR induces apoptosis of lymphocytes, eosinophils and monocytes and protects granulocytes from apoptosis but also stimulates erythropoiesis (13–15). Treatment of non-anaemic patients with prednisolone is associated with increased erythropoiesis, and patients with Cushing’s syndrome (excess circulating glucocorticoids) show elevated haemoglobin and haematocrit (16). Glucocorticoids are also used in the treatment of various forms of anaemia (13, 17–19). GR agonists may stimulate erythropoiesis through a number of mechanisms including the following:

- A direct effect on the erythroid lineage mediated via the GR. Erythroid progenitor cells express the GR and treatment with glucocorticoids has been shown to induce the proliferation and expansion of erythroid progenitor cells in vitro while maintaining their colony-forming capacity and delaying terminal differentiation of erythrocytes (15, 20). In the clinic, children with acute lymphoblastic leukaemia in remission show reticulocytosis and increased haemoglobin concentration following pulse prednisolone therapy (13).
- A secondary response due to marked myelosuppression mediated via the GR. The bone marrow has been shown to respond to marked reductions in the haematopoietic cell volume with a rebound increase in erythropoiesis.

Although GR-mediated increase in erythropoiesis is a plausible mechanism for the induction of MIE and histopathological examination revealed areas where the erythroid population appeared to be enriched, there were no increased numbers of immature erythrocytes seen in either the bone marrow or the peripheral blood. In the bone marrow, it is probable that the number of cells in the areas of erythroid enrichment were not sufficient to increase significantly the overall number in the suspension of cells from the whole femur that were used for flow cytometric analysis. It is also likely that increased numbers were not seen in peripheral blood because the sampling time, 48 h after the first dose, was before the majority of the immature erythrocytes produced after dosing had left the bone marrow. Finally, it is possible that the increase in MIE is not a consequence of increased erythropoiesis but another, unknown effect of GR agonism.

In conclusion, several GR agonists have been shown to increase the frequency of micronuclei in the bone marrow of rodents. The data here show directly comparable responses to AZD2906 and prednisolone in the rat and these are also remarkably similar to those seen with ciclesonide in the mouse (3). Unpublished information from within the pharmaceutical industry indicates that the bone marrow responses to GR agonists have been seen with other compounds in development and it is likely that the negative micronucleus tests for some of the older marketed compounds were not performed to current standards. For example, no evidence of activity was reported in a test with prednisolone farnesylate (9) but subcutaneous injection was used and the data are not available for inspection. Finally, it should be noted that because increases in MIE were seen at all doses of both AZD2906 and prednisolone, NOEL were not established for either compound. Further work would be necessary to establish whether the margin between any intended therapeutic level of AZD2906 and the NOEL in the bone marrow is similar to those for prednisolone and other GR agonists. It has been established previously that increased erythropoiesis following prior toxicity to erythroblasts or by direct stimulation of division in these cells can result in increases in bone marrow micronuclear frequency (21). This study with GR agonists indicates that anti-inflammatory pharmacology modulating the erythroid lineage in the bone marrow can have the same effect but without marked effects on simple indicators of erythropoiesis such as increased numbers of immature erythrocytes in the bone marrow or circulating reticulocytes.
Supplementary data

Supplementary Appendices 1–7 are available at Mutagenesis Online.

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References