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Thiadiazolodiazepine Analogues as a New Class of Neuromuscular Blocking Agents: Synthesis, Biological Evaluation and Molecular Modeling Study.

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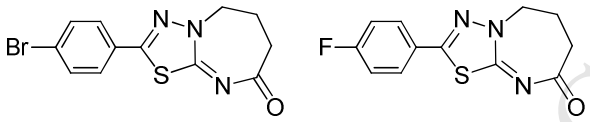

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onset time 3.0 min,
duration 70.0 min

(AAH1), **33**

onset time 10.0 min,
duration 110.0 min

Structures of the most active neuromuscular blocking compounds GS-53 (**30**), and AAH1 (**33**)

Thiadiazolodiazepine Analogues as a New Class of Neuromuscular Blocking Agents: Synthesis, Biological Evaluation and Molecular Modeling Study.[#]

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Abstract

The synthesis, biological evaluation and molecular modeling study of 6,7-dihydro-[1,3,4] thiadiazolo[3,2-*a*][1,3]diazepine analogues as new class of neuromuscular blocking agents are described. The new compounds act via competitive mechanism with ACh which could be reversed by the anticholinesterase - Physostigmine. Compounds GS-53 (**30**) and AAH1 (**33**) induced dose-dependent neuromuscular blockade with onset time of 3 and 10 min, ED_{50} 0.15 and 0.36 mmoles/kg i.p., respectively, in rats. Compound **30** proved to be as twice as potent as **33** with rapid onset and shorter duration ($P < 0.05$). Docking profile of **30** and **33** closely resembles HIE-124 (**3**), in α7β2 nAChR receptor. Molecular modeling analysis indicated that hydrogen bonding to Thr120 and Thr124 beside hydrophobic interactions play effective role incorporating the active ligands to nAChR. The obtained model could be useful for further development of new skeletal muscle relaxants.

Keywords: Synthesis, Thiadiazolodiazepine Analogues, Neuromuscular Blocking Agents, Molecular Modeling Study

[#] Part of the Patents EP 2 514 754 B1 and US 8,846,665 B2, ref. [16, 17]

1. Introduction

Neuromuscular blockade and anesthesia are essential for surgical procedures. The use of neuromuscular blockers or skeletal muscle relaxants will decrease the doses of the general anesthetics. Neuromuscular blockers are used to ease tracheal intubation and to suppress the patient's spontaneous breathing. Other uses of skeletal muscle relaxants include prevention of fractures during electroshock therapy, suppression of titanic convulsions and diagnosis of myasthenia gravis. The history of these drugs goes back to the use of curare, the alkaloid extract of *Chardrodendron tomentosum* which followed by the introduction of Atracurium besylate (**1**) and Succinylcholine chloride (**2**), Figure 1 [1-7]. Neuromuscular blockers can be classified according to their mechanism of action into competitive and depolarizing blockers. Most of the drugs currently in use are of the competitive type. Competitive neuromuscular blockers block the nicotinic acetylcholine receptor (nAChR) located postsynaptic on skeletal muscle membranes. They compete with acetylcholine and prevent its action on evoking muscular contraction and thus muscle relaxation ensues gradually. The depolarizing blockers mimic ACh in their ability to activate the postsynaptic nicotinic receptors on the skeletal muscles but differ from ACh in their ability to induce persistent depolarization of the skeletal muscles rendering them insensitive to any released ACh. The only drug of this group that is still in use is Succinylcholine (**2**) due to its rapid action and short duration (up to 5 minutes following bolus injections at 0.5-2 mg/kg i.v.), [2-4]. The competitive neuromuscular blockers are mostly used in medicine due to their antagonism by anticholinesterases e.g. Neostigmine, Pyridostigmine or Edrophonium. However, those drugs suffer from various side effects e.g. apneas, non-neuromuscular blockade, release of histamine with consequent hypotension, broncho-constriction and excessive mucus secretions, and headache as observed with **1** [5-7], stimulation of sympathetic autonomic ganglia, induction of tachycardia, elevation of the arterial pressure, increase in the intraocular pressure and induction of hyperkalemia as observed with **2** [2,3]. An ideal neuromuscular blocker should possess a rapid onset of action, reasonable duration, and rapid reversibility after ending its use together with freedom from non-neuromuscular blockade side effects. The majority of selective nicotinic acetylcholine receptor (nAChR) antagonists are natural products with marked complexity in size which makes them not suitable as lead compounds for drug discovery. To date, 17 nAChR subunits have been cloned, and among them five are of muscle-type. Development of selective agonists or antagonists may therefore result in new and potentially useful therapeutic agents [8, 9].

Recently, 6,7-dihydro-[1,3,4]thiadiazolo[3,2-*a*][1,3]diazepine analogues were synthesized as structure modification derivatives of the patented "HIE 124, **3**" [10-14]. The obtained compounds proved to possess short acting hypnotic activity in addition to *in vivo* potentiating effect toward the

known ultra-short acting hypnotic "Thiopental sodium" [15]. Some of the studied derivatives, at doses around 0.4 mmole/kg, showed respiratory depression to the tested animals as a sign of sever muscle relaxation. The use of lower doses of those derivatives led to the discovery of a new class of neuromuscular blocking compounds discussed in the present study. New structure modified derivatives were synthesized which showed competitive neuromuscular blocking activity, devoid of the disadvantages of the previous generations of muscle relaxants, with reasonable onset and intermediate duration of action which can be rapidly reversed by anticholinesterases. The obtained results contributed in part to the issuance of European and US patents [16, 17].

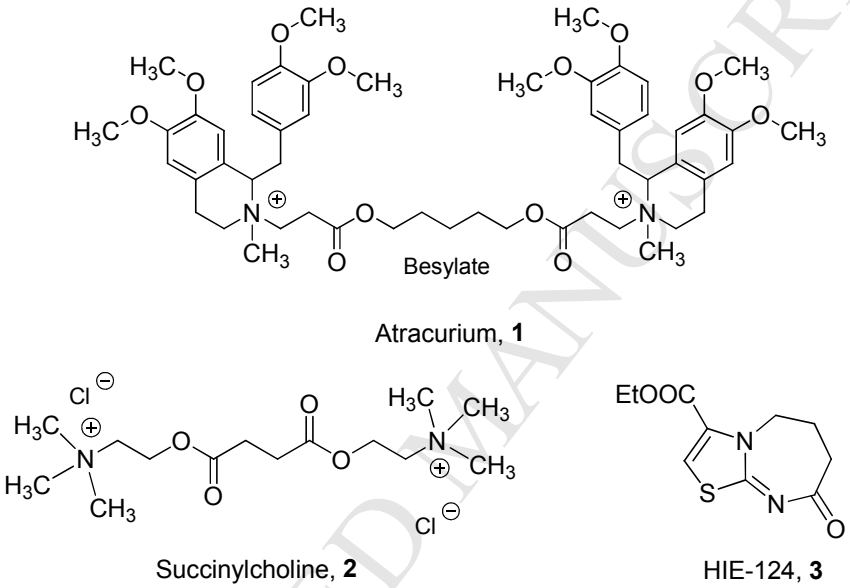


Figure 1: Structures of literature neuromuscular blocking agents (**1**, **2**), and the ultra-short acting hypnotic **3**.

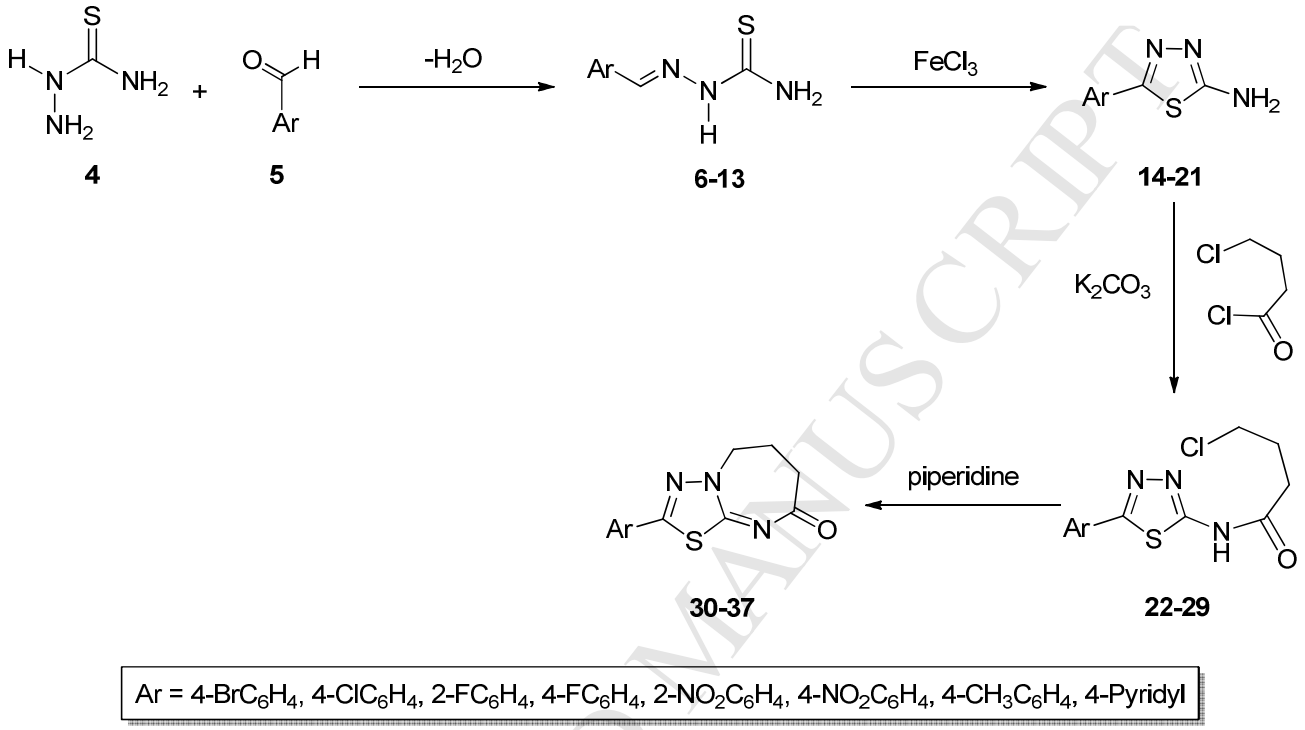
2. Results and Discussion

2.1 *Chemistry*

1,3,4-Thiadiazolo[3,2-*a*][1,3]diazepine as a heterocyclic system, usually obtained according to literature procedures [18,19]. Compounds of the present study (**30**-**37**) were synthesized according to the inventive method showed in Schemes 1 and 2. The proper aldehyde and thio-semicarbazide were mixed in alcohol with stirring to get the required thiosemicarbazones **6**-**13** which were suspended in $FeCl_3$ solution to get the required 2-amino-5-substituted-1,3,4-thiadiazole **14**-**21** [20]. Compounds **14**-**21** were acylated with 4-chlorobutyryl chloride in toluene at room temperature to afford 4-Chloro-*N*-(5-substituted-1,3,4-thiadiazol-2-yl)butanamide analogues **22**-**29**, which were purified by silica gel and neutral alumina chromatography. The butanamide analogues **22**-**29** were then heated under reflux with piperidine in toluene to give the cyclized derivatives 2-substituted-6,7-dihydro-thiazolo[3,2-*a*][1,3]diazepin-8(5*H*)-ones **30**-**37**, (Scheme 1). Structure elucidation of the new

synthesized intermediates and final products was attained by the aid of elemental analysis, ^{1}H, ^{13}C NMR, and Mass spectrometry.

Scheme 1: Synthesis of the target compounds **30-37**



2.2 Evaluation of the Neuromuscular Blocking Activity

The neuromuscular blocking activity of the test compounds **30-37** was performed by measuring its effect on rat tibialis muscle and the sciatic nerve as well as its effect in chicks using standard reported procedures [21, 22].

2.2.1. The neuromuscular blocking activity of test compounds on rat tibialis muscle electrically-induced twitches *and the sciatic nerve*

Each of the test compounds (**30-37**), Atracurium besylate (**1**), and Succinylcholine (**2**); as positive controls, or vehicles were administered (i.p.) at various doses. The effect of each dose on the rat tibialis muscle twitches response was measured as a percentage of the pre-drug amplitude. The ED_{50} and the dose that produced 90% of twitches depression were calculated for each drug. The response of the muscle to titanic stimulation was tested before administration of any drug and following 90% inhibition of the twitches. To investigate the reversibility of any neuromuscular block, the anticholinesterase Physostigmine was injected in a single bolus injection of 100 μg/kg. The onset time of reversal and the percentage of reversal were monitored and calculated, respectively. The

duration of each block was monitored. Only compounds GS-53 (**30**) and AAH1 (**33**) showed remarkable activity, the rest of the test compounds proved to be devoid of neuromuscular blocking activity. Administration of compounds **30** and **33** in various doses into the anaesthetized rats induced dose-dependent inhibitions of the twitches i.e. neuromuscular blockade on skeletal muscle relaxation. The ED_{50} were 0.15 and 0.36 mmoles/kg i.p., respectively. The corresponding values for the competitive neuromuscular blocker Atracurium besylate (**1**) and the depolarizing neuromuscular blocker Succinylcholine chloride (**2**) were 0.016 and 0.03 mmoles, respectively. The onset times of **30** and **33** were 3 and 10 min, with duration of 70 and 110 min; whereas those of **1** and **2** were 5 and 2 min, with duration of 30 and 20 min, respectively. Compound GS-53 (**30**) seemed to be twice as potent as AAH1 (**33**) ($P < 0.05$) with rapid onset and shorter duration ($P < 0.05$). GS-53 (**30**) blockade was completely reversed following administration of Physostigmine while that of AAH1 (**33**) was not. The potency of **30** seemed to be $1/10^{th}$ that of Atracurium (**1**) and 20% that of Succinylcholine (**2**). The duration of action of **30** seemed to be 2.3 times, whereas the duration of **33** was more than 3 times that of Atracurium (**1**), Table 1.

Table 1: Effects of GS-53 (**30**), AAH1 (**33**), Atracurium (**1**) and Succinylcholine (**2**) on the rat tibialis electrically-induced twitches together with the effects of tetanus and Physostigmine at a dose of 100 mg/kg i.p. (N = 4-8 animals).

***Parameter**	GS-53 **(30)**	AAH1 **(33)**	**Atracurium**	**Succinyl choline**
ED_{50} (mmole/kg i.p.)	0.15	0.36	0.016	0.03
Time of onset of Block (min)	3 ± 0.9	10 ± 2	5 ± 0.8	2 ± 0.1
Time of 50% Block (min)	7 ± 0.6	18 ± 3	8 ± 0.2	3 ± 0.1
Time of 90% Block (min)	10 ± 3	28 ± 2.7	12 ± 1.3	5 ± 0.2
Effect of Tetanus (30 Hz)	Non-Maintained	Non-Maintained	Non-Maintained	Well-Maintained
Effect of Physostigmine	100% Reversal	No Reversal	100% Reversal	No Reversal
Time of onset of Reversal (min)	1.0	-	2.0	-
Time for 100% Reversal (min)	6 ± 1.2	-	3 ± 0.4	-
Duration of the Block (min)	70 ± 12	110 ± 17	30 ± 6	20 ± 1.8

*The results reported were the mean±S.E., N = number of animals used. Significant differences between the various treatments were performed using paired or un-paired t-test. P values < 0.05 were considered significant.

2.2.2. The neuromuscular blocking activity of test compounds in chicks

The effects of the test compounds GS-53 (**30**) and AAH1 (**33**) together with those of the standard drugs Atracurium (**1**) and Succinylcholine (**2**) were administered to 4-day old chicks to confirm the mode of the neuromuscular blockade [22, 23]. For this purpose submaximal dose of each of the test compounds and the standards, as revealed in the rats tibialis muscle twitches studies, were

administered (i.p) to the chicks and the type of paralysis was noted. Compounds GS-53 (**30**, 0.15 mmoles/kg) and AAH_1 (**33**, 0.3 mmoles/kg) induced flaccid paralysis. The paralysis started with head movement, head drop, and then complete flaccid paralysis. The onset of head drops were 15 sec and 11 min, whereas the flaccid paralysis was 1 and 15 min, respectively following administration. Atracurcium (**1**, 0.016 mmole/kg) induced similar head drop after 5 min and flaccid paralysis of the chicks after 10 min. However, Succinylcholine (**2**, 0.03 mmole/kg) induced rapid spastic paralysis characterized by neck and limbs extension with rigidity of the muscles within 30 seconds. These experiments revealed similar mechanisms for both of **30** and **33** with the clear difference of the rapidity of onset in case of **30** ($P < 0.05$). The mechanism of action of both drugs seemed to be similar to that of **1**. The onset of action of **30** was more rapid than that of **1** and almost similar to that of **2** ($P < 0.05$), Table 2.

Table 2: Effects of GS-53 (**30**), AAH1 (**33**), Atracurium (**1**) and Succinylcholine (**2**) in chicks (N = 4-8 animals).

***Parameter**	GS-53 **(30)**	AAH1 **(33)**	**Atracurium**	**Succinyl choline**
ED_{50} (mmole/kg i.p.)	0.15	0.3	0.016	0.03
Onset of head drop (min)	0.25 ± 0.1	11 ± 2	5 ± 0.8	-
Onset of muscle relaxation (min)	1 ± 0.08	15 ± 1.9	10 ± 0.8	0.5 ± 0.02
Type of neuromuscular paralysis	Flaccid	Flaccid	Flaccid	Spastic
Mechanism of action	Competitive	Competitive	Competitive	Depolarizing

*The results reported were the mean ± S.E., N = number of animals used. Significant differences between the various treatments were performed using paired or un-paired t-test. P values < 0.05 were considered significant.

2.3 Mode of Action of the Test Compounds Induced Neuromuscular Blocking Activity

The experiments performed in this study both in rats and chicks clearly pointed to the neuromuscular blocking action of the two test compounds GS-53 (**30**) and AAH1 (**33**). The results obtained in rats following tetanus application suggest that both compounds (**30** and **33**) acted via competitive mechanism with acetyl choline released following electrical stimulation of the sciatic nerve to the postsynaptic nicotinic receptors on the tibialis muscle membrane. In presence of competitive blockers, tetanus is not well maintained. Such a competitive mechanism is supported by the studies in chicks in which both compounds induced head drop and flaccid paralysis of the limbs [22, 24]. The complete reversal of **30** induced blocks by the anticholinesterase-Physostigmine confirms the competitive mechanisms of action of this compound; while the effect of **33** was not reversed by the anticholinesterase which gives the impression that **33** induced neuromuscular blockade was not competitive in nature [21, 25, 26]. The LD_{50} value of compound GS-53 (**30**) was performed adopting

known procedures [27]. The calculated LD_{50} was found to be 195.0 mg/kg with 95% confidence limits of 185.25-204.75 mg/kg.

Structure activity correlation of the obtained results showed that the type of substituents on the phenyl ring attached to position 2- of the 1,3,4-thiadiazolo[3,2-*a*][1,3]diazepine nucleus affected the onset and duration times of the neuromuscular blocking activity. Introduction of 4-bromo or 4-fluoro function to the phenyl ring produced GS-53 (**30**) and AAH1 (**33**) with remarkable activity. The presence of 4-nitro (**35**) or 4-methyl (**36**) groups, moving those functions to position 2- (**32**, **34**), or the isosteric replacement of the phenyl ring with 4-pyridyl function (**37**) led to the complete loss of the neuromuscular blocking activity.

3. Molecular Modeling Study

3.1. Docking Study

Molecular modeling provides important data in the rational drug design; it can predict the bonding affinity, spatial orientation and total binding energy of the small molecule drug candidates to the active site of their target enzymes. AChE's active site is located on the bottom of a long and narrow gorge; acetylcholine and substrate guidance down the gorge is facilitated by cation–pinteractions with aromatic side-chains residues [28, 29]. In a valuable addition to the present research work, it was thought worthwhile to search for the binding mode of the active compounds into the nicotinic acetylcholine receptors (nAChRs) and hence rationalize the results of the *in vivo* experiments and compare them against the inactive compounds.

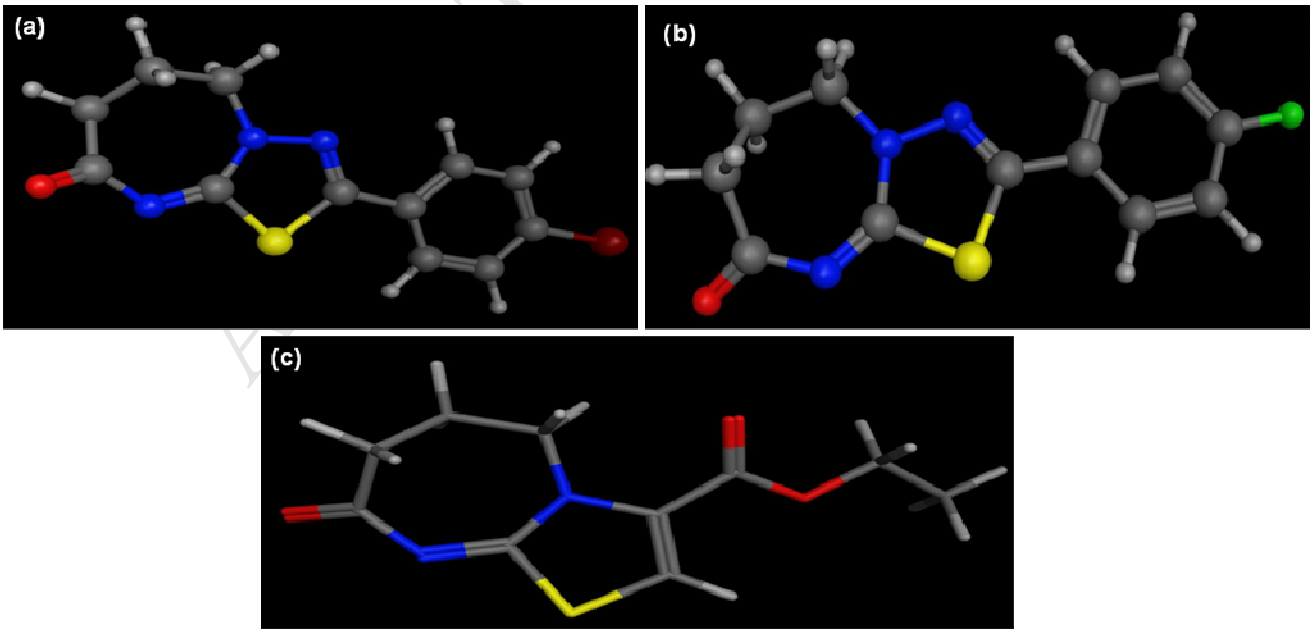


Figure 2: Lowest energy conformers of the active compounds (a) **30** and (b) **33** and (c) HIE-124 (**3**) with balls and cylinders rendering

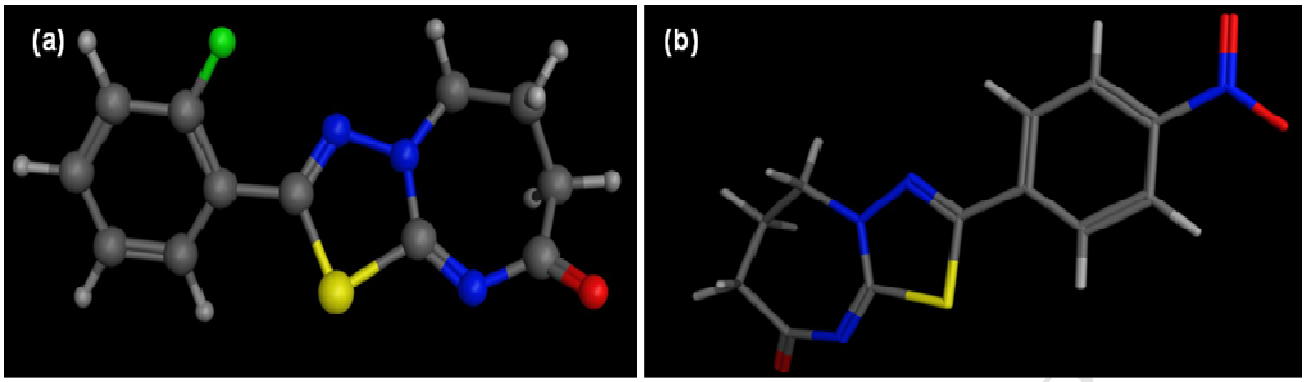


Figure 3: Lowest energy conformers of the inactive compounds (a) **32** and (b) **35** with balls and cylinders rendering.

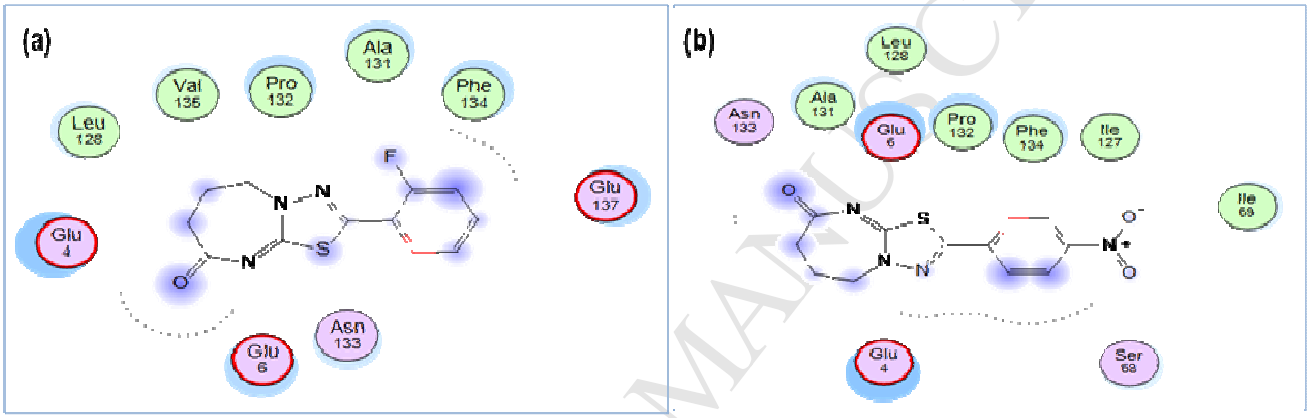


Figure 4: 2D Binding mode of Compounds (a) **32** and (b) **35** docked to the model of α7β2 nAChR receptor.

Docking of the most active muscle relaxant derivatives **30** and **33**, as well as the least active derivatives **32** and **35** in comparison to HIE-124 (**3**) into the α7β2 nAChR (PDB ID: 2MAW) have been performed [30]. Conformational analysis of the selected compounds by AM1 calculation produced the most stable; and least energetic structures which are favorable to start the docking experiment (Figures 2 and 3). The best calculated pose for **3** was assessed, docked and resulted in the recognition of two amino acid residues, Thr120 and Thr124 which bind *via* hydrogen bonds through interaction of its carbonyl oxygen by 41% and 36% respectively, showing pronounced high affinity (binding energy -11.32 kcal/mol), supplementary Figure a. Compound **30** binds to nAChR site *via* Thr124 by hydrogen bonding interaction, the same as **3**, *via* carbonyl oxygen (binding energy -10.25 kcal/mol), supplementary Figure b**,** while **33** binds with Thr124 residue by hydrogen bonding through its diazepine ring carbonyl oxygen by 22 % (binding energy -10.34 kcal/mol), supplementary Figure c. On that basis, it could be stated that **30** and **33** displayed their muscle relaxant action by nicotinic binding site antagonism at AchE receptor. When comparing **3** and the inactive compounds **32** and **35,** it is clearly seen that the compounds did not display any binding

residues at the used model (Figure 4 a,b); and expected to be deprived of any activity. Therefore, the docking study results, could explain the variance in the activity of the tested compounds although of their similar chemical structure.

3.2. Flexible Alignment

Ligands alignment at active site is a famous most used technique for the structural interpretation of protein–ligand interactions. A successful alignment is stated if the strain energy for every molecule is small, with similar shape and overlap of their aromatic moieties [31]. The aim of the alignment is to maximize the similarity between those ligands, after using their best found conformations. The similarity testing of the 3D structures among the most active compounds **30** and **33**, then the inactive **32** and **35**, followed by comparing the active **30** against the inactive **35** were done using MOE/MMFF94 flexible alignment which smoothly produced superposition of the compounds under study in minimal user bias [32]. 200 conformers of each compound were given then minimized by a distance-dependant dielectric model. The lower energy set of 100 was used for further results analysis. It was quite remarkable to note the similarity in flipping mode attitude and direction of the active candidates and the inactive analogues in Figure 5a,b respectively, while there was a strong deviation in alignment angle between compound **30** and **35** and therefore they could not have the same activity pattern (Figure 5c).

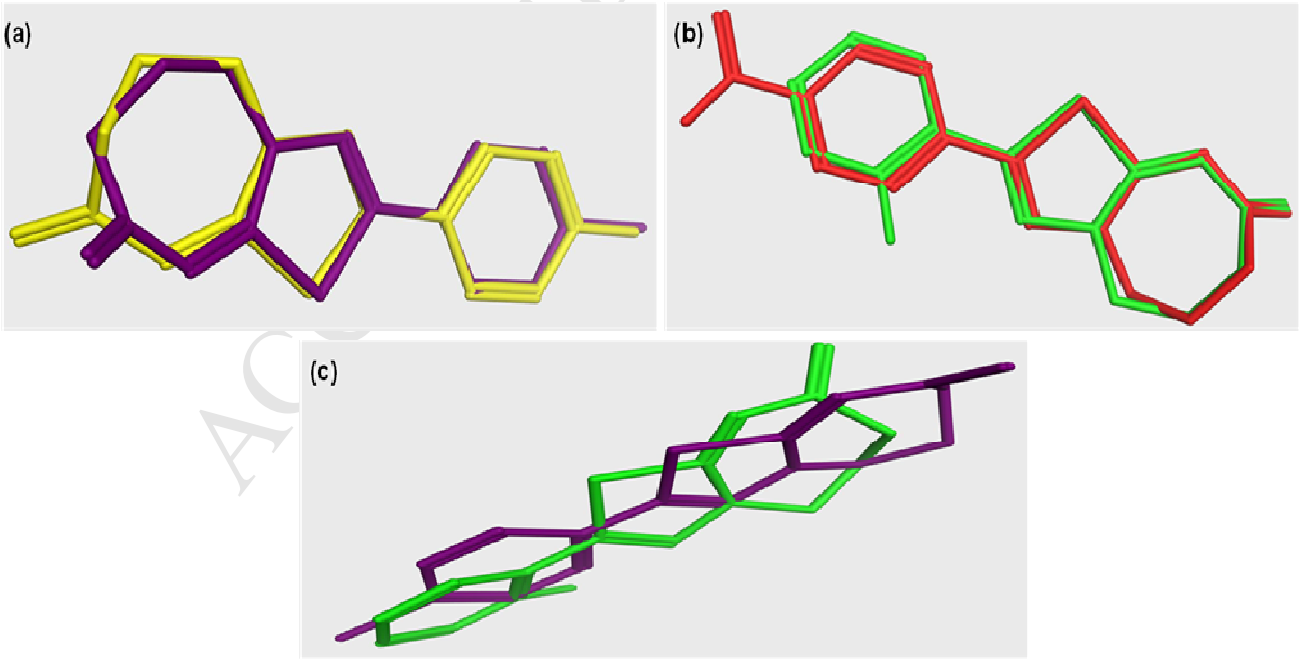


Figure 5: Flexible alignments of (a) the most active compounds **30** (violet) and **33** (yellow), (b) the least active compounds **32** (red) and **35** (green), (c) the most active compound **30** (violet)) against least active **35** (green).

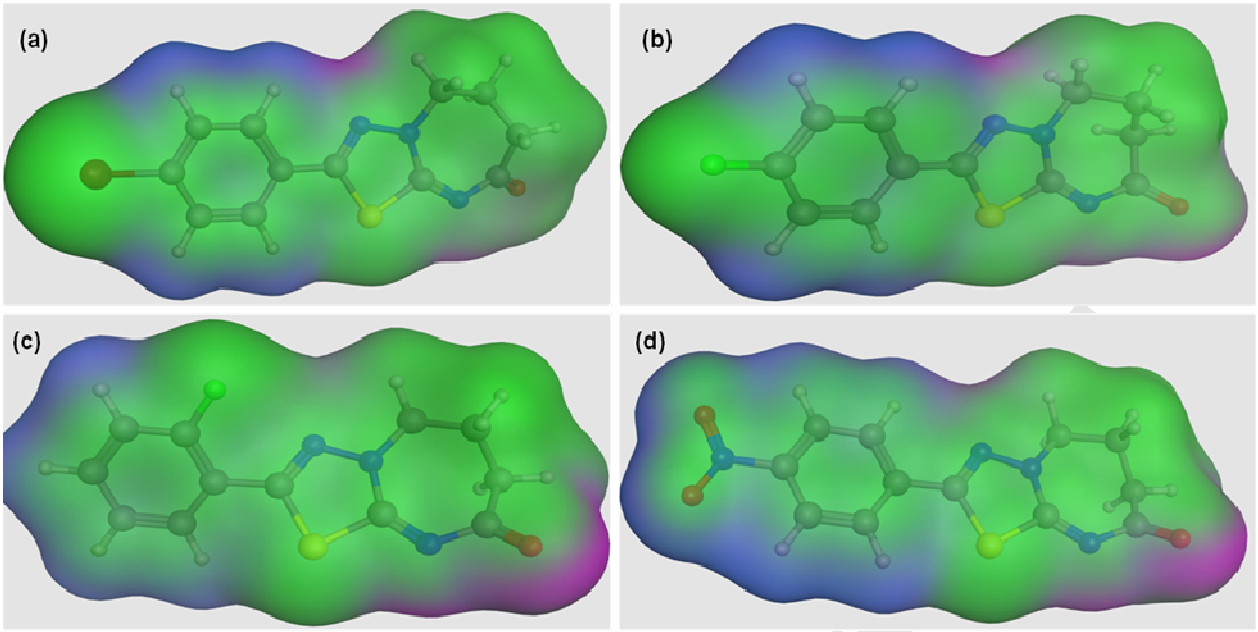


Figure 6: Surface map for compounds (a) **30**, (b) **33**, (upper), (c) **32** and (d) **35** (lower). Pink: hydrogen bond, blue: mild polar, green: hydrophobic regions.

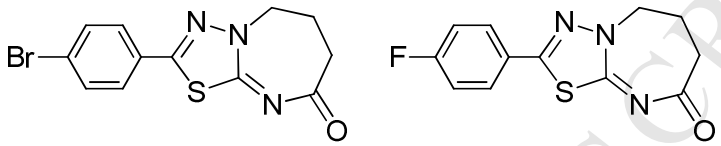
3.3. Surface Mapping

It could be noted that the difference in the aromatic substituent seems to have an impact on the interactions with the nAChRs, so in a valuable extension to this work, surface mapping comparing study between the most active **30** and **33**, against least active counterparts **32** and **35** was performed. Compounds **30** and **33** showed some hydrogen bond acceptor–donor region on the 4-carbonyl (hydrophilic region in red), *N*-1, non-polar area located at the thiazole ring (in green), in addition to the mild non polar center on the aryl moiety (Figure 6a,b). It can be recognized that higher lipophilic distribution at the 4- position of the active candidates (Figure 7a,b) than the inactive analogues which have less lipophilic at position 4- in comparison to position 2- (Figure 6c,d). There is a clear difference in the distribution of surface mapping among the most and the least active derivatives especially at 4- position of the aromatic substitution. These differences could play some role in the binding affinities of the compounds at the nAchR receptor site and hence the biological activity.

4. Conclusion

The biological evaluation of the new compounds revealed that compounds GS-53 (**29**) and AAH1 (**32**) are neuromuscular blockers (Figure 7). The obtained results clearly point to the discovery of a new group of muscle relaxants acting via competitive mechanism with ACh which could be reversed by the anticholinesterase Physostigmine. Compounds **30** and **33** induced dose-dependent neuromuscular blockade with onset time of 3 and 10 min, ED_{50} 0.15 and 0.36 mmoles/kg i.p., respectively, in rats. Compound **30** seemed to be as twice as potent as **33** ($P < 0.05$) with rapid onset

and shorter duration ($P < 0.05$). Thus, the discovery of the neuromuscular blocking action of the 2-(4-bromophenyl)-6,7-dihydro-[1,3,4]thiadiazolo[3,2-*a*][1,3]diazepin-8(5*H*)-one (GS-53, **30**) and 2-(4-fluorophenyl)-6,7-dihydro-[1,3,4]thiadiazolo[3,2-a][1,3]diazepin-8(5*H*)-one (AAH1, **33**) mark the era of introduction of a new group of intermediate acting skeletal muscle relaxants. Molecular docking of **30** and **33** into nAChR receptor provided the binding mode to be through hydrogen bonding interaction with Thr120 and Thr124. The obtained model could be useful for further development of new neuromuscular blocking agents.



(GS-53),**30** onset time 3.0 min, duration 70.0 min

(AAH1), **33** onset time 10.0 min, duration 110.0 min

Figure 7: Structures of the most active neuromuscular blocking compounds GS-53 (**30**) and AAH1 (**33**).

5. Experimental Part

Melting points (°C) were determined on Mettler FP80 melting point apparatus and are uncorrected. Microanalyses were performed on a Perkin-Elmer 240 elemental analyzer at the Central Research Laboratory, College of Pharmacy, King Saud University. All of the new compounds were analyzed for C, H and N and agreed with the proposed structures within ± 0.4% of the theoretical values. ^{1}H, ^{13}C-NMR spectra were recorded on a on Bruker 500 MHz FT spectrometer (the Central Research Laboratory, College of Pharmacy, King Saud University); chemical shifts are expressed in δ ppm with reference to TMS. Mass spectral (MS) data were obtained on a Perkin Elmer, Clarus 600 GC/MS and Joel JMS-AX 500 mass spectrometers. Thin layer chromatography was performed on pre-coated (0.25 mm) silica gel GF_{254} plates (E. Merck, Germany), compounds were detected with 254 nm UV lamp. Silica gel (60–230 mesh) was employed for routine column chromatography separations. Compounds **6**-**13**, **14**-**21** [20], **22**, **27**, **28**, **30**, **34**, and **36** [15] were previously reported. Adult male Wistar rats (250 g) and 4-day old chicks (50 g) were used to conduct the neuromuscular blocking activity evaluation. They were housed in cages and kept at a temperature of 20 ± 2°C and a relative humidity of 55 ± 5% with a light-dark cycle of 12 h. The animals were provided with Purina rodent's chow pellets supplied by Grain silos and Flour Mills Organization, Riyadh, Saudi Arabia

and had both food and water *ad libitum*. The chicks were bought from the local market in Riyadh. Male Wistar rats (250 g) were randomly divided into various groups (N = 4-8 animals). The animals were prepared following modifications of the reported method [21, 22]. All the procedures involving animals were conducted in conformity with the Institutional guidelines that are in compliance with National and International laws and policies.

5.1. Chemistry

*5.1.1. General procedure for the preparation of 4-Chloro-N-[5-(substituted phenyl)-1,3,4-thiadiazol-2-yl]butanamide (**22-29**)*

A mixture of 5-(substituted)-1,3,4-thiadiazol-2-amine (**14-21**, 0.04 mol), 4-chloro-butyryl chloride (11.3 g, 9.0 ml, 0.08 mol) and potassium carbonate (5.5 g, 0.04 mol) in toluene (100 ml) was heated under reflux for 4 h. The toluene was then evaporated under reduced pressure. The residue was then quenched with water, stirred, and filtered then purified by silica gel and neutral alumina chromatography. The obtained solid was washed, dried and recrystallized to give the required products **22-29**.

*5.1.1.1 N-(5-(4-Bromophenyl)-1,3,4-thiadiazol-2-yl)-4-chlorobutanamide (**22**).* White solid (Toluene), Yield 87%, m.p. 187-9^{o}C [15].

*5.1.1.2. 4-Chloro-N-[5-(4-chlorophenyl)-1,3,4-thiadiazol-2-yl]butanamide (**23**).* White solid (EtOH), Yield 75%, m.p. 125-7^{o}C, ^{1}H NMR ($CDCl_3$): δ 2.29-2.31 (m, 2H, -CH_2), 2.91 (t, *J* = 7.2 Hz, 2H, -CH_2), 3.72 (t, *J* = 7.2 Hz, 2H, -CH_2), 7.47-7.90 (dd, *J* = 8.4, 8.4 Hz, 4H, ArH), 11.83 (br s, 1H, NH). ^{13}C NMR: δ 27.6, 33.2, 44.0, 123.6, 128.4, 129.5, 132.3, 156.5, 161.7, 171.7. MS *m/z* (%): 316 (13.4, M^+), 318 (5.5, M+2). Calcd. for $C_{12}H_{11}Cl_2N_3OS$**.**

*5.1.1.3. 4-Chloro-N-[5-(2-fluorophenyl)-1,3,4-thiadiazol-2-yl]butanamide (**24**).* White gum, Yield 48%, ^{1}H NMR (DMSO-d_6): δ 1.65 (t, *J* = 6.5 Hz, 2H, -CH_2), 3.01 (t, *J* = 7.5 Hz, 2H,-CH_2), 3.58 (t, *J* = 6.5 Hz, 2H, -CH_2), 7.17-7.25 (m, 3H, ArH), 8.14-8.20 (m, 1H, ArH), 12.43 (s, 1H, NH). ^{13}C NMR: δ 28.9, 32.1, 45.3, 117.3, 125.1, 126.9, 131.6, 145.1, 162.3, 163.4, 172.1, 175.3. MS *m/z* (%): 299 (15.2, M^+), 301 (5.7, M+2). Calcd. for $C_{12}H_{11}ClFN_3OS$**.**

*5.1.1.4. 4-Chloro-N-[5-(4-fluorophenyl)-1,3,4-thiadiazol-2-yl]butanamide (**25**).* White solid (EtOH), Yield 81%, m.p. 143-5^{o}C, ^{1}H NMR ($CDCl_3$): δ 2.31-2.37 (m, 2H, -CH_2), 2.75 (t, *J* = 8.4 Hz, 2H,-CH_2), 4.82 (t, *J* = 8.4 Hz, 2H, -CH_2), 7.15-7.21 (m, 2H, ArH), 7.93-7.96 (m, 2H, ArH), 12.44 (s, 1H,

NH). ^{13}C NMR: δ 27.7, 33.3, 47.9, 116.4, 126.8, 129.3, 157.4, 162.9, 165.1, 173.7. MS *m/z* (%): 299 (18.6, M^+), 301 (6.7, M+2). Calcd. for $C_{12}H_{11}ClFN_3OS$

*5.1.1.5. 4-Chloro-N-[5-(2-nitrophenyl)-1,3,4-thiadiazol-2-yl]butanamide (**26**).* Yellowish white gum, Yield 57%, ^{1}H NMR (DMSO-d_6): δ 2.15 (t, *J* = 6.5 Hz, 2H, -CH_2), 2.32 (t, *J* = 7.5 Hz, 2H, -CH_2), 4.17 (t, *J* = 6.5 Hz, 2H, -CH_2), 7.83-7.73 (m, 2H, ArH), 8.09-8.07 (m, 1H, ArH), 8.88 (d, *J* = 8.5 Hz, 1H, ArH), 12.96 (s, 1H, NH). ^{13}C NMR: δ 28.2, 33.6, 45.8, 124.8, 126.6, 128.9, 132.0, 142.9, 147.9, 148.6, 165.2, 172.4. MS *m/z* (%): 326 (25.2, M^+), 328 (8.5, M+2). Calcd. for $C_{12}H_{11}ClN_4O_3S$.

*5.1.1.6. 4-Chloro-N-[5-(4-nitrophenyl)-1,3,4-thiadiazol-2-yl]butanamide (**27**).* White solid (EtOH), Yield 54%, m.p. 135-7^{o}C [15].

*5.1.1.7. 4-Chloro-N-[5-(p-tolyl)-1,3,4-thiadiazol-2-yl]butanamide (**28**).* White solid (EtOH), Yield 66%, m.p. 139-41^{o}C [15].

*5.1.1.8. 4-Chloro-N-[5-(pyridin-4-yl)-1,3,4-thiadiazol-2-yl]butanamide (**29**).* White solid (EtOH), Yield 59%, m.p. 111-4^{o}C, ^{1}H NMR (DMSO-d_6): δ 2.17-2.28 (m, 2H, -CH_2), 2.71 (t, *J* = 6.5 Hz, 2H, -CH_2), 4.16 (t, *J* = 6.5 Hz, 2H, -CH_2), 7.95-7.96 (m, 2H, ArH), 8.74-8.75 (m, 2H, ArH), 12.10 (s, 1H, NH). ^{13}C NMR: δ 17.9, 30.5, 37.1, 48.7, 120.8, 137.8, 150.9, 160.8, 174.8. MS *m/z* (%): 282 (15.7, M^+), 284 (5.1, M+2). Calcd. for $C_{11}H_{11}ClN_4OS$.

*5.1.2. General procedure for the preparation of 2-(Substituted phenyl)-6,7-dihydro-[1,3,4]thiadiazolo[3,2-a][1,3]diazepin-8(5H)-ones (**30**-**37**)*

A mixture of 4-chloro-*N*-[5-(substituted)-1,3,4-thiadiazol-2-yl]butanamide (**22**-**29**, 0.004 mol) and piperidine (0.7 g, 0.8 ml, 0.008 mol) in toluene (50 ml) was heated under reflux for 3 h. The reaction mixture was cooled, poured into water and stirred. Toluene was separated dried and evaporated to give a crude product which was purified by repeated silica gel and neutral alumina column chromatography eluting with EtOAc/hexane (50:50 v/v) and $CHCl_3$/hexane (80:20 v/v). The solid obtained was washed, dried and recrystallized to give the required products **30**-**37**.

*5.1.2.1. 2-(4-Bromophenyl)-6,7-dihydro-[1,3,4]thiadiazolo[3,2-a][1,3]diazepin-8(5H)-one (**30**).* White yellowish solid (EtOH), Yield 78%, m.p. 204-7^{o}C [15].

*5.1.2.2. 2-(4-Chlorophenyl)-6,7-dihydro-[1,3,4]thiadiazolo[3,2-a][1,3]diazepin-8(5H)-one (**31**).* Yellow solid (EtOH), Yield 64%, m.p. 127-4 ^{o}C, ^{1}H NMR (DMSO-d_6): δ 2.21-2.24 (m, 2H, -CH_2), 2.65 (t, *J* = 7.5 Hz, 2H, -CH_2), 4.13 (t, *J* = 7.5 Hz, 2H, -CH_2), 7.60-8.00 (dd, *J* = 8.5, 8.5 Hz, 4H,

ArH). ^{13}C NMR: δ 17.7, 30.7, 47.8, 128.7, 128.9, 129.4, 135.3, 157.4, 161.2, 174.2. MS *m/z* (%): 279 (10.4, M^+), 281 (3.6, M+2). Calcd. for $C_{12}H_{10}ClN_3OS$**.**

5.1.2.3. 2-(2-Fluorophenyl)-6,7-dihydro-[1,3,4]thiadiazolo[3,2-a][1,3]diazepin-8(5H)-one ***(32)****.* White solid ($CHCl_3$/Hex), Yield 91.2%, m.p. 88-90 ^{o}C, ^{1}H NMR (DMSO-d_6): δ 2.39 (t, *J* = 6.5 Hz, 2H, -CH_2), 2.45 (t, *J*=6.5 Hz, 2H, -CH_2), 4.02 (t, *J*=8.5 Hz, 2H, -CH_2), 7.37-7.46 (m, 3H, ArH), 8.34 (d, *J* = 8.5 Hz, 1H, ArH). ^{13}C NMR: δ 17.4, 31.3, 44.8, 116.2, 124.9, 127.8, 131.7, 144.4, 160.2, 162.2, 162.9, 172.5. MS *m/z* (%): 263 (18.0, M^+). Calcd. for $C_{12}H_{10}FN_3OS$.

5.1.2.4. 2-(4-Fluorophenyl)-6,7-dihydro-[1,3,4]thiadiazolo[3,2-a][1,3]diazepin-8(5H)-one ***(33)****.* White solid (EtOH), Yield 51%, m.p. 180-2 ^{o}C, ^{1}H NMR ($CDCl_3$): δ 2.21-2.24 (m, 2H, -CH_2), 2.67-2.70 (m, 2H, -CH_2), 4.11-4.14 (m, 2H, -CH_2), 7.37-7.41 (m, 2H, ArH), 8.02-8.05 (m, 2H, ArH). ^{13}C NMR: δ 17.7, 30.7, 47.7, 116.5, 129.4, 157.2, 161.3, 162.4, 164.4, 174.1. MS *m/z* (%): 263 (22.0, M^+). Calcd. for $C_{12}H_{10}FN_3OS$.

5.1.2.5. 2-(2-Nitrophenyl)-6,7-dihydro-[1,3,4]thiadiazolo[3,2-a][1,3]diazepin-8(5H)-one ***(34)****.* White yellowish solid ($CHCl_3$/Hex), Yield 72%, m.p. 108-7^{o}C [15].

5.1.2.6. 2-(4-Nitrophenyl)-6,7-dihydro-[1,3,4]thiadiazolo[3,2-a][1,3]diazepin-8(5H)-one ***(35)****.* Yellowish browen solid (EtOH), Yield 70%, m.p. 154-6 ^{o}C, ^{1}H NMR (DMSO-d_6): δ 2.28 (t, 2H, *J* = 15.5 Hz, CH_2), 2.72 (t, 2H, *J* = 14.5 Hz, CH_2), 4.16 (t, 2H, *J* = 15.0 Hz, CH_2), 8.28 (d, 2H, *J* = 7.0 Hz, Ar-H), 8.36 (d, 2H, *J* = 7.5 Hz, Ar-H). ^{13}C NMR: δ 21.3, 30.7, 47.8, 124.5, 125.0, 126.9, 129.9, 141.3, 163.7, 174.1. MS *m/z* (%): 290 (25.2, M^+). Calcd. for $C_{12}H_{10}N4O_3S$**.**

5.1.2.7. 2-(p-Tolyl)-6,7-dihydro-[1,3,4]thiadiazolo[3,2-a][1,3]diazepin-8(5H)-one ***(36)****.* White yellowish solid (EtOH), Yield 73%, m.p. 166-8^{o}C [15].

5.1.2.8. 2-(pyridin-4-yl)-6,7-dihydro-[1,3,4]thiadiazolo[3,2-a][1,3]diazepin-8(5H)-ones ***(37)****.* Yellowish white solid (EtOH), Yield 85%, m.p. 145-7 ^{o}C. ^{1}H NMR (DMSO-d_6): δ 2.19-2.28 (m, 2H, -CH_2), 2.71 (t, *J* = 6.5 Hz, 2H, -CH_2), 4.16 (t, *J* = 6.5 Hz, 2H, -CH_2), 7.95-7.96 (m, 2H, ArH), 8.74-8.76 (m, 2H, ArH). ^{13}C NMR: δ 17.7, 30.7, 47.8, 62.9, 120.9, 136.9, 150.8, 160.3, 174.5. MS *m/z* (%): 246 (10.6, M^+). Calcd. for $C_{11}H_{10}N_4OS$.

5.2. Measurement of the Neuromuscular Blocking Activity in Rats

Male Wistar rats were prepared following modifications of the reported methods [21, 22]. The animals were anaesthetized using 25% urethane in water (w/v, 1.25 g/kg i.p). Each animal was laid

on its back and fixed to a surgical board and body temperature was maintained by an over-head lamp. An incision was made on the neck, the trachea was located freed and a cannula was inserted to supply artificial ventilation with room air delivered by Parvalux Electric Motors Ltd rodents' respirator (Wallisdown, Bournermouth, England) at a frequency of 90 breaths per minute and a tidal volume of 20 c.c./kg. Furthermore, an incision was made to remove the skin covering the tibialis and its neighbor muscles on the right leg. The membrane covering the tibialis muscle was removed and its tendon freed from the Knob in the middle of the foot. The tendon was tied with a strong thread, passed through a bully, attached to a force displacement transducer (10-100 g, Narco-Biosystems, Myograph F 2000; USA) which was connected to a Narco Physiograph via Universal Coupler Type 7173. An incision was made on the lateral right side of the animal just above the site of the sciatic nerve supplying the tibialis muscle. The sciatic nerve was freed and the nerve was secured between a platinum electrodes. A strong tie was made on the portion of the nerve nearer to the spinal cord to prevent a generalized electrical stimulation. The electrodes were attached to an electric stimulator (Science and Research Instruments, Ltd, Kent, U.K.). Both of the muscle and the nerve were covered with paraffin oil at 37°C to prevent dryness of the tissues. The Tibialis muscle twitches were induced using the following parameters: supramaximal voltage of 40 volts, at a frequency of 0.3 Hz and 0.5 msec duration. When tetanus was performed the frequency was increased to 30 Hz and the speed of recording was increased from 0.25 cm/sec to 1 cm/sec and stimulation was performed for 5-10 sec. The calibration system built in the transducer was used to measure the tension of the muscle. Changes in the muscle tension (twitches) were expressed as percentage change from the pre-drug values. Test compounds (**30**-**37**), standards or vehicles were administered intraperitoneally dissolved in dimethyl sulfoxide, or water as appropriate in various doses 1-100 mg/kg. The effect of each dose on the twitch response was calculated as a percentage of the pre-drug amplitude. The ED_{50} was calculated for each compound. Also the dose that produced 90% of twitches depression was calculated. The response of the muscle to titanic stimulation was tested before administration of test compounds and following 90% inhibition of the twitches. To investigate the reversibility of any block, the anticholinesterase-Physostigmine was injected in a single bolus injection of 100 μg/kg. The onset time of reversal and the percentage of reversal were monitored and calculated, respectively. The duration of each block was monitored. In all animals rectal temperature was maintained at 37±1°C, (Table 1).

5.3. Measurement of the Neuromuscular Blocking Activity in Chicks

The test compounds (**30**-**37**), the standard drugs Atracurium (**1**) and Succinylcholine (**2**) were administered to 4-day old chicks to confirm the mode of the neuromuscular blockade [22, 23]. Sub-

maximal dose of test compounds and the standards as revealed in the rat's studies were administered (i.p) to the chicks and the type of paralysis was noted. Intraperitoneal administration of test compounds in single dose of 0.15 mmoles/kg, induced flaccid paralysis of the chicks within one minute. The paralysis started with head movement and drop; then complete flaccid paralysis occurred. The onset of head drop was 15 sec whereas the flaccid paralysis was one minute following administration. **1** and **2** were used as positive controls (Table 2).

5.4. Determination of the Lethal Dose (LD_{50}) of GS-53 (**30**).

Male mice were divided into various groups. Compound **30** was administered in various doses ranging from 0.1-5 mmole/kg, intraperitoneal. Following treatments, the animals were observed for up to 6 hours continuously and were then kept under observation for 72 hours. All behavioral changes and death during the observation periods were recorded. The percentage of death at each dose level was then calculated, converted to probits and the LD_{50} values were calculated as outlined [27].

5.5. Molecular Modeling Methodology

Conformational analysis of the selected compounds classified as active muscle relaxant compounds **30** and **33** in addition to the least active counterparts **32** and **35** , together with HIE124 (**3**) as a standard were performed using MMX force field method preceded by AM1 calculation as implied in MOE 2009.10. The model of the nicotinic AchE receptor was used for docking onto the active site of nAChR derived from three-dimensional structure of the enzyme complex (PDB ID: 2MAW), [30]. Three-dimensional structures of the representatives selected thiadiazolo-diazepine derivatives, in their neutral forms were built using the MOE of Chemical Computing Group Inc software. The lowest energy conformers in their 'global-minima' were docked.' The energy-minimized structures were used for molecular modeling studies. Ligand structures were built with MOE and minimized using the MMFF94x force field until an RMSD gradient of 0.05 kcal/mol_A was reached. For every ligand, energy minimizations were done by 1000 steps of steepest descent, followed by conjugate gradient minimization to an RMSD energy gradient of 0.01 kcal/mol_A. The alpha triangle placement method and the London dG scoring method were selected for docking experiments. 300 results for each ligand were generated, excluding the results having RMSD value >3 [32]. The best scored result of the remaining conformations for each ligand was further analyzed. The studied compounds were subjected to flexible alignment. Their geometry was optimized using the MMFF94 forcefield followed by a flexible alignment using systematic conformational search [32, 33]. Surface

mapping calculations were performed using 'Molecular Operating Environment' software (MOE of Chemical Computing Group Inc., on a Core i7, 2.7 GHz workstation).

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Captions

Table 1: Effects of GS-53 (**30**), AAH1 (**33**), Atracurium (**1**) and Succinylcholine (**2**) on the rat tibialis electrically-induced twitches together with the effects of tetanus and Physostigmine at a dose of 100 mg/kg i.p. (N = 4-8 animals).

Table 2: Effects of GS-53 (**30**), AAH1 (**33**), Atracurium (**1**) and Succinylcholine (**2**) in chicks (N = 4-8 animals).

Figure 1: Structures of literature neuromuscular blocking agents (**1**, **2**), and the ultra-short acting hypnotic **3**.

Figure 2: Lowest energy conformers of the active compounds (a) **30** and (b) **33** and (c) HIE-124 (**3**) with balls and cylinders rendering

Figure 3: Lowest energy conformers of the inactive compounds (a) **32** and (b) **35** with balls and cylinders rendering.

Figure 4: 2D Binding mode of Compounds (a) **32** and (b) **35** docked to the model of α7β2 nAChR receptor.

Figure 5: Flexible alignments of (a) the most active compounds **30** (violet) and **33** (yellow), (b) the least active compounds **32** (red) and **35** (green), (c) the most active compound **30** (violet)) against least active **35** (green).

Figure 6: Surface map for compounds (a) **30**, (b) **33**, (upper), (c) **32** and (d) **35** (lower). Pink: hydrogen bond, blue: mild polar, green: hydrophobic regions.

Figure 7: Structures of the most active neuromuscular blocking compounds GS-53 (**30**) and AAH1 (**33**).

Scheme 1: Synthesis of the target compounds **30-37**

Research Highlights

- Synthesis of 6,7-dihydro-[1,3,4]thiadiazolo[3,2-*a*][1,3]diazepines
- Compounds GS-53 (**30**) and AAH1 (**33**) induced neuromuscular blockade
- Compound **30** proved to be as twice as potent as **33** with rapid onset and shorter duration
- Recognition with Thr120 and Thr124 at α7β2 nAChR receptor is essential for activity