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Development of a dual luciferase reporter screening assay for the detection of synthetic glucocorticoids in animal tissues

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Synthetic glucocorticoids belong to the most frequently administered drugs in livestock production. These synthetic hormones are employed for therapeutic purposes against inflammatory reactions, disorders of the musculoskeletal system, bovine ketosis and many other diseases of farm animals. A widespread illegal use of synthetic glucocorticoids to improve feed intake and weight gain has also been observed. To enforce the residue limits imposed on glucocorticoid drugs and preclude their illicit administration as growth promoters, it is necessary to establish high throughput analytical methods that can be applied to the screening of animal tissues. Here, we developed a dual luciferase reporter assay that detects residues or contaminants with glucocorticoid activity. This screening assay is performed by transfection of human cell lines with two reporter constructs followed by the measurement of two distinct luminescence signals, one of which serves as internal control to correct for assay variabilities and unspecific matrix effects. The limit of detection (1.25 μg for dexamethasone in liver) depends on the biological potency of each synthetic glucocorticoid but, with all drugs tested, the maximal response reaches a 20 to 30 fold induction of luciferase activity. In combination with an appropriate sample clean-up method (recovery of 82%), this luciferase assay has been applied to the analysis of liver samples from calves treated with a single therapeutic injection of either dexamethasone or flumethasone. Thus, the dual luciferase reporter assay provides a new screening tool to detect unwanted glucocorticoid activities in animal tissues or other crude biological samples without knowledge of the precise chemical entity of the parent compounds or their metabolites.

Introduction

The adverse health effect of many chemicals is mediated by endogenous nuclear receptors that respond to lipophilic ligands by reprogramming gene expression. The glucocorticoid receptor is a member of the large superfamily of ligand-activated transcription factors that also comprises receptors for estrogens and androgens, as well as receptors for thyroid hormones, retinoic acid, fatty acids or eicosanoids.[1–4] The glucocorticoid receptor is initially sequestered in the cytoplasm through interactions with large protein complexes containing heat shock proteins. The binding of cognate ligands causes the glucocorticoid receptor to dissociate from these complexes and translocate into the nucleus where, as a homodimer, it recognizes specific DNA sequences located in the 5′ region of glucocorticoid-regulated genes.[5,6] Upon interaction with these regulatory sequence elements, additional transcription factors and components of the transcription initiation machinery are recruited to modulate gene expression.[7,8]

This mechanism of ligand-induced transcription of target genes has already been used in many instances to develop test systems that facilitate the detection of drugs, chemicals or contaminants. In fact, a number of different bioassays in yeast, fish or mammalian cells rely on the expression of reporter enzymes (chloramphenicol acetyltransferase or luciferase) under control of the recognition sequence for a particular nuclear receptor.[9–15] Reporter constructs based on estrogen response elements in the transcriptional promoter sequence have been used, for example, to monitor the activity of environmental xenoestrogens (reviewed in ref. 16). A similar bioassay has also been established to compare the relative potency of glucocorticoid drugs, thereby replacing canonical animal tests based on hypothalamic-pituitary-adrenal axis suppression, glycogen deposition, eosinopenia or other *in vivo* parameters.[17] More recently, a luciferase reporter system in COS-1 cells transfected with complementary DNA for the human glucocorticoid receptor has been used to determine glucocorticoid activity in the serum of asthmatic children.[18] Often, these bioassays are performed in cultured cells that are transiently transfected with vector DNA containing an appropriate reporter construct. In other cases, stable reporter cell lines, where the reporter sequence is integrated in chromosomal DNA, have been established to avoid possible uncertainties due to variations in transfection efficiency.[19–22]

A major obstacle to the wide application of reporter assays for screening purposes is their potential susceptibility to unspecific matrix effects. For example, the expression of reporter genes is dependent on host cell viability, but cultured mammalian cells are prone to cytotoxic or apoptotic responses upon exposure to crude extracts or high concentrations of chemical mixtures. Also, some matrix components or contaminants may lead to general stimulation or suppression of RNA synthesis in the absence of any specific ligand interactions with nuclear receptors. Such unspecific matrix effects may generate unacceptably high levels of false positive or negative results that are not compatible with the use of reporter gene assays for general screening purposes. Thus, it is necessary to extend the reporter techniques, used in the past mainly on pure test compounds or serum samples, to the analysis of more complex biological matrices such as tissues of farm animals intended for human consumption. Here, we show that effective screening tools can be developed by exploiting an existing dual luciferase gene strategy, where the induction of a reporter construct in response to hormones or hormone-active chemicals

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is normalized against a constitutively expressed internal standard. The practical advantage of this dual reporter assay was demonstrated by its application to the detection of incurred drug residues in the liver of calves injected with synthetic glucocorticoids.

Experimental procedures

Chemicals

Aldosterone, bisphenol A, dexamethasone, 17β-estradiol, flumethasone, 4-hydroxytamoxifen, genistein, hydrocortisone, methylprednisolone, progesterone, testosterone and triamcinolone were purchased from Sigma Chemical Co. The polychlorinated biphenyl (PCB) congeners 54, 126, 153 and 180 were obtained from EGT Chemie AG; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from the NCI Chemical Carcinogen Reference Sandard Repository. Stocks of these chemicals were prepared in ethanol or dimethyl sulfoxide (DMSO). All organic solvents were purchased from Fluka. *Helix pomatia* juice was from Boehringer Mannheim.

Plasmid construction

Plasmid pGRE-Luc (Clontech) served as starting vector for assembly of the reporter construct. This vector contains a minimal promoter region derived from the *Herpes simplex* virus thymidine kinase gene flanked by three tandem repeats of a glucocorticoid response element (GRE). These repeats were further optimized using the QuikChange site-directed mutagenesis kit (Stratagene) to obtain the GRE consensus sequence (5′-GGTACANANTGTTCT-3′) that had been shown to yield the most effective induction in response to glucocorticoids.[23] A DNA fragment, comprising the TATA box sequence of the thymidine kinase promoter in conjunction with the optimized GRE repeats, was removed by digestion with *Kpn*I and *Nco*I and purified by agarose gel electrophoresis.[24] This minimal promoter fragment was cloned into pGL3-basic (Promega) immediately upstream of the gene encoding the *Photinus pyralis* (firefly) luciferase. This reporter construct is referred to as the p3BGre vector (Fig. 1). A negative control vector containing exactly the same TATA box but without the GRE sequences was constructed by digesting the pTAL-Luc plasmid (Clontech) with *Kpn*I and *Nco*I. The resulting fragment, which includes the TATA box but no GREs, was cloned into plasmid pGL3-basic upstream of the luciferase gene. This control construct is referred to as the p3BTal vector (Fig. 1). The correct assembly of both p3BGre and p3BTal was confirmed by nucleotide sequencing. Another construct that was used to generate the internal standard (pRL-TK from Promega) containing the *Renilla reniformis* luciferase gene under the control of a *Herpes simplex* virus thymidine kinase promoter sequence (Fig. 1).

Cell culture and transient transfections

All components for cell culture and transfections were purchased from Gibco BRL. Routine experiments were performed with HeLa cells (American Type Culture Collection) that were grown as monolayer cultures in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, penicillin G (100 units ml^{-1}) and streptomycin sulfate (100 μg ml^{-1}). Cells were transfected by the lipofection procedure following the manufacturer's instructions. In brief, cells were grown until 90% confluence and washed with serum-free DMEM. A plasmid cocktail (in routine experiments: 0.9 μg p3BGre and 0.1 μg pRL-TK) was diluted with serum-free DMEM, mixed with 6 μl PLUS reagent and 4 μl Lipofectamine, and added to the culture. After 4 h, the medium was replaced by DMEM supplemented with 10% fetal calf serum, penicillin and streptomycin. In the optimized protocol, the test compounds were added 5.5 h after transfection, the final concentration of solvent (ethanol or DMSO) was 0.1% (v/v). Cultures were continued for another 24 h, after which the cells were harvested and assayed for luciferase activity. Each sample was tested in triplicate.

Dual luciferase measurements

Cell lysates were prepared and assayed for luciferase activity using the dual luciferase reporter assay system (Promega) with a MLX microtiter plate luminometer (Dynex Technologies). The presence of two different vectors results in the expression of two distinct reporter enzymes, *i.e.*, firefly and *Renilla* luciferase. Firefly luciferase activity is measured by mixing an aliquot (20 μl) of cell lysates with luciferase assay reagent II. After quantifying the firefly luminescence, the "stop and glo" reagent is used to quench the firefly luciferase and simultaneously activate the *Renilla* luminescence. The results are finally expressed as a ratio between the activities of firefly and *Renilla* luciferase.

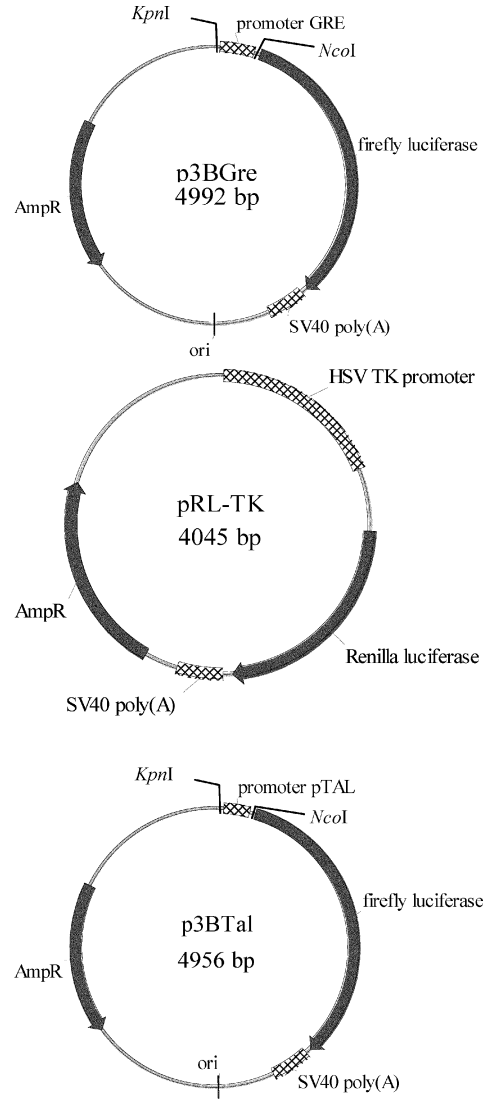

Fig. 1 Maps of the three vectors used to establish the glucocorticoid reporter assay. Plasmids p3BGre and p3BTal encode the firefly luciferase gene under the control of a minimal promoter in the presence (p3BGre) or in the absence (p3BTal) of GRE sequences. Vector pRL-TK encodes the *Renilla* luciferase gene under the control of a constitutive promoter that provides constant expression levels.

Animals, drug administration and tissue sampling

Groups of three calves were selected from two different farms in Switzerland. These animals were diagnosed with respiratory infections on the basis of clinical signs (dyspnoea, nasal discharge and coughing) and treated by repeated oral administration of sulfachlorpyrazidine and trimethoprim in combination with a glucocorticoid injection. The animals were fed a milk replacer and kept with access to water and hay *ad libitum*. The glucocorticoid treatment consisted of a single injection of dexamethasone esters or flumethasone into the neck muscles in amounts corresponding to the common therapeutic range. Three calves were treated with a conventional depot mixture, which contained the short-acting sodium phosphate ester in combination with the long-acting phenylpropionate ester of dexamethasone. The drug dose was 20 μg kg^{-1} body weight for the sodium phosphate and 40 μg kg^{-1} body weight for the phenylpropionate ester, yielding a total amount of 60 μg kg^{-1}. Flumethasone was injected at a lower dose of 20 μg kg^{-1} body weight using a commercially available aqueous preparation intended for fast intramuscular absorption. The animals treated with dexamethasone were slaughtered 72 h after drug administration, while the animals treated with flumethasone were slaughtered 24 h after injection. Several tissue samples of about 50 g were collected from the liver and stored in labelled plastic boxes at −80 °C. Control samples were obtained from 6 additional animals without any previous drug treatment.

Sample clean-up

Aliquots (5 g) of minced liver were homogenized in 10 ml of 3 M acetate buffer (pH 5.2) using a food blender. Each sample was supplemented with 100 μl *Helix pomatia* juice and incubated for 4 h at 40 °C. The hydrolyzates were extracted twice with 2 × 10 ml methanol. The methanol was evaporated and the residues were dissolved in 1 ml of ethanol, then 6 ml of water was added. These solutions were immediately applied onto C_{18} columns (Baker), previously conditioned with 2 × 5 ml methanol and 2 × 5 ml water. After washing the columns with water, acetone/water (20/80; v/v) and 5 ml of hexane, the analytes were eluted with 7 ml diethyl ether. The eluates were mixed with 1 ml 10% Na_2CO_3 and centrifuged for 1 min at 2,000 rpm. The diethylether layers were evaporated under nitrogen at 40 °C and the resulting pellets dissolved in 0.5 ml DMEM. These final extracts were added (in triplicates) at a dilution of 1:25 to the culture medium containing the transiently transfected cells.

Results

Adaptation of the dual luciferase assay to glucocorticoid detection

Dual reporter gene assays have been devised to correct for the variable efficiency of DNA uptake in transient transfection experiments. In many cases, a first vector containing the reporter gene construct, for example firefly luciferase, is combined with a second expression plasmid coding for chloramphenicol transferase or β-galactosidase.[18,25] In the present study, we exploited such a dual reporter strategy to develop a screening assay for the detection of glucocorticoid drugs in complex biological matrices. This analytical method involves cell transfections with two distinct reporter constructs that contain the genes of two related luciferase enzymes. The first luciferase gene is controlled by a minimal promoter coupled to glucocorticoid response elements (GREs), which induce expression upon exposure to glucocorticoid hormones. The second luciferase gene is located upstream of a constitutively active promoter, thereby providing an internal standard that enhances the specificity of the assay.

Three different vectors were used to set up the dual glucocorticoid screening assay (Fig. 1). First, we constructed a reporter vector (p3BGre) that contains the firefly (*Photinus pyralis*) luciferase gene under transcriptional control of a minimal promoter comprising an array of three GREs. The second vector, designated p3BTal, consisted of exactly the same sequence as in p3BGre, except that the GRE arrays were omitted from the promoter region (Fig. 1). This plasmid p3BTal represents a true control vector with the same promoter used in the reporter construct but with the GREs deleted and was, therefore, used to demonstrate that firefly luciferase induction is driven by activation of glucocorticoid receptors and their subsequent binding to GRE sequences. The third vector (pRL-TK), which was included as an internal standard, contained the *Renilla reniformis* luciferase coupled to a constitutive thymidine kinase promoter that is not responsive to glucocorticoid hormones or drugs. This internal standard vector was used to normalize the variable induction of firefly luciferase against the constitutive activity of *Renilla* luciferase.

To set up the experimental conditions for a glucocorticoid reporter assay, we first tested different amounts of vector DNA, between 125 ng and 2 μg per 10^6 cells. DNA constructs (p3BGre or pRL-TK) were transfected into HeLa cells, and firefly luciferase expression was induced 24 h after transfection by adding 1 μM (39 ng ml^{-1}) dexamethasone to the culture medium. Following another incubation time of 24 h, cells were lysed and luciferase activities in each of the different cell extracts were measured. These experiments yielded an approximately 25 fold induction of firefly luciferase in response to dexamethasone, relative to the solvent (0.1% ethanol or DMSO) controls, when the transfections were performed with 0.5 μg DNA per 10^6 cells. Higher amounts of vector DNA led to excessive *Renilla* luciferase expression from the internal standard vector (pRL-TK) in the absence of GRE sequences (Fig. 2A). To avoid such unspecific effects not related to glucocorticoid drug action, all subsequent experiments were performed with a total of 0.5 μg vector DNA per 10^6 HeLa cells.

The next step was to establish an optimal ratio between the p3BGre reporter vector (encoding the firefly luciferase) and the pRL-TK internal standard (encoding the *Renilla* luciferase). HeLa cultures (2 × 10^6 cells) were transfected with 1 μg of DNA (0.5 μg DNA per 10^6 cells) containing the two vectors in various proportions. Subsequently, firefly luciferase expression was induced (1 μM dexamethasone). After cell lysis, the activities of both firefly and *Renilla* luciferase were measured in each sample and expressed as a quantitative ratio of the two luminescence signals (Fig. 2B). As expected, we observed higher levels of firefly luciferase activity when the amount of p3BGre was progressively increased relative to pRL-TK. This enzyme induction was not observed when the reporter vector p3BGre was replaced by p3BTal, which contains the same promoter as p3BGre but without any GRE sequences, confirming that firefly luciferase induction is strictly mediated by activation of the endogenous glucocorticoid receptor (Fig. 2B). These preliminary experiments showed that an optimal induction of firefly luciferase from the p3BGre reporter vector, with minimal background expression from the p3BTal control, could be detected at a plasmid ratio of 9:1. Also, this particular ratio yielded an approximately equal level of firefly and *Renilla* luciferase activity in cells that were not exposed to glucocorticoid drugs. Thus, a molar ratio between p3BGre reporter vector and pRL-TK internal standard of 9:1 was used for the following transfections.

In subsequent experiments, we determined the influence of different recovery and induction periods on assay performances (Fig. 3A). Transfection experiments were conducted with 0.5 μg of vector DNA per 10^6 cells (ratio between p3BGre and

pRL-TK of 9:1). Following variable recovery times after DNA transfection, firefly luciferase expression was induced (1 μM dexamethasone). The induction time before cell lysis and luminescence measurements was kept at 24 h. Final results were expressed as a quantitative ratio between the two distinct luminescence signals induced by firefly and *Renilla* luciferase. Fig. 3B shows that maximal induction of firefly luciferase was observed when cells were exposed to dexamethasone immediately after the transfection step. This finding indicates that there is no requirement for a longer recovery period following cell transfection, thereby shortening considerably the total length of the assay procedure. Moreover, we assessed the optimal induction time. The transfection conditions were as described before (recovery time of 24 h), and HeLa cells were exposed to 1 μM dexamethasone for different periods, between 6 and 36 h. These experiments showed that highest firefly luciferase activities are achieved after exposure times of 24–32 h (Fig. 3C). Exactly the same peak of enzyme induction was observed when the initial recovery time was reduced to 1.5 h (data not shown). Thus, a combination of 1.5 h of recovery and 24 h of induction was chosen for the glucocorticoid detection procedure.

Characterization of dual glucocorticoid reporter assay

Firefly luciferase expression was induced with increasing concentrations of synthetic glucocorticoids to determine the limit of detection and EC_{50} of each single compound. The limit of detection is defined as the concentration of glucocorticoids at which the firefly luciferase activity exceeds the one obtained with the vehicle control plus three times the standard deviation (determined from 6 independent experiments). The EC_{50} defines the effective concentration of each glucocorticoid at which 50% of maximal firefly luciferase induction is reached. These parameters were determined by transfecting HeLa cultures (2×10^6 cells) with 1 μg of DNA (p3BGre/pRL-TK ratio of 9:1). Firefly luciferase expression was induced 1.5 h after transfection by exposure to synthetic glucocorticoids for 24 h. The results are expressed as quantitative ratios of the two luminescence signals generated by firefly and *Renilla* luciferase. Fig. 4A shows typical dose response curves for cortisol, dexamethasone and flumethasone. The limit of detection was reached at a dexamethasone concentration in the assay medium of approximately 1 nM and the EC_{50} was 13 nM. Flumethasone was the most potent glucocorticoid with a limit of detection of 0.2 nM, while the EC_{50} was in the range of 2 nM. The quantitative parameters of six different glucocorticoids are listed in Table 1.

To confirm the specificity of the dual glucocorticoid reporter assay, HeLa cultures (2×10^6 cells) were transfected with 1 μg of vector DNA (p3BGre/pRL-TK ratio of 9:1) and firefly

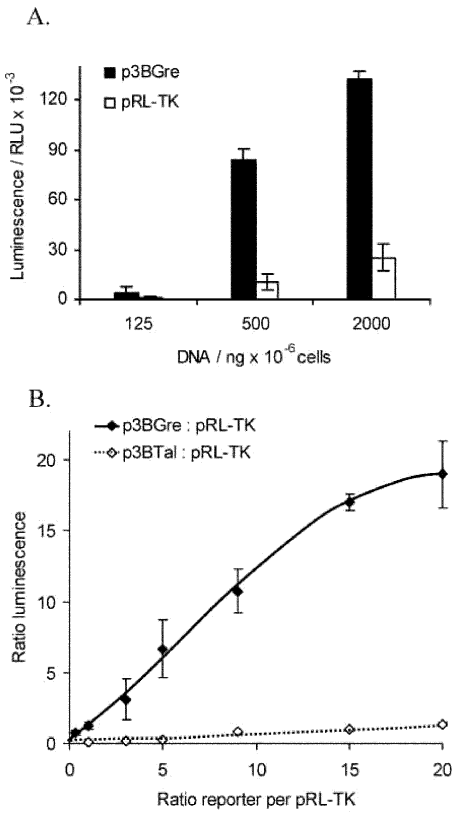

Fig. 2 Transfection conditions for glucocorticoid reporter assay. (A) The optimal amount of vector DNA is 1 μg. HeLa cultures (10^6 cells) were transfected with increasing amounts of p3BGre (black bars) or pRL-TK (white bars). Luciferase expression was induced 24 h after transfection. After another 24 h, the luminescence in each of the different extracts was measured. RLU, relative light unit. (B) The optimal ratio between reporter vector and internal standard is 9:1. HeLa cultures were transfected with different ratios of p3BGre (plain line), p3BTal (dashed line) and the internal standard (pRL-TK). Luciferase expression was induced 24 h after transfection and the two distinct luminescence signals were measured. The results are expressed as ratios of firefly to *Renilla* luciferase activity (mean of 3–6 independent transfections).

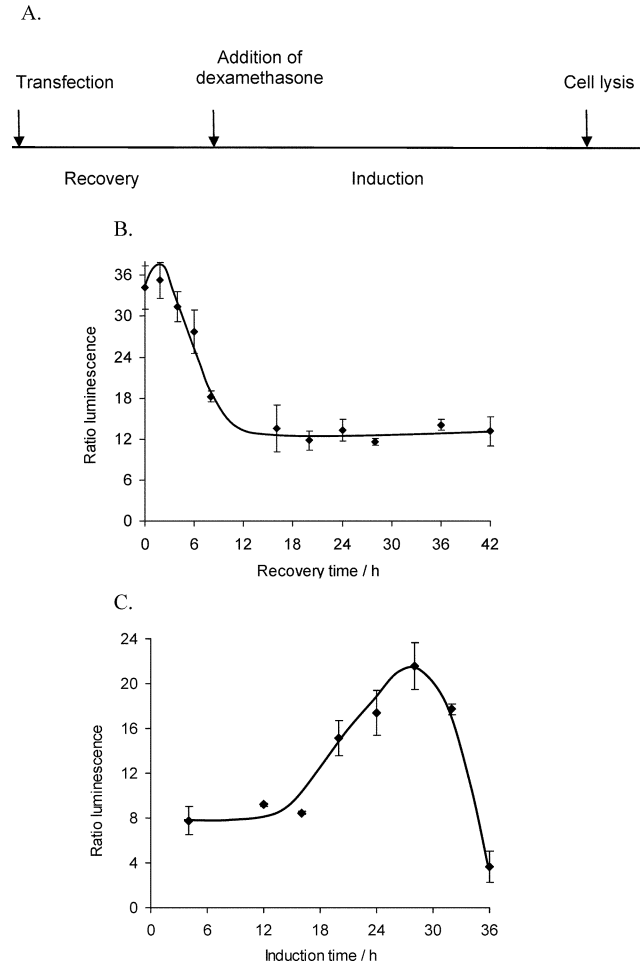

Fig. 3 Incubation times for glucocorticoid reporter assay. (A) Scheme illustrating the experimental protocol established for the dual reporter assay. (B) The optimal recovery period is 1.5 h. HeLa cultures (10^6 cells) were transfected with 1 μg total DNA (9:1 ratio between p3BGre and pRL-TK). Luciferase expression was induced after variable recovery times for a period of 24 h (1 μM dexamethasone). (C) The optimal induction period is 24 h. HeLa cultures (10^6 cells) were transfected with 1 μg total DNA as before. After 24 h of recovery, luciferase expression was induced (1 μM dexamethasone) for variable intervals. The firefly and *Renilla* luciferase activities were measured and all results are expressed as ratios between the two luminescence signals (mean values of 3–6 determinations).

luciferase expression was induced 1.5 h after transfection by exposure to dexamethasone, 17β-estradiol, testosterone, aldosterone, progesterone, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), genistein, 4-hydroxytamoxifen, bisphenol A, and the polychlorinated biphenyl (PCB) congeners 54, 126, 153 and 180. The final concentration of dexamethasone in the medium was 0.1 μM, while the other hormones and chemicals were tested at 1 μM. The solvent consisted of 0.1% ethanol or DMSO. Following 24 h of exposure, the luminescence was detected in each cell extract and expressed as a quantitative ratio of the two distinct signals generated by firefly and *Renilla* luciferase. As shown in Fig. 4B, there was an approximately 25 fold induction of firefly luciferase in response to dexamethasone, relative to the solvent control, but minimal or no induction following exposure to various estrogens, progesterone, dioxin or PCBs. Only in the presence of high concentrations of testosterone and aldosterone there was minimal induction of firefly luciferase exceeding the background levels.

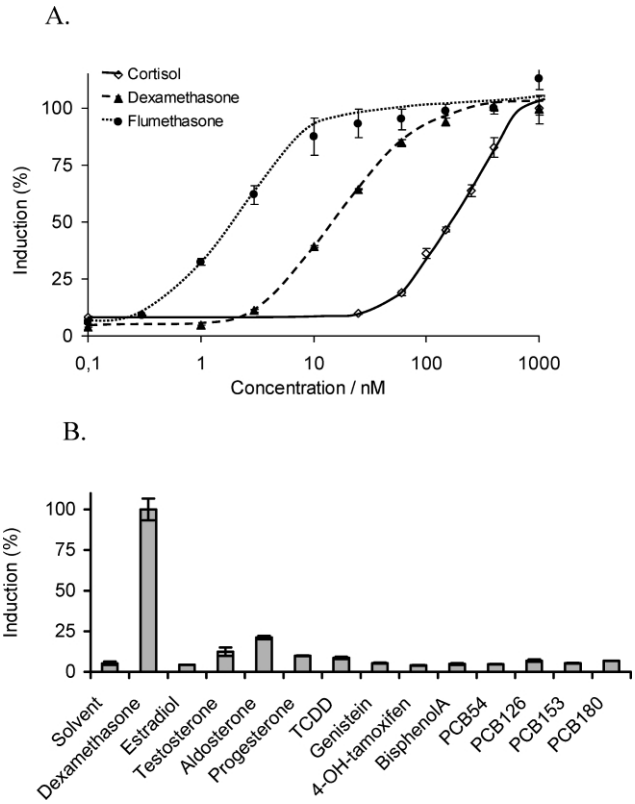

Fig. 4 Induction of firefly luciferase by purified compounds. (A) Dose responses obtained with the optimized dual luciferase assay. Results are expressed as the percentages of the maximal ratio obtained between firefly luciferase and the *Renilla* control. (B) Specificity of the assay. The synthetic glucocorticoid was replaced by the indicated chemicals at a concentration of 1 μM each. Results are shown as the percentages of specific firefly luciferase induction obtained in the presence of 0.1 μM dexamethasone.

Table 1 Comparison of luciferase reporter assay with different glucocorticoids

	LOD[a]/nM	EC_{50}[b]/nM	Maximum[c]/nM
Betamethasone	2	10	25
Cortisol	25	150	1000
Dexamethasone	1	13	60
Flumethasone	0.2	2	10
Methylprednisolone	3	21	150
Triamcinolone	0.3	5	25

[a] Limit of detection. [b] Concentration at which 50% of maximal induction is achieved. [c] Concentration at which maximal glucocorticoid effect is detected.

However, none of these hormones induced firefly luciferase expression at the lower concentration of 200 nM (data not shown). These results indicate that the reporter construct of this study yields a specific response to glucocorticoid activity.

Detection of synthetic glucocorticoids in the liver of treated animals

Two groups of three calves were treated intramuscularly with a therapeutic dose of commercial drug preparations containing either a mixture of dexamethasone esters (60 μg kg body weight^{-1}) or the more potent flumethasone analog (20 μg kg body weight^{-1}). The animals were slaughtered 72 h after dexamethasone treatment or 24 h after the single flumethasone injection. Another group of 6 calves, which were not treated with any glucocorticoid drug for a period of at least two months, were slaughtered to obtain negative control samples. Determinations by enzyme immunoassay and liquid chromatography-tandem mass spectrometry showed that dexamethasone or flumethasone administration yielded higher glucocorticoid levels in the liver compared to kidneys or muscle tissues.[26] Thus, the goal of this study was to develop a practical method to screen liver tissues for synthetic glucocorticoid residues using the dual reporter system. To reduce toxic effects and avoid microbiological contamination of cell cultures, liver samples were first processed by a rapid clean-up procedure involving liquid extraction of homogenates with methanol, followed by solid phase extraction through C_{18} columns. A mean glucocorticoid recovery of 82 + 14% (n = 24) was determined by adding trace amounts of ^{3}H-dexamethasone to the crude liver homogenates immediately before extraction. Efficient recovery of synthetic glucocorticoids was confirmed using fluorometholone as internal control.[26]

The samples from untreated animals were used to establish standard curves. Extracts from these control livers were supplemented with increasing amounts of dexamethasone or flumethasone, and tested in the optimized dual reporter assay. The presence of liver extracts at a dilution of 1:25 in the culture medium reduced significantly cell viability and overall luciferase activity (Table 2). However, characteristic dose response curves with the expected EC_{50} values could be observed when the assay results were expressed as ratios between firefly and *Renilla* luciferase activity (Table 2). The final dose responses calculated for dexamethasone and flumethasone are depicted in Fig. 5A.

The results obtained with spiked liver extracts led us to apply the same reporter assay procedure to the liver of treated animals.

Table 2 Typical luminescence signals obtained from liver extracts spiked with dexamethasone. The extracts were tested at a final dilution of 1:25 in cell culture medium

Dexamethasone concentration/nM	Firefly luciferase activity/RLUs[a]	*Renilla* luciferase activity/RLUs	Ratio between the two signals
0[b]	8475	10752	0.79
	6597	8332	0.79
0	3511	4515	0.78
	3602	4520	0.80
3	13796	7429	1.86
	9624	5691	1.69
10	28320	5583	5.07
	37948	6607	5.74
25	45062	5974	7.54
	48297	5374	8.99
100	82818	8408	9.85
	77762	8017	9.70
400	57003	5301	10.75
	4006	339	11.82

[a] RLU, relative light unit. [b] Control reaction without liver extract.

The presence of dexamethasone or flumethasone in these incurred samples had been determined by enzyme immunoassay (Table 3). Both drugs could also be detected in the liver of the treated animals by liquid chromatography-tandem mass spectrometry.[26] Interestingly, the dual reporter assay yielded positive screening results with the samples of all six calves injected with either dexamethasone (sample No. 7–9) or flumethasone (No. 10–12). The results are shown again as ratios between the two distinct luciferase activities (Fig. 5B). In all cases, the specific induction of firefly luciferase reached a level that exceeded significantly the limit of detection for dexamethasone and flumethasone. In contrast, the level of firefly luciferase in cells exposed to the liver extracts of untreated animals (No. 1–6) was indistinguishable from that observed with blank controls. These results indicate that the dual reporter gene assay can be employed to screen liver samples for drug residues or metabolites with glucocorticoid activity.

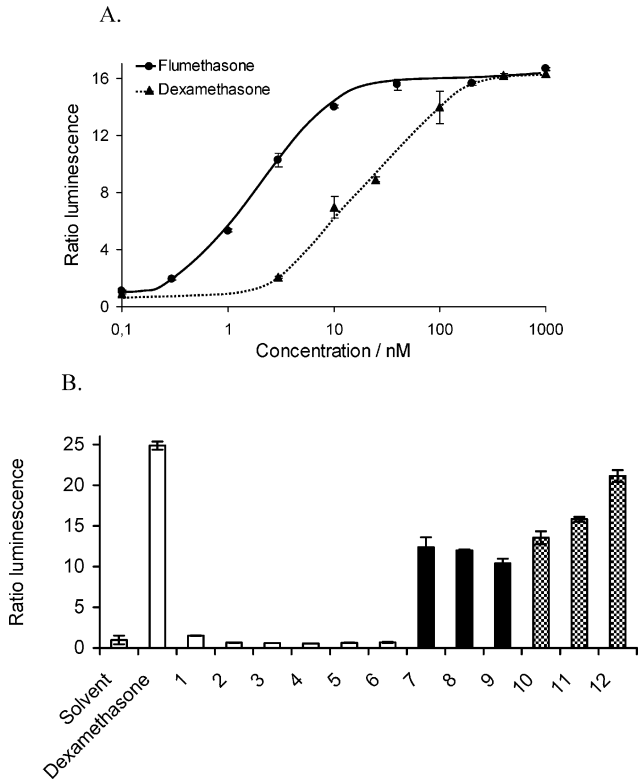

Fig. 5 Application of reporter assay to the detection of glucocorticoid residues in liver extracts. (A) Dose response with spiked samples. Blank liver extracts were added to the HeLa cells at a 1:25 dilution in the presence of the indicated concentrations of flumethasone or dexamethasone. (B) Comparison between liver tissues of untreated and glucocorticoid-treated calves. The tissues were extracted and added to the transfected HeLa cultures. Glucocorticoid inductions are expressed as the ratios between firefly and *Renilla* luciferases (mean results of two independent experiments performed in triplicates).

Table 3 Concentration of synthetic glucocorticoids in the liver of treated animals[a]

	Glucocorticoid injected	Dexamethasone/ μg kg^{-1}	Flumethasone/ μg kg^{-1}
No. 7	Dexamethasone	32.8 ± 7.1	—
No. 8		4.1 ± 1.1	—
No. 9		21.2 ± 1.8	—
No. 10	Flumethasone	—	2.5 ± 0.5
No. 11		—	31.9 ± 4.3
No. 12		—	2.5 ± 0.5

[a] Determined by enzyme immunoassay (mean ± standard deviation; $n = 3$) as described by ref. 26.

Discussion

Drugs or chemicals with hormonal activity are found among many different agrochemical or veterinary products (reviewed in ref. 27). Additionally, food products may be contaminated with a wide spectrum of environmental pollutants or chemical additives that exert hormonal activities. In many countries, the use of hormonal drugs or additives in livestock and food production has been regulated or completely banned. Current control actions to enforce these regulations are based, in the majority of cases, on the detection and identification of single chemicals at unacceptably high concentrations. However, most analytical procedures applied to the monitoring of drug residues or contaminants are slow and focused on a small number of xenobiotic molecules. Thus, it is usually not possible to detect the presence of multiple contaminants or mixtures in an efficient manner. These limitations are aggravated by the possibility that mixtures of chemicals may exert additive or synergistic effects (see for example ref. 14) and by the appearance, in animal tissues, of active metabolites.

The limitations of classical screening methods could be overcome by the development of procedures that determine the adverse activities of contaminants or metabolites in food products, instead of searching for single chemicals or groups of chemicals. Reporter genes may provide such an effect-oriented screening tool because their induction is dependent only on the ability of xenobiotic molecules to activate (or inhibit) cellular receptors. As a consequence, the goal of this study was to explore whether reporter gene assays could be adapted to the analysis of complex matrices like, for example, tissue samples from livestock. Glucocorticoid drugs were targeted in this pilot study because of their wide use in livestock production, their enhanced potency relative to endogenous glucocorticoids, and the deleterious effects that residues of these synthetic hormones may exert on human health, including growth suppression in children.[28]

The approach used in our study was prompted by the hypothesis that existing dual reporter strategies could overcome the unspecific matrix effects that may occur when crude tissue extracts are added to mammalian cell cultures. In standard applications of molecular biology, dual reporter gene assays are employed to correct for variabilities during transfection or the subsequent cell culture and lysis procedures.[25] However, the presence of an internal enzyme standard, which is constitutively expressed at a constant level, may not only eliminate experimental variations caused by differences in cell number, cell viability, transfection efficiency or cell lysis, but also correct for possible influences due to matrix components. The practicability of the dual luciferase screening assay of this study is demonstrated by its successful application to the detection of drug residues in calves that were treated with synthetic glucocorticoids under real farming conditions. The primary goal was to measure glucocorticoid activity in the liver, because this organ was previously found to contain the highest tissue concentration of dexamethasone or flumethasone after treatment with these drugs.[26] The presence of endogenous glucocorticoids did not interfere with the detection of dexamethasone or flumethasone, presumably because of the low level of the endogenous cortisol and the enhanced biological potency of the synthetic derivatives (see Fig. 4A).

Within the European Union, the use of dexamethasone, betamethasone, and prednisolone is approved in livestock only for therapeutic indications and, for that purpose, appropriate maximal residue limits (MRL's) in tissues and milk intended for human consumption have been determined (EEC Council Directive No. 22/96 and Regulations 508/99, 2593/99 and 2535/00). In the case of dexamethasone, toxicological studies in rats yielded a no-effect level of 1.5 μg kg body weight^{-1}, while higher drug doses led to tyrosine aminotransferase induction in the liver.[29] After correction with a safety factor of 100, this no-

effect level has been converted to an acceptable daily intake of 0–0.015 μg kg body weight^{-1}, yielding the following MRL's for dexamethasone in cattle: 2 μg kg^{-1} in liver, 0.75 μg kg^{-1} in kidney and muscle, and 0.3 μg kg^{-1} in milk.[30] The value of 2 μg kg^{-1} in the liver translates to a dexamethasone tissue concentration of approximately 5 nM. After accounting for sample dilution and the extraction recovery of about 80%, 2 μg kg^{-1} in the liver corresponds to a dexamethasone concentration in the reporter assay medium of 1.6 nM, which is still above the limit of detection (1 nM for dexamethasone). The unexpectedly high level of glucocorticoid activity in the liver of calf No. 8, which according to immunoassay and tandem mass spectrometry contained only about 4 μg kg^{-1} dexamethasone (Table 3), is consistent with the possible appearance of an unidentified metabolite that remains to be characterized by mass spectrometry. Additional tests with fortified blank matrices are necessary to determine the detection capability (CCβ) as requested for screening methods by the Commission Decision 2002/657/EC.

In summary, the analysis of liver samples by reporter assays provides a practical screening method to control compliance with current regulations on the use of synthetic glucocorticoids in livestock. The main advantage of the reporter gene assay is that it provides a test system that is able to screen for glucocorticoid activity in animal tissues without knowledge of the chemical properties of the compounds of interest. Also, sample preparation and reporter assays are amenable to automated robotics, thereby providing a high-throughput format necessary to process a large number of samples. As with any other screening assay, only positive results need to be confirmed by more specific and laborious analytical methods that eventually lead to identification of the chemical nature of the contaminants. Future efforts will be devoted to improve the sensitivity of the glucocorticoid reporter assay and to extend the range of matrices to other edible tissues and urine.

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