0022-3565/10/3331-281–289$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 333, No. 1
162487/3569835
Printed in U.S.A.

JPET 333:281–289, 2010



# Differential In Vivo Effects on Target Pathways of a Novel Arylpyrazole Glucocorticoid Receptor Modulator Compared with Prednisolone[S]

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Received November 12, 2009; accepted January 8, 2010

## ABSTRACT

Glucocorticoids are widely prescribed to treat autoimmune and inflammatory diseases. Although they are extremely potent, their utility in clinical practice is limited by a variety of adverse side effects. Development of compounds that retain the potent immunomodulating and anti-inflammatory properties of classic glucocorticoids while exhibiting reduced adverse actions is therefore a priority. Using heavy water labeling and mass spectrometry to measure fluxes through multiple glucocorticoid-responsive, disease-relevant target pathways in vivo in mice, we compared the effects of a classic glucocorticoid receptor (GR) ligand, prednisolone, with those of a novel arylpyrazole-based compound, L5 {[1-(4-fluorophenyl)-4a-methyl-5,6,7,8-tetrahydro-4*H*-benzo-[f]indazol-5-yl]-[4-(trifluoromethyl)phenyl]methanol}. We show for the first time that L5 exhibits clearly selective actions on disease-relevant pathways compared with prednisolone. Prednisolone reduced bone collagen synthesis, skin collagen synthesis, muscle protein synthesis, and splenic lymphocyte counts, proliferation, and cell death, whereas L5 had none of those actions. In contrast, L5 was a more rapid and potent inhibitor of hippocampal neurogenesis than prednisolone, and L5 and prednisolone induced insulin resistance equally. Administration of prednisolone or L5 increased expression comparably for one GR-regulated gene involved in protein degradation in skeletal muscle (*Murf1*) and one GR-regulated gluconeogenic gene in liver (*PEPCK*). In summary, L5 dissociates the pleiotropic effects of the GR ligand prednisolone in intact animals in ways that neither gene expression nor cell-based models were able to fully capture or predict. Because multiple actions can be measured concurrently in a single animal, this method is a powerful systems approach for characterizing and differentiating the effects of ligands that bind nuclear receptors.

Glucocorticoids act as ligands for the glucocorticoid receptor (GR) and are among the most widely prescribed drugs in contemporary medicine. Glucocorticoids are used for the treatment of many common acute and chronic inflammatory and autoimmune diseases, including asthma, rheumatoid arthritis, dermatological conditions, inflammatory bowel disease, and collagen-vascular diseases. An important limitation of glucocorticoid therapy, however, is that the desired anti-inflammatory and immunomodulating effects frequently are accompanied by undesirable effects, such as osteoporosis, loss of muscle mass, redistribution of body fat, skin thinning and bruisability, insulin resistance or diabetes mellitus, and neuropsychiatric disturbances, including depression, cognitive dysfunction, and mood lability (Stanbury and Graham, 1998; Buttgereit et al., 2005b).

Until recently, the classic teaching had been that adverse effects of glucocorticoid treatment could not be pharmacologically dissociated from beneficial effects (Stanbury and Graham, 1998). A new therapeutic concept has emerged, however, in the past few years: in particular, different ligands for nuclear receptors can in principle exhibit selectivity for phenotypic actions caused by receptor subtype specificity or variations in cofactor recruitment (Coghlan et al., 2003; Webb et al., 2003). Selective GR modulators (SGRMs), for example, might exhibit desirable anti-inflammatory and immunomodulating effects to a greater

This work was supported in part by KineMed, Inc. and the State of California Discovery Program [BioStar Grant 02-10294].
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.109.162487.
[S] The online version of this article (available at http://jpet.aspetjournals.org) contains supplemental material.

**ABBREVIATIONS:** GR, glucocorticoid receptor; SGRM, selective GR modulator; TG, triglyceride; DNL, de novo lipogenesis; $^2$H-GDT, $^2$H-glucose disposal test; GC-MS, gas chromatography-mass spectrometry; *m/z*, mass to charge; $^2H_2O$, heavy water; L5, [1-(4-fluorophenyl)-4a-methyl-5,6,7,8-tetrahydro-4*H*-benzo[f]indazol-5-yl]-[4-(trifluoromethyl)phenyl]methanol; AUC, area under the curve.

extent than the undesirable biologic actions. The typical discovery strategy for SGRMs is to screen chemical libraries by using high-throughput screening strategies followed by medicinal chemistry optimization or by using structure-guided rational drug design approaches (Schacke et al., 2007). Subsequent investigations of phenotypic selectivity of SGRMs have generally been based on in vitro ligand-receptor binding assays or selective activation or repression of gene transcription in cell-based assays (Schacke et al., 2007).

A group of novel arylpyrazole compounds have recently been identified as potential SGRMs (Shah and Scanlan, 2004). Each of the compounds carries a different substituent at a single position in a common arylpyrazole backbone, and all 15 compounds (named L1–L15) have binding affinities for the GR similar to potent synthetic glucocorticoid agonists, such as dexamethasone and prednisolone, and endogenous glucocorticoid agonists, such as cortisol. Figure 1 shows the chemical structures and GR binding affinities of L5, prednisolone, and dexamethasone for comparison (Wang et al., 2006). The pleiotropic effects of these arylpyrazole agents have been characterized in cell culture-based systems (Wang et al., 2006). Among the arylpyrazole SGRMs studied, compound L5 exhibited a pattern of favorable anti-inflammatory effects and a reduced side-effect profile in glucocorticoid-responsive cell types. Like dexamethasone, although to a lesser extent, L5 inhibited gene expression of the proinflammatory cytokine genes *IL-8*, *RANTES*, *GRO1*, *MCP1*, *GM-CSF*, and *IL-6* in A549 human lung epithelial cells induced with tumor necrosis factor α (Wang et al., 2006). In contrast to dexamethasone, which potently inhibits differentiation of MC3T3-L1 preosteoblasts to osteoblasts and potently induces differentiation of 3T3-L1 preadipocytes to adipocytes, characteristics that may reflect the negative side effects of glucocorticoid agonists on bone and adipose tissue, respectively, L5 did not inhibit differentiation of MC3T3-L1 preosteoblasts and only modestly induced differentiation of 3T3-L1 preadipocytes.

Here, we systematically characterized and compared the effects of the compound L5 and prednisolone on a number of disease-relevant pathways that are targets of GR ligands, using a heavy water ($^2H_2O$) labeling approach with mass spectrometric analysis (Busch et al., 2006, 2007; Shankaran et al., 2006; Gardner et al., 2007; Turner et al., 2007). The target pathways were selected based on their hypothesized role as "disease-modifying" processes (Stanbury and Graham, 1998; Canalis, 2003; Buttgereit et al., 2005b), i.e., driving forces underlying pathogenic or therapeutic actions of GR ligands. The $^2H_2O$ labeling approach allows concurrent measurement of flux rates through numerous disease-relevant pathways, including bone collagen synthesis (relevant to osteoporosis-related actions); skin collagen synthesis (skin thinning and bruisability); insulin-mediated glucose utilization and pancreatic β cell compensation to insulin resistance (diabetes mellitus and metabolic syndrome); pancreatic islet cell proliferation (diabetes); skeletal muscle protein synthesis (muscle wasting); triglyceride (TG) synthesis, lipolysis, and de novo lipogenesis (DNL) rates in adipose tissue (fat redistribution); hippocampal neurogenesis rate (disorders of cognition and mood); and lymphocyte proliferation and death (immunosuppressive actions). These measurements were able to be performed in living mice with a relatively small amount of L5 material (<50 mg), because of the high sensitivity and reproducibility of in vivo $^2H_2O$ labeling (Neese et al., 2002; Kim et al., 2005; Chen et al., 2007; Gardner et al., 2007). We also analyzed the expression of genes known to be regulated by glucocorticoids, including *Murf1* in skeletal muscle and *PEPCK* in liver, to confirm that L5 was demonstrating glucocorticoid-like effects on gene expression in tissues of living mice.

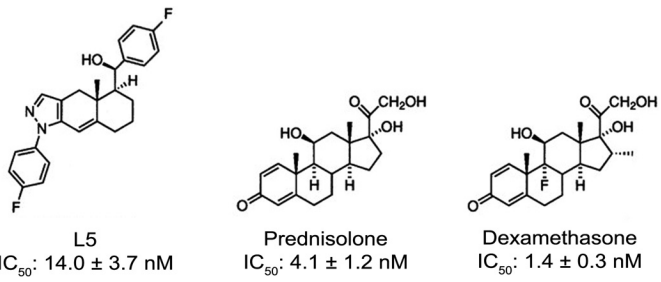


**Fig. 1.** Structures and GR binding affinities of L5 (Left), prednisolone (Center), and dexamethasone (Right) (Wang et al., 2006).

## Materials and Methods

**Animals.** Forty-two C57BL/6J mice (male, 7–8 weeks old, Charles River Breeding Laboratories, Portage, MI) were housed in groups of no more than five under temperature-controlled conditions with a 12-h light/12-h dark cycle. Mice were fed a semipurified AIN-93M (Bio-Serv, Frenchtown, NJ) diet ad libitum. All studies received prior approval from the Animal Care and Use Committee at the University of California Berkeley.

**$^2H_2O$ Labeling Protocol.** Thirty C57BL/6 mice were labeled with $^2H_2O$ throughout the study as described elsewhere (Neese et al., 2002). In brief, mice were given two priming intraperitoneal bolus injections of isotonic 99.9% $^2H_2O$ (Cambridge Isotope Laboratories, Inc., Andover, MA) to raise the $^2H_2O$ concentration in body water to approximately 5%. Body $^2H_2O$ enrichments of approximately 5% were then maintained by ad libitum administration of 8% $^2H_2O$ drinking water throughout the 7-day labeling period, as described previously (Busch et al., 2007).

**GR-Ligand Treatment Protocol.** Mice labeled with $^2H_2O$ were randomized into five groups ($n = 6$ per group). Mice were administered vehicle [0.5% (w/v) carboxymethylcellulose in water], 5 or 30 mg/kg/day prednisolone succinate dissolved in vehicle, or 5 or 30 mg/kg/day L5 dissolved in vehicle for 7 days. Half of the daily dose of each drug was administered by b.i.d. oral gavage. Vehicle-treated animals were administered vehicle by b.i.d. oral gavage. Compound L5 was provided by T.S.S.

**Tissue Collection.** Mice labeled with $^2H_2O$ were anesthetized by using isofluorane. Blood was collected by cardiac puncture and stored on ice. Water distillate was collected from plasma, and $^2H$ enrichment was measured by gas chromatography-mass spectrometry (GC-MS) after conversion to tetrabromoethane, as described in detail elsewhere (Collins et al., 2003; Turner et al., 2003). Mice were killed by cervical dislocation and immediately decapitated and all four limbs were removed. The brain was removed, and the hippocampus was dissected, minced, and placed in Hibernate A medium (BrainBits LLC, Springfield, IL) on ice. Hippocampal neural progenitor stem cells were isolated as previously described (Shankaran et al., 2006). The spleen was dissected, minced with scissors, and strained through 35-μm nylon mesh. $CD4^+$ and non-$CD4^+$ T cells, B cells, and non-B/T cells from spleen were counted and isolated by flow cytometry and cell sorting. Bone marrow was isolated from dissected femurs by perfusing the central cavity with ice-cold PBS. Pancreatic islet cells were isolated from pancreati perfused with Hanks' balanced salt solution containing collagenase as previously described (Chen et al., 1994). DNA was isolated from several cell types (hippocampal progenitor cells, splenic T cells, splenic B cells,

remaining spleen cells, bone marrow cells, and pancreatic islet cells), using QIAGEN kits (QIAGEN, Valencia, CA). Purified DNA then was hydrolyzed to free deoxyribonucleosides, and the deoxyribose moiety of purine deoxyribonucleosides was derivatized to pentane tetra-acetate and analyzed by GC-MS to quantify DNA synthesis rates (cell proliferation rates) as described elsewhere (Neese et al., 2001,2002; Varady et al., 2007). TG-glycerol and fatty acids were isolated from dissected epididymal and inguinal adipose tissue depots and prepared for GC-MS analysis as described previously (Turner et al., 2003, 2007). L-4-(*O-tert*-butyldimethylsilyl)-hydroxyproline pentafluorobenzyl ester was prepared from dissected femur and dorsal skin flap and analyzed by GC-MS to quantify collagen synthesis rates as previously described (Gardner et al., 2007). Pentafluorobenzyl-*N*,*N*-di(pentafluorobenzyl)-alanine was prepared from acid hydrolysates (6 normal HCl, 110°C, 12 h) of mixed muscle proteins from dissected quadriceps and analyzed by GC-MS to quantify protein synthesis rates as previously described (Busch et al., 2006).

**$^2$H-Glucose Disposal Test.** The $^2$H-glucose disposal test ($^2$H-GDT) was used in a separate subset of mice to measure insulin-mediated glucose utilization (insulin resistance) and pancreatic β-cell compensation as previously described (Beysen et al., 2007). Twelve animals were randomly assigned to three groups ($n = 4$ per group). Each group was administered vehicle, 30 mg/kg/day prednisolone, or 30 mg/kg/day compound L5 for 7 days.

**MS.** GC-MS was performed by using a 5890 gas chromatograph attached to either a 5971 or 5973 mass spectrometer (Hewlett Packard, Palo Alto, CA; Agilent Technologies, Santa Clara, CA). Unlabeled compounds of interest, along with their singly and doubly labeled mass isotopomers ($M_0$, $M_1$, and $M_2$ for each compound of interest), were analyzed by selected ion monitoring. For pentane tetra-acetate derivatives from DNA [mass to charge (*m/z*) ratios 435–436], GC was performed with a DB-17 GC column (Agilent Technologies, Santa Clara, CA), and MS was performed in negative chemical ionization mode. For glycerol-triacetate (*m/z* ratios 159–161), GC was performed with a DB-225 GC column, and MS was performed in positive chemical ionization mode. For fatty acid-methyl esters (*m/z* ratios 270–272), GC was performed with a DB-225 GC column, and MS was performed in electron impact ionization mode. For L-4-(*O-tert*-butyldimethylsilyl)-hydroxyproline pentafluorobenzyl ester (derivatized hydroxyproline) and pentafluorobenzyl-*N*,*N*-di(pentafluorobenzyl)-alanine (*m/z* ratios 424–426 and 448–450, respectively) GC was performed with a DB-225 GC column, and MS was performed in negative chemical ionization mode.

**Statistical Analyses.** Statistical analyses were performed with SigmaStat 3.0 (SPSS Inc., Chicago IL). One-way ANOVA was performed within groups administered L5 and vehicle or prednisolone and vehicle to assess statistically significant differences between group means. If a significant difference between groups was found ($P < 0.001$, all levels), a Bonferroni *t* test was used to compare test groups with controls. $P < 0.05$ was considered to be significant for the post hoc test.

**Additional Methods.** Detailed methodology is described in Supplemental Methods.

## Results

**Effect of L5 Versus Prednisolone on Bone and Skin Collagen Synthesis.** Reductions in bone collagen synthesis represent a primary mechanism underlying reduced bone mass during glucocorticoid treatment (Canalis, 2003). The binding affinity of L5 to the GR is similar to that of prednisolone (Shah and Scanlan, 2004; Wang et al., 2006), but the absorption and pharmacokinetics of L5 have not been established. Accordingly, we gave identical doses (mg/mg) of L5 and prednisolone. Two doses were tested (5 and 30 mg/kg/day) to cover a range of potential activity, and administration was twice a day to increase tissue exposure in case the half-life of L5 is shorter than that of prednisolone. The doses of each compound were by intent high to increase the likelihood of observing robust changes in multiple tissues over a 1-week dosing period.

Prednisolone had a dose-dependent inhibitory effect on collagen synthesis in the femur, whereas L5 had no effect (Fig. 2). The group that received 30 mg/kg/day of prednisolone for 7 days showed a significantly lower rate of bone collagen synthesis compared with controls ($P < 0.05$). In contrast, groups administered L5 at either dose showed rates of bone collagen synthesis that were not significantly different from controls.

Skin collagen synthesis was also measured in a subset of animals from each group (data not shown). Groups that received 5 and 30 mg/kg/day of prednisolone showed approximately 50 and 75% reductions, respectively, in the rate of skin collagen synthesis compared with controls. Rates of skin collagen synthesis in groups administered either dose of L5 were similar to controls.

Thus, L5 demonstrated no significant effect on bone collagen and skin collagen synthesis rates, whereas prednisolone reduced both.

**Effect of L5 Versus Prednisolone on Skeletal Muscle Protein Synthesis.** Stimulation of proteolysis is likely the primary mechanism by which glucocorticoids reduce skeletal muscle mass, but reductions in muscle protein synthesis are also observed (Odedra et al., 1983; Louard et al., 1994; Long et al., 2001). Prednisolone exhibited modest effects on skeletal muscle protein synthesis (Fig. 3), with significant reductions observed in the group administered 5 mg/kg/day for 7 days compared with controls ($P < 0.05$). In contrast, L5 did not decrease skeletal muscle protein synthesis at either dose.

**Effect of L5 Versus Prednisolone on Insulin Sensitivity.** Measurement of peripheral tissue insulin sensitivity by $^2$H-GDT (Beysen et al., 2007) involved a separate set of animals than the $^2H_2O$ protocol used for the other target pathways (see *Materials and Methods*). To conserve L5 compound, only one dose of the agents was given. Administration of L5 or prednisolone at 30 mg/kg/day in divided doses for 7

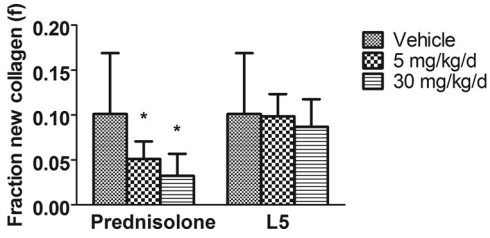


**Fig. 2.** Fraction (f) of new bone collagen (hydroxyproline) synthesized and retained over the course of 1 week in femur. Data are shown as mean ± S.D. $n = 6$ per group. *, $P < 0.05$ compared with vehicle control.

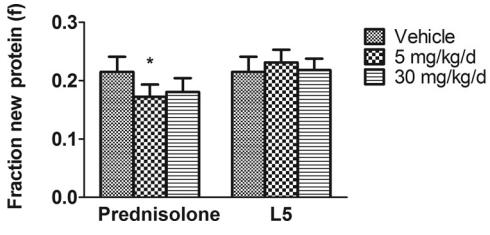


**Fig. 3.** Fraction (f) of new protein (alanine) synthesized and retained over the course of 1 week in quadriceps muscle. Data are shown as mean ± S.D. $n = 6$ per group. *, $P < 0.05$ compared with vehicle control.

days resulted in a significantly lower insulin sensitivity index [(% glucose load metabolized/area under the curve (AUC) insulin) (Fig. 4A)]. Administration of L5 at 30 mg/kg/day also resulted in significantly higher insulin AUC levels than in controls (Fig. 4B). No significant differences in fasting insulin levels, fasting blood glucose levels, total glucose used (representing pancreatic compensation to insulin resistance), or blood glucose AUC (integrated over time points 0–10 min) were observed between groups. Thus, both L5 and prednisolone induced peripheral tissue insulin resistance at 30 mg/kg/day in the intact mouse.

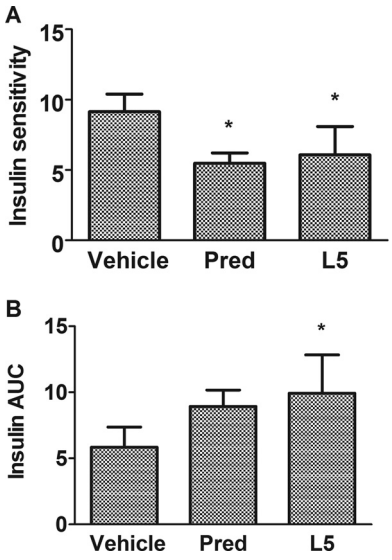


**Fig. 4.** A, insulin sensitivity index, calculated as percentage of glucose load metabolized divided by insulin AUC (Beysen et al., 2007). B, insulin AUC, measured by the trapezoidal method, as average insulin concentration in μg/ml at times 0 and 10 min multiplied by 10 min. Vehicle, vehicle control group; Pred, group treated with 30 mg/kg/day of prednisolone; L5, group treated with 30 mg/kg/day of compound L5. Data are shown as mean ± S.D. $n = 4$ per group. *, $P < 0.05$ compared with vehicle control.

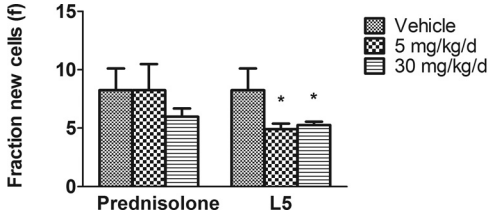


**Fig. 5.** Fraction (f) of newly formed neural progenitor stem cells in the hippocampus. Data are shown as mean ± S.D. $n = 6$ per group. *, $P < 0.05$ compared with vehicle control.

**Effects on Hippocampal Progenitor Cell Proliferation in the Brain.** The proliferation rate of hippocampal progenitor cells is a sensitive measure of neurogenesis in adult rodents (Shankaran et al., 2006). Neurogenesis is altered in models of depression, stress, and cognitive dysfunction and is a drug target for antidepressant and cognition-enhancing agents. Interestingly, L5 was a potent inhibitor of hippocampal progenitor cell proliferation in the hippocampus, whereas prednisolone had no effect (Fig. 5). Hippocampal neurogenesis was significantly lower ($P < 0.05$) in groups administered either 5 or 30 mg/kg/day of L5 for 7 days compared with controls. The groups administered prednisolone at 5 or 30 mg/kg/day showed no statistically significant reduction in hippocampal progenitor cell proliferation compared with controls. We have previously reported that treatment with prednisolone for 4 weeks at 40 mg/kg/day but not 5 mg/kg/day significantly reduced hippocampal progenitor cell proliferation, whereas treatment for 1 week had no effect (Shankaran et al., 2006). Accordingly, L5 is a more potent and faster-onset inhibitor of hippocamapal neurogenesis than prednisolone has been shown to be.

**Effect of L5 Versus Prednisolone on Splenic Lymphocyte Counts, Proliferation Rates, and Death Rates.** Prednisolone reduced numbers of T cells, B cells, and total cells in spleen, whereas L5 had no effect on cell counts (Fig. 6). The effects of prednisolone on cell counts were dose-dependent for all types of cells analyzed. Counts were significantly lower at the prednisolone dose of 30 mg/kg/day compared with controls for total spleen cells and non-$CD4^+$ T cells and at the prednisolone dose of 5 mg/kg/day for B cells and $CD4^+$ T cells.

Prednisolone decreased both fractional and absolute proliferation rates of splenic T and B cell subpopulations dose dependently, with no significant reduction in proliferation rates of total spleen cells (Figs. 7 and 8). The effects of prednisolone on splenic T cell proliferation were statistically significant at the dose of 30 mg/kg/day for both fractional and

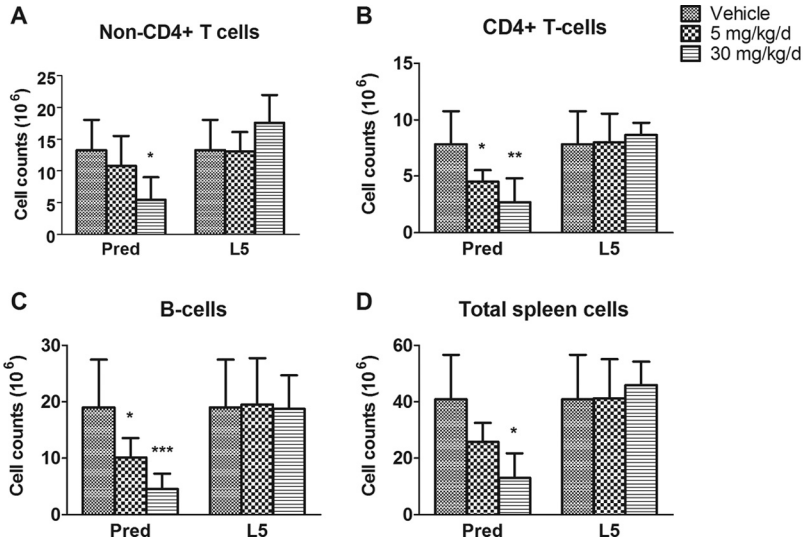


**Fig. 6.** Cell counts (millions) after 1 week of treatment with vehicle or 5 or 30 mg/kg/day of prednisolone (Pred) or compound L5 for splenic non-$CD4^+$ T cells (A), $CD4^+$ T cells (B), B cells (C), and total spleen cells (D). Data are shown as mean ± S.D. $n = 6$ per group. *, $P < 0.05$ compared with vehicle control. **, $P < 0.01$ compared with vehicle control. ***, $P < 0.001$ compared with vehicle control.

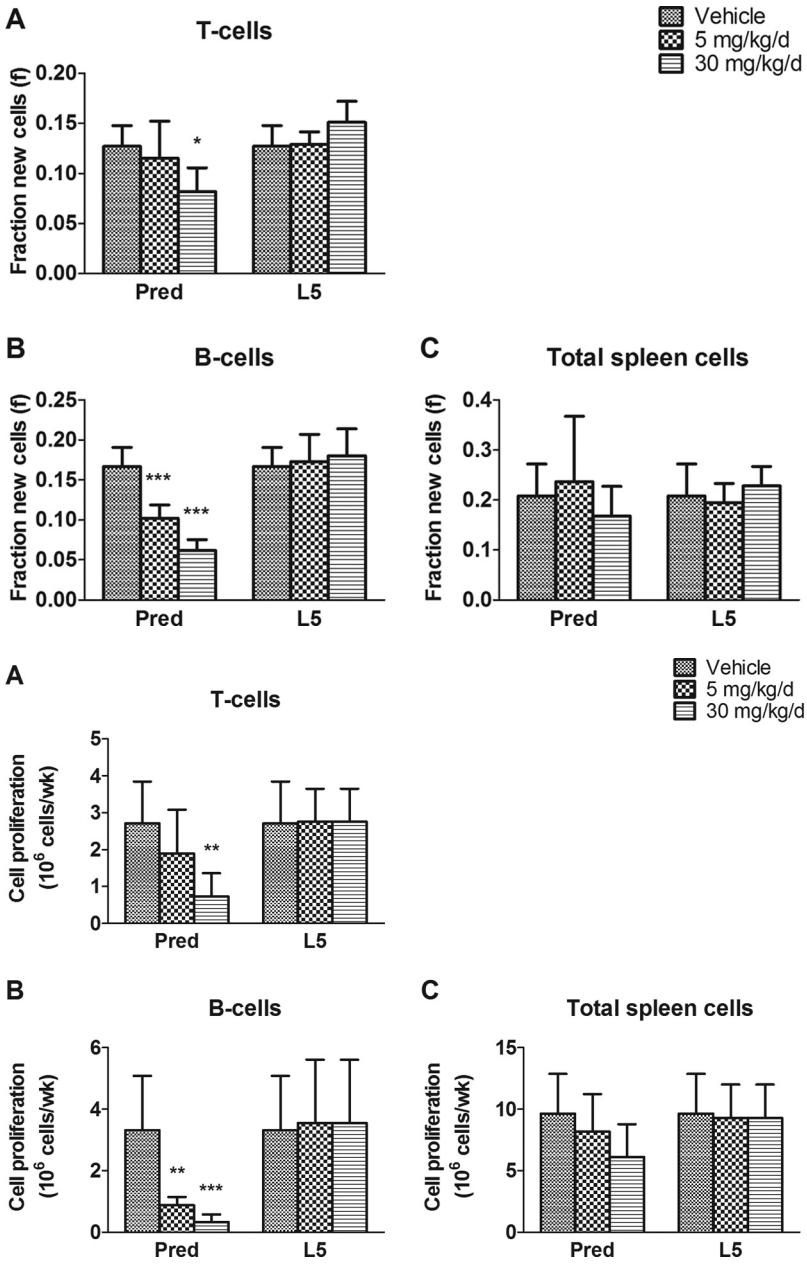


**Fig. 7.** Fraction of newly formed splenic T cells (A), B cells (B), and total spleen cells (C) throughout the course of 1 week of treatment with vehicle or 5 or 30 mg/kg/day of prednisolone (Pred) or compound L5. Data are shown as mean ± S.D. $n = 6$ per group. *, $P < 0.05$ compared with vehicle control. ***, $P < 0.001$ compared with vehicle control.




**Fig. 8.** Proliferation (millions of cells) of splenic T cells (A), B cells (B), and total spleen cells (C) throughout the course of 1 week of treatment with vehicle or 5 or 30 mg/kg/day of prednisolone (Pred) or compound L5. Data are shown as mean ± S.D. $n = 6$ per group. **, $P < 0.01$ compared with vehicle control. ***, $P < 0.001$ compared with vehicle control.

absolute proliferation rates ($P < 0.05$ and 0.01, respectively). The effects of prednisolone on splenic B cell proliferation were statistically significant at the dose of 5 mg/kg/day for both fractional and absolute proliferation rates ($P < 0.001$ and 0.01, respectively). In contrast, L5 treatment had no effect on the fractional or absolute proliferation rates of any cell type analyzed in spleen.

Cell death rates in the total cell, B cell, and T cell subpopulations in spleen increased in a dose-dependent manner in response to prednisolone administration. Cell death rates for splenic T cells, splenic B cells, and total spleen cells were more than 5-, 4-, and 3-fold greater, respectively, in the high-dose prednisolone group than in the control group. L5 had no effect on cell death rates in spleen (Fig. 9).

In summary, prednisolone potently reduced numbers, reduced proliferation rates, and increased death rates of lymphocytes in the spleen, whereas L5 exhibited none of these effects.

**Effect of L5 Versus Prednisolone on Pancreatic Islet Cell Proliferation.** Pancreatic islet cell proliferation rates are stimulated in several rodent models of chronic insulin resistance (Bonner-Weir et al., 1989; Bruning et al., 1997; Paris et al., 2003). We measured pancreatic islet cell proliferation rates in control animals (Chen et al., 2007) and animals given 30 mg/kg/day of either prednisolone or L5 (Fig. 10). No significant differences in islet cell proliferation were observed, however, after 7 days of treatment with prednisolone or L5.

**Effect of L5 Versus Prednisolone on TG Metabolism in Adipose Tissue.** Because glucocorticoids are widely known to alter adipose tissue fat metabolism, we measured fat pad weights, fractional TG turnover, absolute TG synthesis rates, net lipolysis rates, DNL, and glyceroneogenesis in the abdominal epididymal fat depot (Table 1) and the subcutaneous inguinal fat depot (Table 2). Fractional TG turnover in the high-dose prednisolone group was slightly lower ($P < 0.05$) compared with controls in the epididymal depot

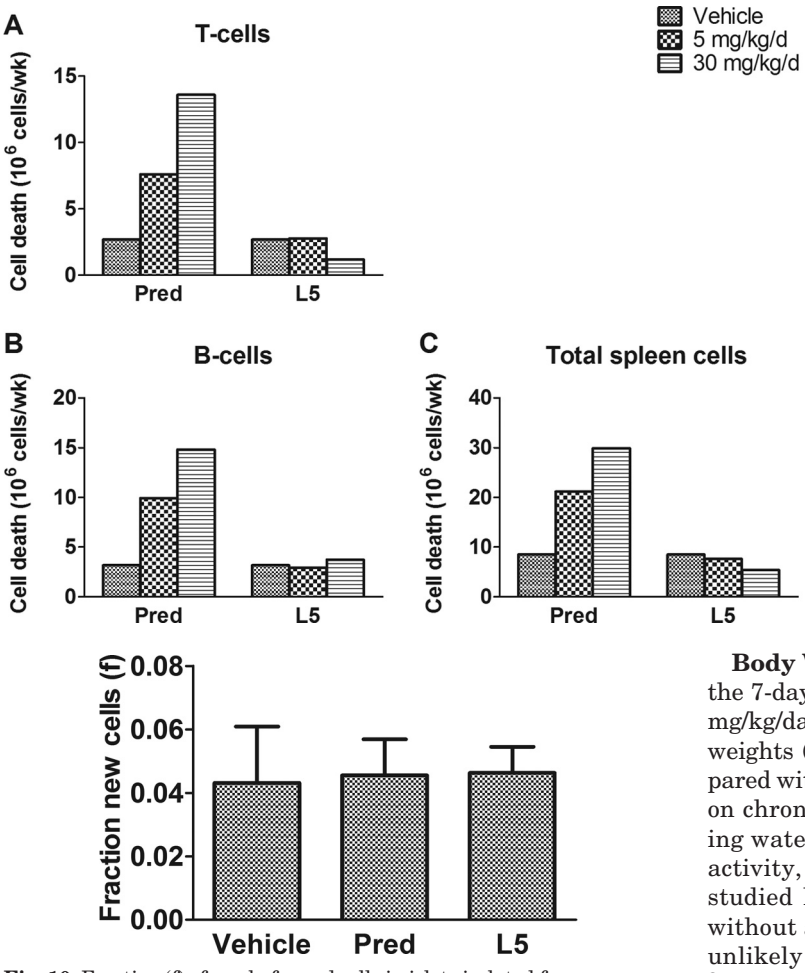


**Fig. 9.** Calculated group average cell death in millions of cells of splenic T cells (A), B cells (B), and total spleen cells (C) throughout the course of 1 week of treatment with vehicle or 5 or 30 mg/kg/day of prednisolone (Pred) or compound L5. Data are shown as calculated group means only.

**Fig. 10.** Fraction (f) of newly formed cells in islets isolated from pancreas. Pred, prednisolone. Data are shown as mean ± S.D. $n = 6$ per group.

(32% versus 36% for the high-dose prednisolone group versus the control group, respectively). Animals administered 30 mg/kg/day of L5 had on average significantly more inguinal fat ($P < 0.05$) than controls (283 versus 200 mg). All other groups administered either L5 or prednisolone showed values similar to those for the control group for all other metrics (Tables 1 and 2). Thus, short-term treatment with neither prednisolone nor L5 induced major changes in adipose tissue lipid dynamics, although long-term exposure to elevated endogenous glucocorticoids markedly increases adipose TG turnover (C. Harris, D. J. Roohk, R. Farese, and M. K. Hellerstein, unpublished work).

**Effect of L5 Versus Prednisolone on Gene Expression in Liver and Skeletal Muscle.** Expression of the gluconeogenic gene *PEPCK* in liver was induced more than 2-and 3-fold on average in mice administered either dose of prednisolone or L5, respectively, compared with controls (Fig. 11). Expression of the gene *Murf1* in muscle, a gene involved in ubiquitin-based protein degradation pathways, was also elevated relative to control in animals administered either dose of prednisolone or L5. *Murf1* was induced on average between 2- and 3.5-fold over controls in response to either dose of prednisolone or L5. These results are consistent with comparable absorption and systemic tissue exposure for both L5 and prednisolone, at least with regard to genes expressed in liver and muscle.

**Body Weights.** No group gained weight over the course of the 7-day treatment period. The groups given vehicle and 30 mg/kg/day of prednisolone had significantly lower body weights ($P < 0.01$ and $< 0.001$, respectively) on day 7 compared with day 0. Rodents have previously been maintained on chronic $^2H_2O$ intake at up to 30% enrichment in drinking water without effects on growth, food intake, behavior, activity, or fertility (Koletzko et al., 1997), and we have studied hundreds of mice on 8% $^2H_2O$ in drinking water without any effect on body weight gain. It is therefore very unlikely that the absence of weight gain here is caused by $^2H_2O$ administration but may relate to the daily gavage procedure.

## Discussion

In this study, we systematically characterized in vivo the pleotropic effects of a putative SGRM, compound L5, in comparison with a classic GR ligand, prednisolone. The actions of these GR ligands were measured by a $^2H_2O$ labeling strategy that allows measurement of fluxes through multiple target metabolic pathways concurrently, including the dynamics of lipids, proteins, and cells, in the whole organism (Neese et al., 2002; Kim et al., 2005; Busch et al., 2006, 2007; Shankaran et al., 2006; Chen et al., 2007; Gardner et al., 2007; Turner et al., 2007). Target pathways were selected based on their hypothesized relevance to the pathophysiology of major adverse effects of glucocorticoids. By measuring flux rates through these disease-modifying pathways that may underlie the initiation or progression of undesired outcomes we were able to demonstrate convincing evidence of side-effect relevant metabolic selectivity for a putative SGRM. Importantly, because multiple pathway fluxes could be measured concurrently in the same animal and with great sensitivity by the $^2H_2O$ labeling approach (Neese et al., 2002; Turner et al., 2003, 2007; Kim et al., 2005; Busch et al., 2006, 2007; Shankaran et al., 2006; Chen et al., 2007; Gardner et al., 2007), this systematic characterization was possible in whole animals with only a limited amount of compound L5 (less than 50 mg).

TABLE 1

Effect of L5 vs. prednisolone (Pred) on TG metabolism in adipose tissue-abdominal fat pad

Data shown are mean (S.D.). $n = 6$ per group.

| | Fat Pad Weight | Fractional Replacement of TG | Absolute TG Synthesis | Absolute Lipolysis | DNL-Fractional Replacement of Palmitate | DNL-Absolute Palmitate Synthesis | Glycero-Neogenesis |
|---|---|---|---|---|---|---|---|
| | *mg* | *%/wk* | *mg/wk* | *mg/wk* | *%/wk* | *mg/wk* | *%* |
| Vehicle | 313 (32) | 36 (3) | 101 (13) | 118 (16) | 20 (8) | 10.4 (4.8) | 21 (8) |
| Pred 5 mg/kg | 293 (112) | 36 (2) | 93 (37) | 106 (39) | 29 (20) | 10.9 (3.7) | 16 (6) |
| Pred 30 mg/kg | 308 (49) | 32 (1)* | 89 (14) | 109 (17) | 15 (2) | 7.2 (1.6) | 11 (8) |
| L5 5 mg/kg | 358 (74) | 35 (2) | 113 (27) | 119 (29) | 22 (4) | 12.9 (4.6) | 13 (9) |
| L5 30 mg/kg | 402 (69) | 35 (2) | 127 (21) | 131 (20) | 23 (3) | 14.7 (2.9) | 16 (9) |

*, $P < 0.05$ compared with vehicle control.

TABLE 2

Effect of L5 vs. prednisolone (Pred) on TG metabolism in adipose tissue- subcutaneous fat pad

Data shown are mean (S.D.). $n = 6$ per group.

| | Fat Pad Weight | Fractional Replacement of TG | Absolute TG Synthesis | Absolute Lipolysis | DNL-Fractional Replacement of Palmitate | DNL-Absolute Palmitate Synthesis | Glycero-Neogenesis |
|---|---|---|---|---|---|---|---|
| | *mg* | *%/wk* | *mg/wk* | *mg/wk* | *%/wk* | *mg/wk* | *%* |
| Vehicle | 200 (60) | 46 (4) | 83 (30) | 94 (33) | 47 (10) | 15.5 (7.7) | 24 (8) |
| Pred 5 mg/kg | 228 (48) | 46 (4) | 97 (33) | 107 (32) | 55 (10) | 19.7 (5.7) | 17 (6) |
| Pred 30 mg/kg | 202 (53) | 42 (4) | 76 (23) | 91 (30) | 45 (10) | 11.4 (7.2) | 12 (8) |
| L5 5 mg/kg | 223 (50) | 46 (4) | 92 (24) | 96 (23) | 50 (5) | 17.7 (4.5) | 16 (10) |
| L5 30 mg/kg | 283 (48)* | 44 (2) | 112 (20) | 115 (19) | 50 (6) | 22.5 (3.9) | 16 (8) |

*, $P < 0.05$ compared with vehicle control.

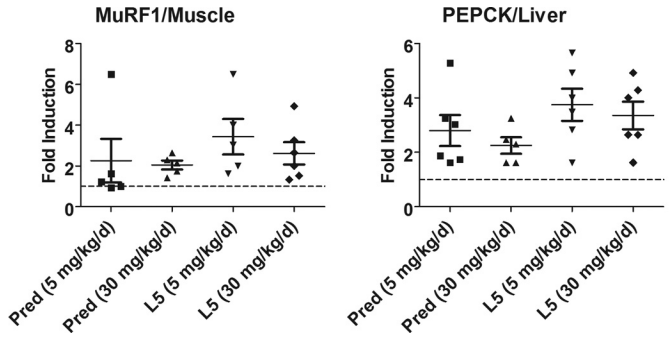


**Fig. 11.** Induction of selective glucocorticoid target genes by prednisolone (Pred) and L5 in vivo. Mice were treated with vehicle, prednisolone (5 or 30 mg/kg/day), or L5 (5 or 30 mg/kg/day) for 7 days. At the end of treatment, liver and skeletal muscle were harvested. Total RNA was prepared and subjected to cDNA synthesis. The cDNA was then analyzed by quantitative polymerase chain reaction to measure the relative mRNA levels of two glucocorticoid target genes using gene-specific primers. Data represent the S.E.M. of the fold induction over that of the vehicle control group (prednisolone- or L5-treated samples divided by vehicle-treated samples) from at least five mice in each group.

The data clearly demonstrate that prednisolone and L5 exert different patterns of activity on these disease-modifying pathways (Table 3). Prednisolone potently inhibited bone and skin collagen synthesis, reduced splenic lymphocyte proliferation rates, increased splenic lymphocyte death rates, and modestly reduced muscle protein synthesis and TG turnover in abdominal fat, whereas L5 had no effect on any of these processes. However, L5 treatment for 7 days significantly reduced hippocampal progenitor cell proliferation, whereas prednisolone treatment had no significant effect (but has previously been shown to have significant effects on hippocampal progenitor cell proliferation after 4 weeks, at a higher dose) and prednisolone and L5 induced insulin resistance equally. Finally, L5 and prednisolone treatment resulted in similar induction of the expression of GR-target genes *PEPCK* in liver and *Murf1* in muscle, and neither exhibited significant effects on pancreatic islet cell proliferation after 7 days of treatment.

TABLE 3

Summary of prednisolone and L5 actions on flux through several disease-relevant glucocorticoid-responsive pathways

| Pathway | Prednisolone | L5 |
|---|---|---|
| Immunosuppression (lymphocyte birth and death) | + + | No effect |
| Muscle protein synthesis | − | No effect |
| Bone and skin collagen synthesis | − − | No effect |
| Insulin resistance (insulin-mediated glucose utilization) | + + | + + |
| Hippocampal neurogenesis | No effect | − − |
| Lipolysis (abdominal fat pad) | No effect | No effect |
| TG turnover (abdominal fat pad) | − | No effect |

++, large stimulatory effect; +, moderate stimulatory effect; −, moderate inhibitory effect; − −, large inhibitory effect.

These results answer several key issues regarding selectivity of the SGRM, compound L5. First, L5 clearly has glucocorticoid agonist effects in vivo in mice, based on the observed glucocorticoid-like effects on insulin resistance (Fig. 4), hippocampal neurogenesis (Fig. 5), and gene expression in muscle and liver (Fig. 11). Accordingly, the compound was absorbed and got to at least some target tissues. Although we did not measure uptake or volume of distribution, these results make it unlikely that pharmacokinetic differences can explain the absence of effect of L5 on other pathways altered by prednisolone.

Second, L5 has some apparent advantages over prednisolone, particularly its lack of inhibition of bone and skin collagen synthesis or muscle protein synthesis. L5 also has potential disadvantages compared with prednisolone, including an apparent lack of immunosuppressive actions (as evidenced by absence of effects on lymphocyte counts, proliferation, or death rates) and its increased potency toward suppression of hippocampal neurogenesis. An important class of actions of GR ligands that we did not evaluate here was anti-inflammatory effects (Buttgereit et al., 2005a; Rosen and Miner, 2005; Schacke et al., 2007), because an animal model of inflammation is required, whereas all the

parameters that were studied here could be performed in animals in their baseline health status.

Third, in vitro comparisons of actions of L5 and the potent GR agonist dexamethasone in cell-based models (Wang et al., 2006) correlated well, but not perfectly, with in vivo actions on target pathways. Differential effects of dexamethasone and L5 on differentiation of MC3T3-E1 preosteoblasts to osteoblasts (Wang et al., 2006) parallel our observation of differential effects of prednisolone and L5 on bone collagen synthesis (Fig. 2). Similar inhibitory effects of L5 and dexamethasone on insulin-stimulated glucose uptake were also observed in OP9 adipocytes (J. C. Wang, unpublished results), consistent with our finding that both L5 and prednisolone reduce whole-body insulin-mediated glucose disposal (Fig. 4). In contrast, dexamethasone and L5 both repressed transcription of several proinflammatory genes, including *RANTES*, *MCP1*, and *IL-6*, in tumor necrosis factor α-induced A549 cells (Wang et al., 2006), with L5 doing so to a lesser degree. But these cytokine-inhibitory effects did not predict the different effects of prednisolone and L5 on lymphocyte proliferation and death rates or counts in vivo (Figs. 6–9). Nor was the finding of increased lipolysis by dexamethasone but not L5 in OP9 cells (J. C. Wang, unpublished results) supported in vivo by our data, at least after 7 days of treatment (Tables 1 and 2).

The use of ex vivo cell-based models for predicting in vivo phenotypic selectivity in the whole organism is therefore potentially informative, but results have to be interpreted cautiously. The differentiation of MC3T3-E1 preosteoblasts to osteoblasts appears to predict effects of GR-ligands on bone collagen synthesis in mice, for example, suggesting that this cell model may be useful in screening for SGRMs that do not reduce bone mass. As a general principle, however, intact organisms must be used to establish definitively whether GR ligands induce differential actions in vivo.

Moreover, different metabolic pathways exhibit ligand-specific dose-response relationships. For prednisolone, dose-response effects on T cell dynamics (Figs. 6, A and B, 7A, 8A, and 9A) and muscle protein synthesis (Fig. 3) are quite different, for example. Studies to establish therapeutic index should therefore include a number of target pathways.

An important practical feature of the strategy used here is that a broad array of physiological effects could be quantified simultaneously in a relatively small number of animals. This feature is enabled by the sensitivity and reproducibility of mass spectrometric flux measurements after $^2H_2O$ administration (Hellerstein, 2004; Hellerstein and Murphy, 2004). Our results here show that some metabolic pathways are rapidly affected by administration of GR ligands, whereas others may require more prolonged exposure. Insulin-mediated glucose disposal, lymphocyte proliferation and death rates, bone collagen synthesis, and skin collagen synthesis responded to 7 days of prednisolone treatment. In contrast, inhibition of hippocampal neurogenesis requires longer administration of prednisolone (Shankaran et al., 2006) but not L5 (Fig. 5), and we have observed marked changes in adipose tissue lipid dynamics in transgenic mice overproducing endogenous GCs from birth (C. Harris, D. J. Roohk, R. Farese, and M. K. Hellerstein, unpublished observations), but not in response to 7 days of prednisolone treatment. Similarly, increased pancreatic islet cell proliferation associated with insulin resistance was observed after 4 weeks of prednisolone treatment (S. Chen and M. K. Hellerstein, unpublished observations) but not after 7 days of treatment.

In a general sense, our results demonstrate that in vivo phenotypic selectivity on disease-modifying target pathways can be achieved for GR ligands. Compound L5 is undeniably selective in its effects on GR-mediated pathways, whether or not L5 represents an attractive drug candidate. These findings are encouraging for other putative SGRMs. When incorporated into existing clinical and preclinical screening strategies, this approach can also inform upstream selection of models and assays.

There are a number of future directions that may help to optimize the use of in vivo pathway measurements for evaluating selectivity of GR ligands. Testing the effects of SGRMs in animal models of inflammation and including pathways related to inflammation may extend the findings generated here in healthy animals. Many of the parameters described here can also be translated directly into clinical studies in humans by using the $^2H_2O$ labeling approach (Hellerstein, 2004; Hellerstein and Murphy, 2004; Beysen et al., 2007). Data generated in this manner may also allow quantitative comparison of dose-response effects of leads, rapid evaluation of different doses and regimens, and the capacity to evaluate the relevance of preclinical models to actions in humans. It also will be instructive to correlate gene expression, pharmacokinetics of tissue drug exposure, and in vivo phenotypic outcomes in detail. Target pathways interrogated by flux measurements can also be expanded.

In summary, we show here for the first time that a novel arylpyrazole compound, L5, exhibits selective actions on disease-modifying target pathways of GR ligands in vivo in intact animals. The net pattern of activities for L5 exhibits both disadvantages and advantages compared with prednisolone. The capacity to decouple inhibition of bone collagen synthesis, skin collagen synthesis, and muscle protein synthesis from induction of insulin resistance and suppression of neurogenesis, however, is encouraging for the long-term goal of creating SGRM ligands for targeted disease indications. Neither gene expression nor cell-based models fully captured the phenotypic selectivity of L5 compared with prednisolone. In vivo measurement of dynamic fluxes in a wide array of target pathways represents an efficient and powerful systems approach for interrogating the phenotypic selectivity of ligands that bind nuclear transcription factors.

**Acknowledgments**

We thank Simply Florcruz for help during the treatment administration phase of the study and Shoreh Noori and Madhav Vissa for assistance with laboratory analyses for compound L5.

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