CHARACTERIZATION AND SYNTHESIS OF NEW MODEL OF DERIVATIVE COLONAZEPAM AND CLINICAL TRIAL TO INSPECTION OF ADVERSE EFFECT IN MALE MICE

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ABSTRACT

Prepare new derivatives 5-(3-Fluoro-biphenyl-2-yl)-7-nitro-1,3-dihydro-benzo[e][1,4]diazepin-2-ol (88)and 5-Nitro-2'-(7-nitro-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-5-yl)-biphenyl-3-carboxylic acid (89)compound from clonazepam and identified by spectrum of 13C-NMR and showed spectrum 1H-NMR. Clonazepam has massive adverse effect on prostate, lung, liver, kidney and spleen 3- new derivatives 5-(3-Fluoro-biphenyl-2-yl)-7-nitro-1,3-dihydro-benzo[e][1,4]diazepin-2-ol (88) has limited effect and there is no clear changes in most organs studies. 4-5-Nitro-2'-(7-nitro-2-oxo-2,3-dihydro-1Hbenzo[e][1,4]diazepin-5-yl)-biphenyl-3-carboxylic acid (89)compound has significant adverse effect but still less potent than clonazepam.

OBJECTIVE

To characterization and synthesis of new drugs related to clonazepam as well as to overcome massive adverse effect of clonazepam and increase the potency and half live of drugs.

Key words: Adverse effect, New generation, Reproductive damage.

1.INTRODUCTION

1.1 Background

Infertility is one of the major problems notable via some humanism around the world and the male counterpart contributes half of the infertility cases [1]. Agree to USA. Androgens lack in men may appears signs such as shortage libido, erectile dysfunction and azospermia [2]. The diagnostic trial can be take place from the history of treatment used, physical examination and of course, semen biochemical analyze [3]. According to [4] sperm characteristics estimated has been highly substantial in reproductive research. Clonazepam is a long-acting that is used as an anticonvulsant and for anxiolytic, sedation, and muscle relaxant. clonazepam is one of the major popular benzodiazepines used for anxiety [5, 6]. The severity of clonazepam induced disadvantages and be carefully to adverse effects when prescribing this drug [5]. Newly report found that clonazepam derivative's act as Ca channels blocker as they can output complete discouragement of voltage-dependent Ca uptake [7]. Other propose that the Na+/Ca2+ exchange carrier in mitochondria may be a public receptor for clonazepam and calcium channel blockers [8]. The endocrine observation of Leydig cell steroidogenic activity by luteinizing hormone (LH) or follicle-releasing hormone (FSH) has been exerted through their respective receptors coupled to the Ca2+ mediated signaling pathway [9, 10]. Calcium ion is implicated in various cellular functions in both germ cells and somatic cells in the testis, specially, potentiate the reply to pituitary - hypothalamic hormones and local regulators in genital tracts [11]. Investigator have given massive awareness to studies of clonazepam derivatives for its assets of medical and biological effectiveness, antitumor drugs treatment of schistosomal, treatment-HIV, Bradykinin blocking, anti-arrhythmic agents and antimalarial [12, 13].

Recent studies have shown that some BDZs compounds have been synthesized and the results have presented the important role of the drugs as therapeutic drugs used specifically as anti-hepatitis B virus [14, 15]. Finally clonazepam derivatives compound 88,89 have been synthesis to evaluate adverse effect on reproductive function and body system but may be very slightly in 94 compound.
2. MATERIALS AND METHODS:

2.1 Instrumentation

Melting points are uncorrected and were measured on a Büchi melting point apparatus B-545 (BüchiLabortechnik AG, Switzerland). NMR data were obtained on 400 and 600 MHz (1H) and 150.91 MHz (13C) spectrometers (Avance III, Bruker, Germany) with TMS as internal standard and on the δ scale in ppm. Heteronuclear assignments were verified by 1H, 13C HMBC and 1H, 13C HSQC NMR experiments. Micro-analytical data were obtained with a Vario, Elemental analyzer (Shimadzu, Japan). Analytical silica gel TLC plates 60F254 were purchased from Merck. All reagents were obtained from commercial suppliers and were used without further purification.[16]

General procedure for the synthesis of the biaryl derivatives of clonazepam via Suzuki Cross-coupling reaction (88,89).

To a solution of clonazepam (3) (60 mg, 0.20 mmol) in mixture of chloroform (10 mL) with MeOH (5 mL), aryl boronic acid (0.2 mmol) was added, then the mixture was stirred for 15 min at suitable temperature then adding Pd(0)(PPh3)4 (100 mg, 5% mmol) and aqueous solution of 2 M sodium carbonate (5 mL). The mixture was heated under reflux for 12-14 h. After cooling phase, water (5 mL) was added and the mixture was partitioned with ethyl acetate (3 × 10 mL) and the combined organic extracts which were washed with aqueous solution of 5% Na2CO3 (3 × 10 mL), and dried with sodium sulfate and then evaporated in vacuum. The residue was then filtered on a short SiO2 column using hexane: ethyl acetate (3:2, v:v) as diluents to get the desired product(1) Graphical Abstract (Scheme 1)

| Comp No. | Formula | M.Wt | m.p. (°C) | Yield % | Color | Rf |
|----------|---------|------|-----------|----------|-------|----|
| 88       | C21H14FN3O3 | 375.36 | 252 | 56 | Light brown | 0.52 |

Figure 1. 5-(3-Fluoro-biphenyl-2-yl)-7-nitro-1,3-dihydro-benzo[e][1,4]diazepin-2-ol (88) according to[16].

| Comp No. | Formula | M.Wt | m.p. (°C) | Yield % | Colour | Rf |
|----------|---------|------|-----------|----------|--------|----|
| 89       | C22H14N4O7 | 446.37 | 258-260 | 65 | brown | 0.55 |

2.2 Animals

Mature male mice weighting range from 55-80 g body weight and two month age were purchased from laboratory animal colony of AlQassim University/ veterinary college. The animals were kept under normal conditions in plastic cages with [16] light nearly and feed on pellets contain milk power free of fat and water was given ad labium during all period of study.
2.3 Drugs
Clonazepam was purchased from neuraxpharm, Germany Company as standard.

2.4 Experimental Design
Twenty male mice were randomly divided randomly into four equal groups:
Group 1 as control. Second group: animal were given with clonazepam at dose (30mg/kg/orally three times weekly for 4 weeks. Group 3: mice given new drugs prepared named (89) at dose (30mg/kg/day B.W.) orally three times weekly for 4 weeks.
Group: mice were treated with new drugs (88) at dose 30mg/kg/TID three times weekly for 4 weeks via stomach tube. Finally the tissue organs were collected to evaluate of clonazepam and new drugs on body tissue organ.

3. Finding
General procedure for the synthesis of the biaryl derivatives of clonazepam via Suzuki–ross–coupling reaction according to[17]a-prepare 88 and 89 compound.

Figure 3. Refer to spectrum of (FT-IR) for 88 new compounds.

Figure 4. Showed the spectrum of 89 compound.
Figure 5. Showed spectrum 1H-NMR for 88 compounds.

Figure 6. Refer spectrum of 13C-NMR for 88 compounds.
Figure 7. Refer spectrum of HSQC of 88 compounds.

Figure 8. Spectrum of $^{1^\text{H}}\text{FT-IR}$ for 89 compounds.
General procedure for the synthesis of the biaryl derivatives of clonazepam via Suzuki cross–coupling reaction to prepare 88, 89 compound according to[18, 19]Suzuki Cross–coupling reaction has been used in the preparation of new clonazepam analogues. Thus, treatment of clonazepam (4),with the appropriate arylboronic acids (e.g.: 4-methylsulfanylphenyl-, 3-cyanophenyl-, 4-fluorophenyl-, 4-tri methylsilylphenyl-, 3,4-dimethoxyphenyl-, 5-nitro-3-carboxy phenyl boronic acid. The structure of compounds (88,89)were assigned on the basis of their 1H and 13C NMR which showed similar patterns of aliphatic proton and carbon atoms .The 1H NMR spectra of compound (88,89) were characterized by the presence of additional aromatic protons and carbon atoms, indicative for arylation of clonazepam backbone. The aromatic proton appeared at the region δ 6.62-8.67 ppm, the diazepine ring protons (CH2) appeared at δ7.32-7.56 ppm, the other aliphatic protons and substituents have been fully identified[20].

This procedure was prepared two compounds then many tests was done to record and named as new compound related with benzodiazepine group 1H-NMR 13C-NMR, according to action was classified as long-acting type.
4. Histopathological results.

**Figure 11.** Histopathological section of kidney of one animal treated with (89 compounds) expressed dilated Bowman’s capsule with 3G shows mononuclear cells aggregation around blood vessels and glomeruli which expressed dilated Bowman space (H and stain 400X).

**Figure 12:** Section in the kidney of one animal treated with (88 compound) shows no clear lesions (H and E stain 400X).

**Figure 13:** Kidney section of one animal treated with clonazepam shows inflammatory cells aggregation in the interstitial tissue with acute cellular degeneration of epithelial cells (H and E stain 400X).

**Figure 14:** Section in the kidney of one normal animal shows no clear lesions (H and E stain 400X).
Figure 15: Prostate section of one animal treated with (89 compound) shows vacuoles and desquamation of epithelial cells lining prostate (H and E stain 400X).

Figure 16: Section of prostate gland in one animal treated with (88 compound) expressed dilated shows no clear lesions (H and stain 400X).

Figure 17: Section in the prostate gland of one animal treated with clonazepam shows destruction of epithelial cells (H and stain 400X).

Figure 18: Section in the prostate of one animal treated with clonazepam shows abscess surrounded by pyogenic membrane (H and stain 400X).
Figure 19: Section of prostate of one animal treated with clonazepam shows abscess surrounded by pyogenic membrane infiltrated by neutrophils (H and E stain 100X).

Figure 20: Section in the liver of one animal treated with clonazepam shows granulomatous lesion consisting from aggregation of mononuclear cells in the liver parenchyma and mononuclear cells aggregation around congested blood vessels (H and E stain 100X).

Figure 21: Histopathological section of liver in one animal treated with (89 compound) shows mononuclear cells aggregation around blood vessels (H and E stain 400X).

Figure 22: Liver section of one normal animal shows no clear (H and E stain 400X).
**Figure 23**: Liver of one normal animal shows no clear (H and E stain 400X).

**Figure 24**: Section in the lung of one normal animal shows no clear (H and E stain 400X).

**Figure 25**: Pathological section in the lung of animal treated with clonazepam shows inflammatory cells in congested blood vessels and in the interstitial aggregation in the interstitial tissue with acute cellular degeneration of epithelial cells (H and stain 400X).

**Figure 26**: Section in the lung of one animal treated with (89 compounds) shows hyperplasia of lymphoid tissue around blood vessels (H and E stain 400X).
Figure 27: Histopathological section of lung shows thickening of alveolar septa with inflammatory cell infiltration 88 compounds.

Figure 28: Section in the spleen of one animal treated with (clonazepam) shows depletion of white pulp (H and E stain 400 X).

Figure 29: Section of spleen of one animal treated with (88 compound) shows moderate hyperplasia of white pulp (H and E stain 400 X).

Figure 30: There are only mild changes in spleen section in animal treated with 89 compounds (H and E stain 40 X).
5. Discussion, limitation and recommendation

General procedure for the synthesis of the biaryl derivatives of clonazepam via Suzuki cross-coupling reaction to prepare 88, 89 compound according to Suzuki Cross-coupling reaction has been used in the preparation of new clonazepam analogues. Thus, treatment of clonazepam (4), with the appropriate arylboronic acids (e.g.: 4-methylsulfanylphenyl-, 3-cyanophenyl-, 4-fluorophenyl-, 4-tri methylsilylphenyl-, 3,4-dimethoxyphenyl-, 5-nitro-3-carboxy phenyl boronic acid) The structure of compounds (88,89) were assigned on the basis of their 1H and 13C NMR which showed similar patterns of aliphatic proton and carbon atoms. The 1H NMR spectra of compound (88,89) were characterized by the presence of additional aromatic protons and carbon atoms, indicative for arylation of clonazepam backbone. The aromatic proton appeared at the region $\delta$ 6.62-8.67 ppm, the diazepine ring protons (CH$_2$) appeared at $\delta$ 7.32-7.56 ppm, the other aliphatic protons and substituents have been fully identified.

This procedure was prepared two compounds then many tests was done to record and named as new compound. The current results reveal prostate gland of one animal treatment with clonazepam appear destruction of epithelial cells and abscess surrounded by pyogenic membrane as well as pyogenic membrane infiltrated with neutrophils these result was agree with who indicated a high percent of abnormality sperms include both shape and size of the sperm head and tail, on the other hand recorded that new 88 compound substantiation improvement live sperm. Diazepam induces oxidative stress in goat epididymis sperms and as well as alter percent of active motility and viability of sperm that due to reduce the activity of mitochondria. Diazepam had antifertility effects on male rats through attenuating steroidogenesis and androgens production by diminish the pituitary gonadal axis hormones and the StAR gene expression through interfere with calcium ions our result also indicated. That mice receive clonazepam for long period of time shows inflammatory cells aggregation in the interstitial tissue with acute cellular degeneration of epithelial while animal receive new drugs specially 89 kidney of expressed dilated Bowman capsule with shows mononuclear cells aggregation around blood vessels and glomeruli which expressed dilated Bowman space, on the other aspect animal treated with (88 compound) appear there is no clear lesions.

The safety and efficacy of clonazepam in patients with renal failure has not been studied. Clonazepam and its metabolites are removed by the kidneys; to prevent further accumulation, admonition should be utilized when taken the drug to patients suffering from impaired renal function.

The researchers concluded that Rat receive diazepam at dose 124.5 mg/kg BW reveal severe situation of renal cortex. Large numbers of cells were likely to experience both reversible and irreversible cell damage. Hemorrhagic and congestion were also recorded commonly in renal cortex. Epithelial abrasion becomes a prominent histopathological filed in renal cortex. Furthermore, there are many hyper segmented and crampy glomerulus in renal cortex. This state can be due to mesangial cells defect cause membrane proliferative glomerulonephritis also glomerulosclerosis.
Diazepam cause hypotension and decrease blood flow to the kidney. This situation may lead to an ischemic condition of kidney which produce hypoxia in renal cortex cells[10][23]. In conclusion, diazepam treatment on rats for 28 days increased the levels of urea and creatinine in the urine compare to control group. Furthermore diazepam treatment caused histological damage in the kidneys of rats. Since test only done in 28 days it couldn’t analyze chronic effect of diazepam; thus, longer treatment is needed to get more comprehensive data.

Histopathological result recorded there is many harmful effect in the lung of animal treated with clonazepam can listed by inflammatory cells in congested blood vessels and in the interstitial aggregation in the interstitial tissue with acute cellular degeneration of epithelial cells as well as histopathological section of lung shows thickening of alveolar septa with inflammatory cells infiltration. Moreover the lung I of one animal treatment with (89 compound) reveals hyperplasia of lymphoid tissue around blood vessels. Improvement was highly significant in animal histopathological section of lung treated with 88 compound it showed thickening of alveolar septa with inflammatory cell infiltration.

Conclusions
1-prepare new derivatives 5-(3-Fluoro-biphenyl-2-yl)-7-nitro-1,3-dihydro-benzo[e][1,4]diazepin-2-ol (88)according to and 5-Nitro-2-'(7-nitro-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-5-yl)-biphenyl-3-carboxylic acid (89)compound from clonazepam identified by spectrum of 13C-NMR and showed spectrum 1H-NMR.
2-Clonazepam has massive adverse effect on prostate, lung, liver,kidney and spleen.
3-New derivatives 5-(3-Fluoro-biphenyl-2-yl)-7-nitro-1,3-dihydro-benzo[e][1,4]diazepin-2-ol (88) has limited effect and there is no clear changes in most organs studies.
4-5-Nitro-2-'(7-nitro-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-5-yl)-biphenyl-3-carboxylic acid (89)compound has significant adverse effect but still less potent than clonazepam.

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Conflict of Interest:
The authors confirm that there are no conflicts of interest to disclose.

REFERENCE

[1] Miyamoto, T., et al., 2012. Male infertility and its causes in human. Advances in urology.
[2] JOSE-MILLER, A.B., J.W. BOYDEN, and K.A. FREY, Infertility is defined as one year of frequent, unprotected intercourse during.
[3] Stahl, P.J., D.S. Stember, and M. Goldstein, 2012. Contemporary management of male infertility. Annual review of medicine. 63: p. 525-540.
[4] Schulte, R.T., et al., 2010. Sperm DNA damage in male infertility: etiologies, assays, and outcomes. Journal of assisted reproduction and genetics, 27(1): p. 3-12.
[5] Glowinski, J. and L.L. Iversen, 1966. Regional studies of catecholamines in the rat brain-I: the disposition of [3H] norepinephrine,[3H] dopamine and [3H] dopa in various regions of the brain. Journal of neurochemistry, 13(8): p. 655-669.
[6] Bellomo, R., J.A. Kellum, and C. Ronco, 2012. Acute kidney injury. The Lancet, 380(9843): p. 756-766.
[7] Taft, W.C. and R.J. DeLorenzo, 1984. Micromolar-affinity benzodiazepine receptors regulate voltage-sensitive calcium channels in nerve terminal preparations. Proceedings of the National Academy of Sciences, 81(10): p. 3118-3122.
[8] Matlib, M.A. and A. Schwartz, 1983. Selective effects of diltiazem, a benzothiazepine calcium channel blocker, and diazepam, and other benzodiazepines on the Na+-Ca2+ exchange carrier system of heart and brain mitochondria. Life sciences, 32(25): p. 2837-2842.
[9] Mendelson, C., M. Dufau, and K. Catt, 1975. Gonadotropin binding and stimulation of cyclic adenosine 3', 5'-monophosphate and testosterone production in isolated Leydig cells. Journal of Biological Chemistry, 250(22): p. 8818-8823.
[10] Sullivan, M.H. and B.A. Cooke, 1986. The role of Ca2+ in steroidogenesis in Leydig cells. Stimulation of intracellular free Ca2+ by lutropin (LH), lufiberin (LHRH) agonist and cyclic AMP. Biochemical journal, 236(1): p. 45-51.
[11] Tomic, M., et al., 1995. Calcium signaling in single rat Leydig cells. Endocrinology, 136(8): p. 3422-3429.
[12] Meneses, C.M., et al., 2012. Synthesis, Biological Evaluation, and Structure–activity Relationship of Clonazepam, Meclonazepam, and 1, 4-Benzodiazepine Compounds with Schistosomical Activity. Chemical biology & drug design, 79(6): p. 941-949.
[13] Cascade, E. and A.H. Kalafi, 2008. Use of benzodiazepines in the treatment of anxiety. Psychiatry (Edgmont), 5(9): p. 21.
[14] Ban, E.N., et al., 2008. The anticonvulsant and sedative properties of stems of Cissus quadrangularis in mice. African Journal of Pharmacy and Pharmacology, 2(3): p. 042-047.
[15] Salman, M.A.A.-H. and N.A. Abdul-Rida, 2016. Synthesis of new derivatives of aryliclonazepam via Suzuki Cross-coupling reaction. European Journal of Chemistry, 7(2): p. 152-155.
[16] Mahdi, K.M., N.A. Abdul-Reda, and N.A. Al-Masoudi, 2015. Exploration of new 3α-pregnenolone ester analogues via Mitsunobu reaction, their anti-HIV activity and molecular modeling study. European Journal of Chemistry, 6(11): p. 1-7.
[17] Taher, M.A. and Z.N. Anber, 2015. Effect of diazepam on the reproductive system in male rats. World J. Pharm. Pharm. Sci., 4: p. 60-78.

[18] Al-Masoudi, N.A., et al., 2015. New biaryl-chalcone derivatives of pregnenolone via Suzuki–Miyaura cross-coupling reaction. Synthesis, CYP17 hydroxylase inhibition activity, QSAR, and molecular docking study. Steroids, 101: p. 43-50.

[19] Jassim, A.M., et al., 2016. Study efficacy of new model of derivative clonazepam on hypnotic, sedative, blood hematolgy and evaluation reproductive function in male mice. Journal of Contemporary Medical Sciences, 2(8): p. 126-132.

[20] Miyaura, N. and A. Suzuki, 1995. Palladium-catalyzed cross-coupling reactions of organoboron compounds. Chemical reviews, 95(7): p. 2457-2483.

[21] Setiawan, P.G.M., W.A.S. Tunjung, and L. Nurhidayat, 2016. Effect of diazepam on kidney function and histological structure of white rat’s kidney. Journal of BIOLOGICAL RESEARCHES| Volume, 22(1).

[22] Ullah, I., et al., 2015. Prevalence of Tuberculosis in Kohat and.

[23] Khajuria, A., et al., 2014. Anesthetics attenuate ischemia–reperfusion induced renal injury: Effects and mechanisms. Acta Anaesthesiologica Taiwanica, 52(4): p. 176-184.