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Differential Potency of Beclomethasone Esters In-vitro on Human T-lymphocyte Cytokine Production and Osteoblast Activity

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Abstract

Beclomethasone dipropionate is an inhaled corticosteroid, used for the treatment of asthma. It is metabolised to 17-beclomethasone monopropionate, which has greater affinity for corticosteroid receptors than the parent compound, and to beclomethasone. We investigated the potency of beclomethasone dipropionate, 17-beclomethasone monopropionate and beclomethasone (compared with dexamethasone as a reference steroid) in two different human cell types, peripheral blood mononuclear cells and osteoblasts.

We found that beclomethasone dipropionate, 17-beclomethasone monopropionate (EC50 10^{-14} M) and beclomethasone (EC50 approx. 10^{-12} M) were much more potent than dexamethasone (EC50 10^{-8} M) in inhibiting interleukin-5 production by peripheral blood mononuclear cells. In contrast, beclomethasone dipropionate, 17-beclomethasone monopropionate and beclomethasone were equipotent with dexamethasone (EC50 range 0·3–$1{\cdot}2\times10^{-9}$ M) in affecting several functional assays of osteoblasts (e.g. alkaline phosphatase activity and osteocalcin synthesis).

These results show that the relative bioactivities of corticosteroids vary between different human cell types, and that affinities observed in receptor binding assays are not necessarily predictive of the bioactivity in cell populations, such as peripheral blood mononuclear cells and osteoblasts, which are putatively relevant to efficacy and side effects respectively.

The introduction of the inhaled corticosteroid, beclomethasone dipropionate, almost 30 years ago was a major advance in the treatment of asthma because patients who had previously required long-term oral prednisone could switch to this new inhaled corticosteroid without any apparent unwanted systemic activity, provided that the inhaled dose was no more than 2000 μg daily (Wilcox & Avery 1973). Beclomethasone dipropionate undergoes hydrolysis to the more polar products, 17-beclomethasone-monopropionate, beclomethasone-21-monopropionate and beclomethasone. It was initially reported that the degradation of beclomethasone dipropionate was an inactivation step because 17-beclomethasone-monopropionate and 21-beclomethasone-monopropionate were thought to be less potent than the parent beclomethasone dipropionate (Martin et al 1974). More recent studies have demonstrated that 17-beclomethasone-monopropionate binds to steroid receptors with a greater affinity than beclomethasone dipropionate so it appears that beclomethasone dipropionate is a pro-drug which is hydrolysed to a more active species, 17-beclomethasone-monopropionate (Würthwein & Rohdewald 1990).

When corticosteroids are administered by the inhaled route, they are delivered directly to the target organ without significant unwanted systemic activity, provided that clinically recommended doses are not exceeded. With the development of sensitive assays it has been possible to detect beclomethasone in the plasma after the inhalation of doses as low as 400 μg (Seale & Harrison 1998).

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In some patients this systemic absorption of inhaled corticosteroids can cause unwanted effects such as easy bruising of the skin (Capewell et al 1990). Whether or not inhaled corticosteroids affect bone metabolism and predispose to the development of osteoporosis, a well recognised side effect of long-term oral prednisone, is disputed. Changes in markers of bone formation such as serum osteocalcin have been demonstrated in short-term studies of inhaled corticosteroids. The clinical significance of these findings is uncertain as the reductions in serum osteocalcin were paralleled by reductions in bone mineral density in one study (Hanania et al 1995), but not in another (Boulet et al 1994).

In clinical studies the efficacy of an administered dose of beclomethasone dipropionate will be the net result of the parent drug and its metabolites on the inflammatory response in the lung. Similarly, any unwanted systemic activity will be due to the net activity of the absorbed parent drug and its active metabolites. In the absence of any data to the contrary, it has been assumed that the rank order of potency of corticosteroids at receptors in one cell type would predict the rank order at receptors in other cell types. Thus, the most potent steroid in models of inflammation would be the most potent in terms of target cells for side effects.

We have investigated whether this assumption is valid by determining the rank order of potency of beclomethasone dipropionate and its metabolites, using two separate human cell types in-vitro. We chose peripheral blood mononuclear cells, and their stimulated release of interleukin-5 (IL-5) to reflect an aspect of the anti-inflammatory activity of corticosteroids (Rolfe et al 1992). The second cell type was human osteoblasts (Namkung-Matthai et al 1998), which were chosen because of the clinical interest in the possibility of osteoporosis with long-term use of inhaled corticosteroids (Toogood et al 1994). The activity of beclomethasone dipropionate and its esters were compared with a reference corticosteroid, dexamethasone. In addition, we assessed the conversion of the parent compound, beclomethasone dipropionate, to its active metabolite under our experimental conditions.

Materials and Methods

Mononuclear cells

Isolation. Human peripheral blood mononuclear cells were isolated from white cell concentrates using Ficoll-Paque (Pharmacia Biotech AMRAD, Uppsala, Sweden) as described previously by Rolfe et al (1992). Briefly, blood was diluted with an equal volume of phosphate buffered saline (PBS) without magnesium and calcium (Trace Biosciences, Seven Hills, Australia) underlayered with Ficoll-Paque and spun at 707 *g* for 15 min at room temperature. The layer of peripheral blood mononuclear cells at the gradient interface was carefully harvested and washed three times with PBS and resuspended in culture medium (RPMI 1640, 20 mM Hepes, 20 μg mL^{-1} gentamicin, 2 mM L-glutamine and 5% v/v heat inactivated foetal bovine serum) (Trace Biosciences) at a cell density of 2×10^6 cells mL^{-1}/well and plated into 24-well culture plates. Cells were stimulated with 1 μg mL^{-1} of the mitogen, phytohaemagglutinin (PHA) (Murex Biotech Ltd, Dartford, UK) with the exception of the negative controls to which the PHA vehicle (PBS) was added. Immediately afterwards the appropriate concentration of beclomethasone dipropionate, metabolite or vehicle (final ethanol concn 0·00001%) was added. Beclomethasone dipropionate, 17-beclomethasone monopropionate, 21-beclomethasone monopropionate and beclomethasone were added to give concentrations of 10^{-14}–10^{-5} M and dexamethasone was added to give concentrations of 10^{-14}–10^{-9} M. All treatments were performed in triplicate and cells were cultured for 48 h at 37°C in a humidified 5% CO_2 atmosphere.

Determination of interleukin-5 content within samples. Total IL-5 protein content was determined in each well culture following cell lysis by freeze/thawing. A sandwich ELISA using a rat IgG1 anti-human IL-5 capture antibody (TRFK5), a biotinylated rat IgG2a anti-human IL-5 detection antibody (JES1-5A10), recombinant human IL-5 standard and protocols were supplied by PharMingen (San Diego, CA). The lower limit of detection of IL-5 using this protocol was 5 pg mL^{-1}.

Data analysis. All values of IL-5 in pg mL^{-1} were converted to a percentage of the positive control (PHA-stimulated cells) to normalise for the variability between donors. Differences between treatment and control samples were analysed using two-way analysis of variance and the post-hoc Fischer test. $P < 0{\cdot}05$ was considered to be significant.

Osteoblasts

Isolation. Human osteoblasts were prepared as previously described (Namkung-Matthai et al 1998). In brief, the whole bone end was minced and washed extensively using primary growth medium before being plated out onto 25-cm^2 flasks in BGJ medium containing 10% v/v foetal calf serum (FCS) and 44 mg mL^{-1} phosphoascorbate, and

supplemented with 30 μg mL^{-1} penicillin and 40 μg mL^{-1} streptomycin. Cells grown from the explanted chips reached confluence after 3–4 weeks and were subcultured into 24-well plates. Cells were plated at a concentration of 5–8$\times$10^{-4} cells cm^{-2} and maintained in BGJ without antibiotics and supplemented with 10% (v/v) FCS overnight. The cells were maintained in this medium for the duration of the study with medium changes every 48 h. Serum-containing medium was used for assays of [^{3}H]-thymidine incorporation and alkaline phosphatase activity due to poor development of osteoblasts and the high level of osteoblast apoptosis in serum-free medium (Namkung-Matthai et al 1998).

The stock solutions of glucocorticoids were prepared in spectroscopic-grade ethanol so that the final concentration of vehicle did not exceed 0·1% v/v. All steroid treatment commenced 24 h after subculture into 24-well plates and continued for 48 h before assay. The 1a,25-dihydroxyvitamin D3 (1,25(OH)2D3) was prepared in the same manner.

Measurement of proliferation. Cell numbers were counted using a haemocytometer or Coulter counter and [^{3}H]-thymidine incorporation as previously described (Namkung-Matthai et al 1998).

Measurement of differentiated functions. Alkaline phosphatase activity and the secretion of osteocalcin were chosen as indices of bone-derived cell function and assayed as previously described (Namkung-Matthai et al 1998).

Data analysis. Each experiment examining the effect of the 5 glucocorticoids on one parameter was carried out in triplicate with cells obtained from 4 different donors. Data points were expressed as percentage of (vehicle) control, then combined for the 4 cell strains to obtain means $\pm$ s.e.m. The EC50 (concentration of drug at which 50% maximal effect is acheived) for each glucocorticoid in each assay was obtained as previously described (Namkung-Matthai et al 1998).

Conversion of beclomethasone dipropionate to esters

Kinetic experiments, which were carried out for 18 h at 37°C, were initiated by the addition of an ethanolic solution of beclomethasone dipropionate (5 mg mL^{-1}) to cell-culture flasks, either with or without human osteoblasts added, yielding an initial concentration of 10 μM in ethanol–culture medium (0·1 : 99·9, v/v). At predetermined time intervals, 10 mL samples were removed and extracted on the same day as the incubation was performed. The concentrations of parent drug and decomposition products were monitored by HPLC. To each 10-mL sample was added 10 mL of dexamethasone 21-acetate 40 μg mL^{-1} in ethanol (internal standard), and then the mixture was shaken with 40 mL dichloromethane for 15 min. The organic layer was collected and evaporated to dryness in-vacuo at 30°C. The resulting residue was reconstituted in a 1-mL mobile phase and centrifuged (at 15 000 *g* for 2 min) before injection onto the HPLC column (injection volume, 100 μL).

HPLC analysis. Liquid chromatography was performed isocratically at ambient temperature (20°C), using an Alltima C_{18} (250$\times$4·6 mm i.d.) column (Alltech Associates, Baulkham Hills, NSW, Australia), a mobile phase of methanol–water–acetonitrile–acetic acid (352 : 166 : 50 : 1, v/v) and a flow rate of 1·3 mL min^{-1} with UV detection at 242 nm. Under these conditions, retention times were 4·7 min for beclomethasone, 6·0 min for internal standard, 9·0 min for 17-beclomethasone monopropionate and 20·0 min for beclomethasone dipropionate. The assay had been validated for linearity, precision, accuracy and recovery for beclomethasone dipropionate, 17-beclomethasone monopropionate and beclomethasone over the concentration range 1–50 μg mL^{-1}.

Data analysis. Relative peak area ratios were calculated using the standard curves. The apparent decomposition rate constants (k_{app}) were estimated from the slope of log-linear phase of declining concentration versus time plots. The half-lives ($t_{½}$) were calculated using the following equation: $t_{½} = 0{\cdot}693/k_{app}$ (Martin 1993). Results were expressed as the mean $\pm$ s.d.

Materials and chemicals

Beclomethasone dipropionate and its metabolites 17-beclomethasone monopropionate, 21-beclomethasone monopropionate and beclomethasone were supplied by 3M Pharmaceuticals (St Pauls, MN) and were stored in 100% ethanol as 10^{-2} M stock solutions at −20°C. Beclomethasone dipropionate and its metabolites were serially diluted in culture medium until the desired concentration range was achieved. Dexamethasone (Decadron, Merck Sharp and Dohme, Seven Hills, Australia) was stored in PBS as a 10^{-3} M stock solution at 4°C. Dichloromethane (99·9%, HPLC grade) was purchased from Sigma (St. Louis, MO). Acetic acid of analytical reagent grade was obtained from

Rôhne-Poulenc Chemicals (Clayton South, Victoria, Australia). ChromAR HPLC-grade methanol and acetonitrile, as well as ethanol (analytical reagent grade), were purchased from Biolab Scientific (Clayton South, Victoria, Australia).

Results

Mononuclear cells

Interleukin-5 protein production was detected in all donors (n = 6) following stimulation with PHA. The IL-5 production following PHA stimulation was within the range 150–814 pg mL^{-1}. There was no detectable IL-5 production in unstimulated cells.

The parent compound, beclomethasone dipropionate, and its major metabolite, 17-beclomethasone monopropionate, caused significant inhibition of IL-5 protein production at concentrations as high as 10^{-9} M and at concentrations as low as 10^{-14} M ($P < 0{\cdot}0001$ for both). Even at 10^{-14} M, both beclomethasone dipropionate and 17-beclomethasone monopropionate significantly inhibited IL-5 protein secretion by 85% and 80%, respectively, relative to the positive control (Figure 1). The other metabolites, namely beclomethasone and 21-beclomethasone monopropionate, were also able to inhibit IL-5 protein production though not to the same extent as beclomethasone and 17-beclomethasone monopropionate (Figure 1). Beclomethasone, at a concentration of 10^{-12} M, and 21-beclomethasone monopropionate, at a concentration of 10^{-10} M, both significantly inhibited IL-5 protein production by 50% ($P = 0{\cdot}0166$ and $P = 0{\cdot}0038$, respectively).

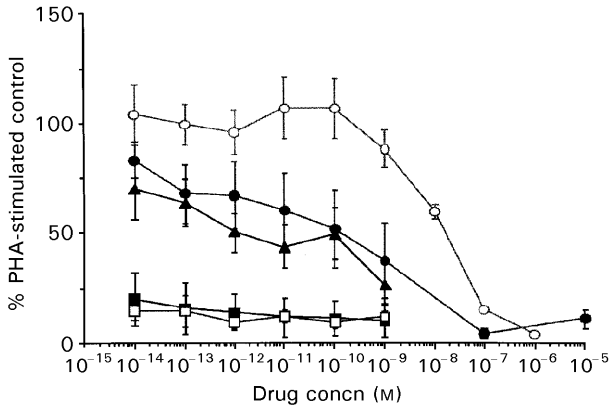

Figure 1. Activity of beclomethasone dipropionate, its metabolites and dexamethasone on IL-5 protein production in human peripheral blood mononuclear cells. Results are expressed as a percentage of the untreated PHA-stimulated control value in each donor. The mean and standard error (vertical bars) of 3–6 experiments are shown. □, Beclomethasone dipropionate (n = 4); ■, 17-beclomethasone monopropionate (n = 4); ▲, beclomethasone (n = 6); ●, 21-beclomethasone monopropionate (n = 6); ○, dexamethasone (n = 3).

In contrast to beclomethasone dipropionate and its metabolites, a concentration of 10^{-8} M dexamethasone was required to inhibit IL-5 secretion by 50% ($P = 0{\cdot}0128$).

Osteoblasts

The cells which grew from the trabecular ends of long bones under these culture conditions exhibited phenotypic characteristics typical of osteoblasts. These included high alkaline phosphatase activity, a marker of osteoblastic differentiation, which was further stimulated by treatment with 1,25-dihydroxyvitamin D and secretion of the bone-marker protein osteocalcin, when treated with 1,25-dihydroxyvitamin D. When maintained in culture for long periods on Thermanox coverslips, the cells form a relatively regular multilayer which has an abundant collagen matrix and which mineralises after about 4 weeks in culture (Slater et al 1994 a, b).

All glucocorticoids tested stimulated alkaline phosphatase activity in bone cells, and all inhibited thymidine incorporation and 1,25-dihydroxyvitamin D-stimulated osteocalcin synthesis by these cells. When subjected to analysis of variance, the dose–response curves for each of the glucocorticoids were not significantly different from dexamethasone for any of the parameters tested in bone cells. All the EC50s were in the nanomolar range (Table 1).

Conversion of beclomethasone dipropionate to esters

Following the incubation of beclomethasone dipropionate as parent drug at 37°C in the cell culture flasks, either with or without the addition of human bone-derived cells, serial formation of 17-

Table 1. EC50 (nM) values for beclomethasone esters in osteoblasts.

Assay	17-Beclomethasone monopropionate	21-Beclomethasone monopropionate	Beclomethasone dipropionate	Beclomethasone	Dexamethasone
Thymidine incorporation	0·6	4·0	0·5	1·7	3·1
Alkaline phosphatase	1·1	1·6	0·3	1·6	0·4
Osteocalcin	0·4	0·7	1·2	0·9	1·6

beclomethasone monopropionate and beclomethasone was observed. In the culture medium alone, beclomethasone dipropionate was relatively stable ($t_{1/2} = 52{\cdot}4 \pm 2{\cdot}7$ h, n = 3, Table 2), with less than 23% of beclomethasone dipropionate being biotransformed after 18 h incubation. In contrast, rapid formation of 17-beclomethasone monopropionate following incubation of beclomethasone dipropionate with human bone-derived cells was observed ($t_{1/2}$ of beclomethasone dipropionate $= 3{\cdot}3 \pm 0{\cdot}2$ h, n = 3, Table 2). The intermediate metabolite, 17-beclomethasone monopropionate was subsequently hydrolysed at a much slower rate ($t_{1/2} = 12{\cdot}6 \pm 0{\cdot}9$ h, n = 3) to form the ultimate product (beclomethasone) with human bone-derived cells (Table 2). The active metabolite, 17-beclomethasone monopropionate was approximately 4-fold more stable than its parent, beclomethasone diproprionate, in the presence of human bone-derived cells. Thus, the overall kinetics following incubation of beclomethasone dipropionate with human bone-derived cells were rate-limited by the decomposition of 17-beclomethasone monopropionate.

Discussion

These experiments were carried out to determine the effects of beclomethasone and its esters in two different human cell populations; firstly, peripheral blood mononuclear cells, comprising mainly T-lymphocytes, as an index of potency in inhibiting release of the pro-inflammatory cytokine, interleukin-5 (IL-5), and secondly, osteoblasts, as an index of potency in a target cell for unwanted systemic activity. Dexamethasone was used as a reference corticosteroid in both cell populations.

Both the peripheral blood mononuclear cells and osteoblasts responded to each of the corticosteroids, although there was considerable variation in sensitivity between cells from different donors. This variability is frequently seen when working with cells from human donors. The EC50 values for dexamethasone were similar for peripheral blood mononuclear cells and osteoblasts. However, the sensitivity to beclomethasone esters differed markedly between the two cell populations. In osteoblasts, the EC50 for beclomethasone dipropionate, 17-beclomethasone monopropionate and beclomethasone were similar to the EC50 for dexamethasone. In contrast, beclomethasone and its esters were much more potent than dexamethasone in inhibiting IL-5 production by peripheral blood mononuclear cells. Peripheral blood mononuclear cells were very sensitive to beclomethasone dipropionate and 17-beclomethasone monopropionate, with concentrations of 10^{-14} M inhibiting IL-5 production by greater than 50%. In general, T-lymphocytes (which are the cells in the population that release IL-5) are very sensitive to the effects of corticosteroids (Robinson et al 1993).

The effects of beclomethasone dipropionate on human T-lymphocytes have been reported previously. Crocker et al (1998) isolated populations of allergen-specific T-lymphocytes after long-term culture (30 days) in the presence of allergens. These cells were then incubated in beclomethasone dipropionate (10^{-7}–10^{-12} M) for 24 h before stimulation with PHA. Beclomethasone dipropionate inhibited IL-5 production in a dose-dependent manner, with an IC50 value of $3{\cdot}6 \times 10^{-9}$ M. The sensitivity of the T-lymphocytes to beclomethasone dipropionate in our experiments was approximately 10 000 times that reported by Crocker et al (1998). It is possible that the 30-day period of incubation with allergens altered the corticosteroid responses of the T-lymphocytes used by Crocker et al (1998). Another possible explanation for the discrepant findings may be that the subsets of allergen-specific T-lymphocytes studied by Crocker et al (1998)

Table 2. Concentration–time data of beclomethasone dipropionate and its metabolites, following the incubation of beclomethasone dipropionate in culture flasks, with and without human osteoblasts at 37°C.

Incubation media	Time (h)	Mean concn (μM, n = 3)		
		Beclomethasone dipropionate	17-Beclomethasone monopropionate	Beclomethasone
Culture flasks	0	10·0	0	0
	1	9·7	0·2	0·1
	6	9·2	0·6	0·2
	18	7·8	1·6	0·5
Culture flasks + osteoblasts	0	10·0	0	0
	1	7·7	2·1	0·2
	6	2·8	3·9	3·2
	18	0·6	2·0	7·3

were less sensitive to beclomethasone dipropionate than the broader population of lymphocytes represented in the peripheral blood mononuclear cells used in our experiments.

The magnitude of the potency ratios for beclomethasone dipropionate : dexamethasone and 17-beclomethasone monopropionate : dexamethasone in peripheral blood mononuclear cells was greater than predicted from binding studies reported by others (Würthwein & Rohdewald 1990). In our experiments, 17-beclomethasone monopropionate was approximately 10 000 more potent than dexamethasone in inhibiting IL-5 production yet it has only 10 times the relative receptor affinity (Würthwein & Rohdewald 1990). There are many steps between steroid receptor binding and cellular response. These findings suggest that, at some or all of these steps, the effects of beclomethasone are amplified or distorted with respect to dexamethasone to produce a final cellular effect ratio which is discrepant from that predicted by receptor binding studies.

In the experiments with peripheral blood mononuclear cells, the effects of beclomethasone dipropionate and 17-beclomethasone monopropionate on IL-5 release were indistinguishable, which may have been due to esterase activity fully metabolising beclomethasone dipropionate to its active metabolite 17-beclomethasone monopropionate during the incubation period of 48 h. The decomposition rate of beclomethasone dipropionate was about 16 times faster in the presence of osteoblasts than in culture medium alone, indicating relatively high esterase activity for beclomethasone dipropionate in osteoblasts. It has been reported that beclomethasone dipropionate is hydrolysed to 17-beclomethasone monopropionate and beclomethasone in the human lung (Martin et al 1974; Andersson & Ryrfeldt 1984; Würthwein & Rohdewald 1990) and gut homogenates (Würthwein & Rohdewald 1990), so it is likely that peripheral blood mononuclear cells possess this esterase activity as well.

In human osteoblasts, this extraordinary differential activity of dexamethasone and beclomethasone dipropionate was not observed. The vast differences in bioactivity of the beclomethasone dipropionate compounds in the two cell types add to evidence that early models of steroid hormone action, in which a ligand was thought to switch the receptor from an inactive to an active state and receptor affinities were thought to be the prime determinants of bioactivity, are clearly oversimplifications. Different oestrogens also have a wide range of activity in different cell types (McDonnell & Norris 1997). It seems likely that binding of the ligand induces shape changes in the activated receptor complex which differ from ligand to ligand and which are modified by the particular receptor-associated proteins present in that cell type. The final effects will also depend on dimerisation characteristics of the receptor–ligand complex, which may involve more than one receptor type, and how that complex interacts with target sequences in the DNA of that cell, modified by the DNA-associated proteins present (Haussler et al 1998). The more complex model predicts that different compounds acting through the same receptor may display different activities in different cells (McDonnell & Norris 1997).

The mono-ester, 21-beclomethasone monopropionate, which has very low steroid receptor binding (Würthwein & Rohdewald 1990) had demonstrable biological activity, particularly in the osteoblasts. This may be due to non-steroid receptor binding effects, which have been reported with other similar agents such as vitamin D (Norman 1998).

Beclomethasone and its esters were far more potent in inhibiting release of the pro-inflammatory cytokine IL-5 from peripheral blood mononuclear cells than in affecting functional responses of osteoblasts, such as alkaline phosphatase activity and osteocalcin release. If one accepts that a putative anti-inflammatory drug should have greater effect on a response which is part of the inflammatory reaction and less effect on a cell involved in unwanted side effects (such as osteoblasts) then the beclomethasone esters fulfill this criterion.

Finally, these results show the importance of examining effects of corticosteroids on cellular responses, as potency ratios observed in receptor binding assays are not necessarily predictive of the magnitude of the potency ratios in cell populations possibly relevant to the efficacy and side effects.

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