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Dexamethasone And Certain Nonsteroidal Drugs Stimulate The Transcriptional Activity Of GR-In The U2-OS Cancer Cell Line

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Abstract

Glucocorticoids are steroid hormones that act as immunosuppressants, immunomodifiers, and anti-inflammatory drugs. A synthetic GC chemical called dexamethasone (Dex) is used for a number of things other just reducing inflammation. Dex stops cell proliferation in leukemia and other cancers. In order to help chemotherapy kill leukemia cells or to minimize side effects from specific chemotherapy drugs, steroids are frequently given along with chemotherapy. The two steroids that are most frequently administered for ALL are prednisolone and dexamethasone. Steroid pills are typically taken. In both people and animals, chronic GC use increases the risk of illnesses of the nephrological system, skeletal system, and metabolism.

The current study sought for strategies to improve the current glucocorticoid medication in order to treat leukemia. The effects of numerous substances on cells were examined using a variety of procedures.

This study investigated the dissociation effects of CPDA TYRAMINE AND THCL to dexamethasone on wild GR and transfected Mutant GR-I628A using the cancer cell line U2-OS and the luciferase assay. The results show that DEX stimulates the transcriptional activity of wild GR. Following Dex treatment, TAT3 luciferase reporter activity was elevated in both mutant and wild-type GR.

The other non-steroidal medications that were tested also demonstrated positive regulation activity, with DEX, T, CpdA, and THCL upregulating TAT3 transcription, in that order. Given that transactivation activity is the only adverse consequence, this implies that other drugs may be less likely to do so than GR. Additionally, residue I628 appears to be essential for interaction because its mutation decreases GR activity when it interacts with all drugs.

Keywords: Leukemmia, GR, GC, Compound A, Luciferase

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Introduction

There are three major approaches to treat cancer: surgery, radiation therapy, or systemic therapy. Stem cell transplantation (SCT) and interferon therapy are additional leukaemia treatments .Leukemia also affects animals besides humans, and some kinds of the disease have a similar course of development [12].

Since more than 50 years ago, synthetic glucocorticoids have been often recommended for a wide range of diseases. Among others, glucocorticoids are the preferred treatment for allergic rhinitis, asthma, chronic bronchitis, cystic fibrosis, emphysema, inflammatory bowel disease, multiple sclerosis, and rheumatoid arthritis [2].

Asthma, rheumatoid arthritis, and allergies are all inflammatory diseases, and GCs are the medicine of choice for treating them [13].

They are crucial to metabolism because they regulate blood sugar levels through the three distinct processes of gluconeogenesis, glucose conservation, and lipolysis. This action is made possible by GR-induced activation of the transcription of the gluconeogenic enzymes glucose-6-phosphatase and phosphoenolpyruvate carboxy kinase (PEPCK). To produce their downstream effects, glucocorticoids bind to their receptor . For some cancer forms, they are also used in cancer therapy or in combination with chemotherapy [6].

Due to their effects on the apoptotic process, GC have been a part of the conventional therapy for patients with ALL for a lot of years. GCs can have no effect or even protect cells from death in other cell types, despite the fact that only a small number of cell types—mostly white blood cells—show these effects on apoptosis. However, prolonged usage of glucocorticoids might have a deleterious impact on the body. Hypertension, glaucoma, osteoporosis, and growth retardation are some of the negative effects . Thioredoxin-interacting protein (TXNIP), which controls thioredoxin, is modulated by glucose, which is one of the primary metabolic drawbacks of GCs. [10].

The body weight, feeding effectiveness, and blood cholesterol levels of chicken were all reduced by Dex, though [3]. adding Dexamethasone to chicken meal caused the body temperature to be rised, the respiration rate to quicken, and the growth hormone to be suppressed [10].

The lipophilic and hydrophobic steroid hormones known as glucocorticoids are produced from cholesterol. These hormones are stress hormones that are released by the adrenal gland, an endocrine gland, in response to stress (for an explanation of the HPA axis, see below). The well-known immunosuppressants, immunomodifiers, and anti-inflammatory steroids known as GCs. In reaction to stress, the adrenal cortex's zona fasciculata secretes GCs, which are then regulated by the HPA through negative feedback loops.

The neurohormone corticotrophin releasing hormone (CRH/CRF), which is secreted by the hypothalamus, promotes the pituitary hormone adrenocorticotropic hormone (ACTH), which in turn increases the release of glucocorticoids (GC) from the adrenal cortex by the adrenal gland. Finally, in a negative feedback loop, these hormones prevent the hypothalamus from producing more hormones [1].

GCs affect cells through binding to the glucocorticoid receptor (GR), a specific GC receptor. The various molecular weight heat shock proteins that are typically found in complexes with GR in the cytosol include HSp 23, HSp70 KDa, and HSp90 KDa. This complex also contains immunophilins such PP5, Cyp44, FKBP51, and FKBP52. The activated GR may either alter

additional genes or proteins in the cytoplasm and modify or modulate the entire pro-inflammatory response when the T-cell receptor signaling pathway is disrupted.

However, the principal mechanism of action of the activated GR homodimer is translocation into the nucleus and binding to a specific consensus sequence in the DNA of GCs sensitive genes. GCs affect cells through binding to the glucocorticoid receptor (GR), a specific GC receptor. The various molecular weight heat shock proteins that are typically found in complexes with GR in the cytosol include HSp 23, HSp70 KDa, and HSp90 KDa. This complex also contains immunophilins such PP5, Cyp44, FKBP51, and FKBP52. [6].

The activated GR may either alter additional genes or proteins in the cytoplasm and modify or modulate the entire pro-inflammatory response when the T-cell receptor signaling pathway is disrupted. However, the principal mechanism of action of the activated GR homodimer is translocation into the nucleus and binding to a specific consensus sequence in the DNA of GCs sensitive genes.

This consensus sequence, known as GRE (from GCs responsive elements), is found in the promoter region of target genes. GR then controls the transcription of these genes by activating or suppressing genes involved in metabolism, inflammation, apoptosis, and several other processes. (16).

Numerous variables, such as GR expression, nuclear translocation and transactivation, and posttranslational changes that result in GC resistance, may affect GC sensitivity [5].

In order to sensitize resistant cells and reverse Dex unresponsiveness, it is crucial to discover novel drugs . In some leukaemia cells, the levels of the GR protein are up-regulated after interaction with the ligand, whereas in the majority of epithelial cells, the levels of the GR protein and mRNA are down-regulated .It is known that GC activates GR, which causes leukaemia cells to undergo apoptosis [9].

Aims

GCs can be used alone or in conjunction with other anti-cancer therapies to treat a range of malignancies. Resistance occurs frequently, though, and the consequences can be severe. The initiative's objective is to have a better understanding of how GCs function so that novel medications and veterinary treatments can be developed. In order to discover if non-steroid synthetic compounds like Compound A or steroid medications like Dexamethasone, alone or in combination, would provide more efficient therapy choices, the study's objectives included additional exploration of GR as a potential therapeutic target.

The particular goals are:

- To ascertain Luciferase experiments and mutant GR derivatives will be used to better examine the molecular processes underlying drug actions.
- The ultimate goal is to employ one of these substances to treat ALL or avian leukosis more effectively.

Methods

Compounds used in study

Several hormones and compounds) were used to mimic the action of natural GC which in human is called cortisol. Four synthetic compounds were used for vitro experiments; Dexamethasone, **Compound A, Tyramine and Tyramine Hydrochloride**, while SEGRM CpdA. T and THCl were used together with CpdA to test their ability to dissociate

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transrepression from transactivation action of GR as most side effects are thought to be due to transactivation action.

Cancer cell lines
U2OS cells are utilized to perform the transfection procedure. This cell line is an osteosarcoma epithelial cell line taken from Caucasian female.

Maintenance of the cells
U-2 OS cells are maintained in DMEM (ATCC® 30-2002TM) supplemented with 10% FCS and 1% penicillin/streptomycin (Labtech). In the incubator (Galaxy S-Wolf laboratories), cells were maintained at 37°C in a humid atmosphere with 5% CO2. Cells were grown in T25 and T75 tissue culture flasks with Fisher supplements.

Bacteriological methods for plasmid preparation , Bacterial transformation
The targeted plasmids were transferred into competent bacteria, DH5α™ bacteria (Sub cloning Efficiency DH5α™ Competent Cells-40 reactions), and propagated DNA harvested from bacteria using Life Technologies Ltd.'s PureLink® HiPure Plasmid Filter Maxiprep Kit-25 preps are all products of that company. Chemically competent cells with a transformation efficiency of more than 1 x 106 cfu/g plasmid DNA are known as DH5α™ Competent Cells. Three types of media are utilized for growing competent bacteria: LB Ampicillin composition (20 grams of Luria Bertani (LB) powder + 1L distilled water,

Agar plates (20 grams of Luria Bertani (LB) agar plus 1 L of distilled water, autoclaved for 15 minutes, and ampicillin (100 g/ml) added. LB broth Ampicillin composition (20 grams of Luria Bertani (LB) powder plus 1 L of distilled water, autoclaved for 15 minutes.

To provide a sterile environment, enough is placed into Petri plates while they are being heated with benzene. On ice, one tube of DH5 is defrosted. For each transformation, 50 µl of cells are aliquoted into a 1.5 ml microcentrifuge tube. Add 2.5 l of DNA to the cells and gently stir by flicking the tubes. 30 minutes of incubation on ice,20 seconds of heat shock treatment on cells in a 42°C water bath, followed by tubes placed for two minutes on ice.,

950 µl of preheated LB broth should be added to each tube. Tubes should then be incubated at 37 °C for an hour at 225 rpm with 200 µl from each transformation. Spread over LB plates that have been preheated and include 100 g/ml of ampicillin. 37°C overnight incubation. One colony was taken from the plate the next day, inoculated into a conical flask of 100ml LB/amp overnight, and E. coli cultures were cultured at 37°C with a centrifuge running at a low speed of 200rpm.

Utilizing the Maxiprep Kit, DNA from converted DH5 cell culture was purified. The Maxiprep procedure included several steps, including harvesting the cells by centrifuging the overnight LB culture at 4000 g for 10 minutes, equilibrating the column and preparing cell lysate, loading the filter column and washing the DNA, and re-suspending the DNA pellet in 200 L TE Buffer (TE), purified DNA store at -20 °C as determined by DNA concentration measured by nano-drop.

Transient Transfection
Following the manufacturer's instructions, the U-2 OS human bone sarcoma (osteosarcoma) epithelial cell line was transiently transfected. The PolyFect Transfection Reagent, item number 301105, improves DNA translocation into the nucleus and ensures effective transfection. The chemical polyfect (dendrimers) affects transfection by forming complexes with the transfected DNA and facilitating its uptake across the cell membrane via endocytosis.

According to table 1, cells were seeded in a 6-well plate 24 hours prior to transfection at a density of 4x105 cells per well and incubated at 37°C and 5% CO2. On the day of transfection, 1.5 ml of old media were replaced with new media. TE buffer was used to dissolve 1.5 g of DNA, 100 µl of plain medium was added, and 10 l of the polyfect transfection reagent were added to the DNA solution. To allow complex formation, samples were mixed by pipetting up and down five times or by vortexing for 10 seconds. They were then incubated for 5–10 min at room temperature. By adding and thoroughly blending 600 µl of complete growth media into the DNA solution, which was then applied to the cells . (fig.1), and gently spun, the process was stopped. The plates were eventually kept inside the incubator for 24 hours [14].

Luciferase Assay

To gauge the transcription activity of GR in response to particular therapy, the Luciferase Assay was used. The assay works on the basis of the luciferin, a luciferase assay system reagent, which emits light when Firefly Luciferase catalyzes its reaction (fig. 2). The best way to measure the reporter's activity is by the amount of light produced.

PBS was used to clean the cells before employing 100 µl of reporter lysis buffer per well to lyse them. Following a 30-minute incubation period on a rocking platform, samples are collected, transferred to Eppendorf tubes, and centrifuged for 15 min. at 15,000 RPM at 40C. Finally, 10 µl of the cellular extract and 100 µl of luciferin are added to each well of a 96-well Grenier plate. Using an Omega luminometer and MARS software, samples were combined and measured right away in less than 10 seconds [4].

Beta-galactosidase assay

The reporter -beta-galactosidase (beta-gal) activity and transfection efficiency are measured using the beta-galactosidase test, a colorimetric technique. Ortho-nitrophenyl-D-galactopyranoside (ONPG), a substrate, was combined with 30 l of cellular extracts and 300 l of B-gal buffer (table 1), mixed, and stored for 60 min at 370 C in the dark. Orthonitrophenol (ONP), which is formed when -galactosidase hydrolyzes ONPG and has a yellow color (fig. 3) and absorbs at 420 nm OD, is produced. After adding 500 µl of deionized water to stop the reaction, the absorbance was measured, and the findings were compared to the outcomes of the luciferase assay [8].

Results

According to the findings, DEX boosts wild GR's transcriptional activity. TAT3 luciferase reporter expression increased in both mutant and wild-type GR after Dex administration (fig. 4). This positive regulation activity was also observed when the other non-steroidal drugs were used, with DEX upregulating TAT3 transcription, followed by T, CpdA, and THCL, respectively. This suggests that other substances might be less likely to cause adverse reactions than GR, in which all negative effects result from transactivation action (fig. 5). Additionally, as its mutation reduces GR activity when compared to Dex, residue I628 appears to be crucial for interaction with all drugs.

Discussion

Tyrosine aminotransferase (TAT3)-luciferase reporter gene expression was detected following Dex administration using the luciferase assay (Figs. 4 and 5). Although less so than Dex, other substances can induce positive control. The findings of Dex were consistent with those of other tests performed on A549 after GR activation, where the authors proposed that this upregulation may be prevented by modulating GR [15].

Our findings suggest that the investigated chemicals may not cause or cause less transactivatin than Dex, suggesting that using the medications in in-vivo models may result in less side effects.

Conclusion

This initiative looked on ways to enhance the standard glucocorticoid therapy used to treat leukemia. Several substances were used, and several tests were used to examine their impact on subcellular structures. However, compared to the steroid Dex, none of the non-steroid substances examined showed as much tat-3 transcriptional activation.

Table 1. Parameters for transient transfection of Hela cells (adapted from polyfect supplier textbook)

Culture format	No. of cells to seed	Volume of medium(ml)	DNA(µg)	Final volume of diluted DNA (µl)	Volume of olyfect Reagent (µl)	Volume of medium to add to cells (ml)	Volume of medium to add to complexes (ml)
Protocol step	1	1	3	3	4	6	7
6-well plate*	4 x 10^5	3.0	1.5	100	20	1.5	0.6

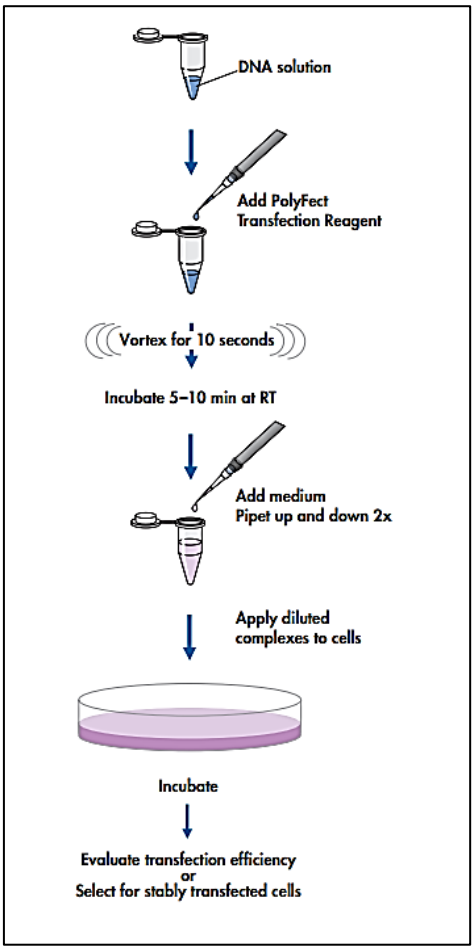

Figure 1 Transfection Procedures for PolyFect Transfection Reagent

by Firefly Luciferase. Light produced is the best way to quantify the reporters activity.

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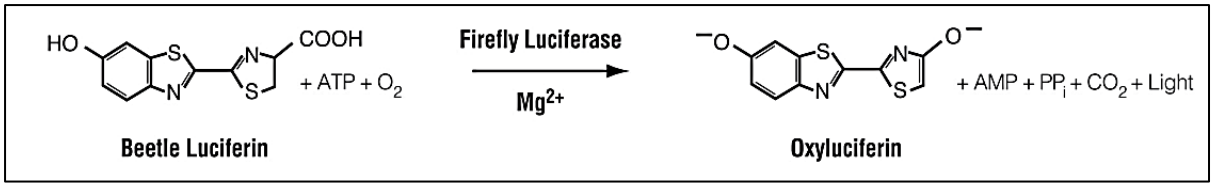

Figure 2 Chemiluminescent reaction principle of luciferase assay (Adapted from Promega bulletin)

Figure 3 Yellow colours as indicator of transfection efficiency

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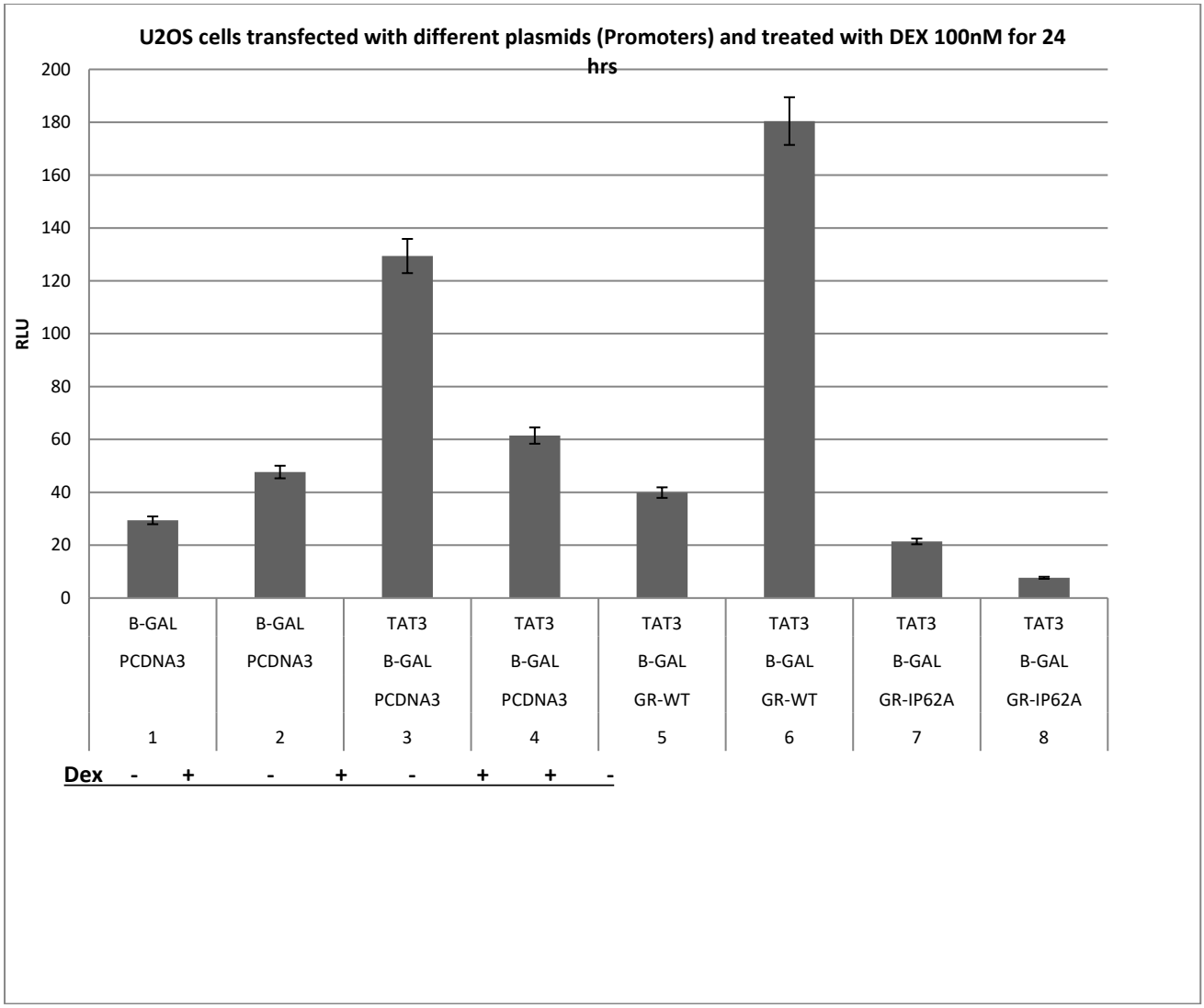

Figure 4 Relative Luciferase Units upon treatment with Steroids

The experiment from1-4 are carried out two times in duplicate and from 5-8 one time in duplicate. GR-I628A mutant kind gift from prof. Marija communications, Wild GR are from Rat species in prof. Marija lab plasmid bank. Cell lysate are undergone B-galactosidase assay first to ensure successful transfection and then luciferase assay , data shown represent luciferase assay units divided by related results of B-galactosidase assay.

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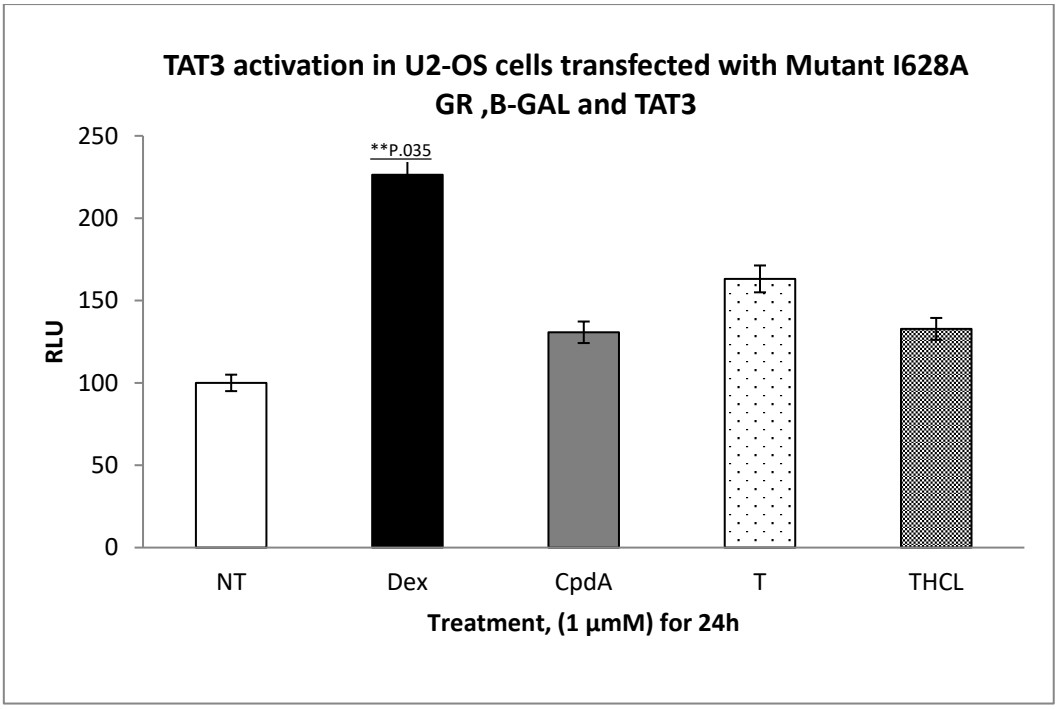

Figure 5 Reveal Relative Luciferase Units upon treatment with different compounds.

U-2 OS cells (Human Bone Osteosarcoma Epithelial Cells) transfected with different plasmids (Mutant I628A GR ,B-GAL and TAT3) and treated with 1 µM DEX, CPDA, T and TH for 24 hrs. Luciferase reporter assay activity was measured using Luminescence and Omega software.

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