Journal of Applied Sciences

ISSN 1812-5654

science alert

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Journal of Applied Sciences 8 (1): 158-162, 2008
ISSN 1812-5654

Synthesis of Dihydropyridine Analogues for Sperm Immobilizing Activity

[1]H.R. Sadeghipour Roodsari, [2]M. Amini, [1]Z. Naghibi Harat, [2]P. Daneshgar, [2]M. Vosooghi and [2]A. Shafiee
[1]Department of Physiology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[2]Department of Medicinal Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran

Abstract: In the present study, the activity of seven newly synthesized dihydropyridine analogues on the motility of sperm were determined and compared to nifedipine activity that was used as standard. Sperm motility reduced value for test compounds 6a-g shows a gradual increase proportional to the size elongation of alkyl ester groups. Consequently the size of alkyl is important in the activity of test compounds and finally increase in the lipophil size of hydrocarbon's ester (R_1) is inversely related to the activity of the synthetic compounds. As a result, the methyl ester of the test compounds with 50% of nifedipine activity (in two hours group) is the most active test compound.

Key words: Acrosomal reaction, sperm motility, male contraception, calcium channel blocker, calcium antagonist

INTRODUCTION

Sperm motility plays an important role in normal fertilization process of mammalian. Successful *in vitro*-oocyte fertilization by artificial insemination technique requires sufficient sperm motility (Yanagimachi, 1966). Role of external factors on regulation of sperm motility has been studied extensively. Ionized calcium ion stimulates sperm motility in some studies (Fakih *et al*., 1986; Lornage *et al*., 1983), meanwhile lack and/or inhibition of activity have been observed and reported by others (Hong *et al*., 1985; Umeyama *et al*., 1986). Versatility of mechanisms involved and/or multiple intracellular sites activity of calcium ions, as well as variation in experimental design could be mentioned as the underlying cause of controversial data and results observed. In order to elucidate the role of ionized calcium in regulation of human sperm motility, an *in vitro* study was conducted to evaluate the activity of exogenous calcium ion, EDTA and diltiazem on mean velocity and percent motility of human sperms (Aaberg *et al*., 1989). The results indicated that a minimum level of extracellular ionized calcium is essential for normal sperm motility and also suggests that regulation of sperm motility are more dependent on the intracellular translocation of calcium ions than the extracellular ionized calcium concentration. A reversible male infertility episode after administration of Calcium Channel Blocker (CCB) to control hypertension has been reported to be responsible for failures in an IVF trial case (Benoff *et al*., 1994). Insertion of lipophilic calcium ion antagonists into the lipid bilayer of the sperm plasma membrane has been proposed as a potential mechanism for inhibition of sperm fertilization.

The possible role of L-type Ca^{2+} channels on the acrosomal reaction of human spermatozoa has also been investigated. The results demonstrated that acrosomal reaction of human spermatozoa is highly associated with L-type Ca^{2+} channels and is mainly mediated by calcium influx through α-1H-T-type Ca^{2+} channels (Weon-Young *et al*., 2000). Diltiazem and Nifedipine are both an L-type calcium channel blocker, that when used, the first one cause a drastic and irreversible inhibitory activity of sperm motility *in vitro* along with significant reduction of sperm viability and the second demonstrated acrosomal inhibitory activity (Wood *et al*., 2003). The efficacy of dihydropyridine compounds as inhibitors of acrosomal reaction and their potential contraceptive activity has been investigated by (Kirkman-Brown *et al*., 2003). The subsequent studies revealed acrosomal reaction is the result of progesterone induction and consequently, Nifedipine inhibits progesterone-induced acrosomal reaction in human sperm. In the present study, the activity of seven newly synthesized dihydropyridine analogues on the motility of sperm were determined and compared to nifedipine activity that was used as standard.

Corresponding Author: M. Vosooghi, Department of Medicinal Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran Tel: 98+21 66406757 Fax: 98+21 66461178

MATERIALS AND METHODS

The candidate compounds were synthesized and evaluated in the department of Medicinal Chemistry and physiology faculties of pharmacy and Medicine respectively, during Dec. 2005- Apr. 2007.

Melting points were determined using a kofler hot stage apparatus and are uncorrected. ^{1}HNMR spectra were obtained on a Bruker FT-80 spectrometer (Bruker, Rheinstetten, Germany). TMS was used as internal standard. Mass spectra were measured with a Finnigan TSQ-70 spectrometer (Finnigan Mat, Breer, Germany) at 70 ev. Infrared spectra were acquired on a Nicolet 550-FT spectrometer (Madison, WI, USA).

Ham's F-10 medium were obtained from Sigma-Aldrich Chemie Gmbh (Deisenhofen, Germany).

Measurement of spermatozoa motility was determined according to the published methods (Gardner *et al*., 2006; Placzek *et al*., 1987).

Chemistry: Methyl, ethyl, i-propyl, i-butyl, t-butyl and benzyl acetoacetate were purchased from Sigma-Aldrich Chemie Gmbh (Deisenhofen, Germany). Cyclohexyl acetoacetate and 2-Chlorothiobenzamide (2) were prepared according to the recommended methods from cyclohexanol (Clemens and Hyatt, 1985) and 2-chlorobenzonitrile (Fairfull *et al*., 1952) and the corresponding reagents respectively (Scheme 1).

Preparation of 4-chloromethyl-2 (2-chlorophenyl) thiazole (3): To a stirred solution of 1, 3-dichloroacetone (0.1 mole) in toluene (100 mL) was added 2-chlorothiobenzamide (2) (0.1 mole) and refluxed at 110°C for 3 h. The reaction was monitored by TLC. The solvent was removed under reduced pressure. To the residue, water (200 mL) was added and the precipitated material was filtered. The crystallization was carried out from methanol to give 3. m.p. 61-63°C, % yield: 68. ^{1}H NMR ($CDCl_3$): δ 4.78 (s, 2H, CH_2), 7.20 (s, 1H, H_5), 7.37-7.8 (m, 4H, phenyl and H_5 thiazole).

Preparation of 4-hydroxymethyl-2- (2-chlorophenyl) thiazole (4): To the compound 3 (10 g) was added a mixture solution of sulfuric acid (50%) and refluxed for 24 h. After cooling, the pH was neutralized with concentrated solution of sodium hydroxide, the precipitation was filtered and recrystallized from chloroform and petroleum ether. m.p. 87-88°C, % yield: 83. ^{1}H NMR ($CDCl_3$): δ 4.88 (s, 2H, CH_2), 5.40 (bs, 1H, OH), 7.06-7.90 (m, 5H, phenyl and H_5 thiazole). IR (KBr): ν 3300 (OH) cm^{-1}.

Preparation of 2- (2-chlorophenyl) thiazol-4-carboxaldehyde (5): To compound 4 (2 g) was added MnO_2 (6 g) in dichloro methane (50 mL) and stirred over night. The mixture was filtered on diatomaceous earth and the solvent removed under reduced pressure. The residue was crystallized from MeOH: H_2O to give aldehyde of 5. m.p. 58-60°C, yield: 70. ^{1}H NMR ($CDCl_3$): δ 7.2-7.7 (m, 3H), 8.15-8.36 (m, 2H), 10.14 (s, 1H, CHO), IR (KBr): ν 1697 (C = O) cm^{-1}.

Preparation of dimethyl 1,4-dihydro-2,6-dimethyl-4- (2-(2-chlorophenyl)thiazol-4-yl)-3,5-pyridinedicarboxylate (6a): To a solution of ammonium acetate (1 g) in methanol (20 mL) was added 5 (2 mmol) and methyl acetoacetate (4 mmol). The mixture was refluxed for 5 h. The solvent was removed under reduced pressure. To the residue was added water (10 mL) and extracted with ethyl acetate (2×30 mL), dried by sodium sulphate and removed under reduced pressure. The residue was crystallized in MeOH: H_2O to give compound 6. m.p. 194-195°C, yield: 48. ^{1}H NMR ($CDCl_3$): δ 2.34 (s, 6H, CH_3), 3.72 (s, 6H, OCH_3), 5.34 (s, 1H, H_4), 5.88 (bs, 1H, NH), 7.01 (s, 1H, H_5-thiazole), 7.20-7.50 (m, 3H, aromatic), 8.01-8.02 (m, 1H, aromatic), IR (KBr): ν 3329 (NH), 1700 cm^{-1} (C = O), MS m/z (%) 419 (100), 359 (30), 355 (7), 224 (30), 149 (8), 131 (30), 116 (22), 71 (14), 59 (49).

Preparation of diethyl 1,4-dihydro-2,6-dimethyl-4- (2-(2-chlorophenyl) thiazol-4-yl) -3,5-pyridine dicarboxylate (6b): Compound of 6b was prepared similar to 6a, m.p. 143-145°C, yield: 48. ^{1}H NMR ($CDCl_3$): δ 1.24 (t, J = 7 Hz, 6H, CH_3), 2.28 (s, 6H, CH_3), 4.15 (q, J = 7 Hz, 4H, CH_2), 5.32 (s. 1H, H_4), 6.27 (bs, 1H, NH), 7.05 (s, 1H, H_5-thiazole), 7.20-7.50 (m, 3H, aromatic), 8.01-8.15 (m, 1H, aromatic), IR (KBr): ν 3338 (NH), 1695 (C = O), MS m/z (%) 447 (15), 373 (29), 359 (15), 278 (15), 252 (86), 224 (100), 196 (30), 150 (20), 77 (15), 57 (30).

Preparation of di-isopropyl 1,4-dihydro-2,6-dimethyl-4- (2- (2 chlorophenyl) thiazol-4-yl) -3,5-pyridine dicarboxylate (6c): Compound of 6c was prepared similar to 6a, m.p. 145-146°C, yield: 47. ^{1}H NMR ($CDCl_3$): δ 1.03-1.29 (two d, J = 6.2 Hz, 12H, CH_3), 2.29 (s, 6H, CH_3), 4.95-5.11 (m, 2H, CH), 5.29 (s, 1H, H_4), 5.92 (bs, 1H, NH), 7.04 (s, 1H, H_5-thiazole), 7.37 (m, 3H, aromatic), 8.06-8.22 (m, 1H, aromatic), IR (KBr): ν 3354 (NH), 1694 (C = O), MS m/z (%) 475 (8), 446 (28), 386 (15), 373 (45), 345 (28), 280 (21), 251 (94), 195 (100), 148 (42), 105 (21), 56 (37).

Preparation of di-isobuthyl 1,4-dihydro-2,6-dimethyl-4- (2- (2-chlorophenyl) thiazol-4-yl) -3,5-pyridine dicarboxylate (6d): Compound of 6d was prepared similar

to 6a, m.p. 101-102°C, yield: 33. ^{1}H NMR ($CDCl_3$): δ 0.91 (d, J = 6.6 Hz, 12H, CH_3), 1.93 (m, 2H, CH (CH_3)$_2$), 2.2 (s, 6H, CH_3), 3.89 (d, J = 6.2 Hz, 4H, OCH_2), 5.38 (s, 1H, H_4), 6.23 (bs, 1H, NH), 7.05 (s, 1H, H_5-thiazole), 7.32 (m, 3H, aromatic), 8.1 (m, 1H, aromatic), IR (KBr): ν 3339 (NH), 1690 (C = O), MS m/z (%) 503 (9), 501 (15), 427 (15), 400 (22), 343 (15), 307 (36), 251 (15), 194 (43), 148 (15), 105 (5), 66 (15), 56 (100).

Preparation of di-tertiarybutyl 1,4-dihydro-2,6-dimethyl-4- (2- (2-chlorophenyl) thiazol-4-yl) -3,5-pyridine dicarboxylate (6e): Compound of 6e was prepared similar to 6a, m.p. 163-165°C, yield: 22. ^{1}H NMR ($CDCl_3$): δ 1.45 (s, 18H, t-Bu), 2.28 (s, 6H, CH_3), 5.32 (s, 1H, H_4), 5.75 (bs, 1H, NH), 7.09 (s, 1H, H_5-thiazole), 7.25 (m, 3H, aromatic), 8.01-8.15 (m, 1H, aromatic), IR (KBr) ν: 3338 (NH), 1691 (C = O), MS m/z (%) 503 (9), 501 (20), 430 (8), 402 (15), 399 (43), 345 (15), 306 (87), 251 (21), 194 (100), 150 (21), 128 (14), 56 (87).

Preparation of di-benzyl 1,4-dihydro-2,6-dimethyl-4- (2-(2- chlorophenyl) thiazol-4-yl) -3,5-pyridine dicarboxylate (6f): Compound of 6f was prepared similar to 6a, m.p. 117-118°C, yield: 28. ^{1}H NMR ($CDCl_3$): δ 2.33 (s, 6H, CH_3), 5.15 (m, 4H, CH_2), 5.35 (s, 1H, H_4), 5.96 (s, 1H, NH), 6.66 (bs, 1H, H_5-thiazole), 7.05-7.45 (m, 13H, aromatic), 8.02-8.22 (m, 1H, aromatic), IR (KBr): ν 3308 (NH), 1695 (C = O), MS m/z (%) 571 (15), 503 (58), 435 (29), 427 (86), 401 (94), 327 (37), 307 (100), 253 (29), 196 (65), 195 (100), 150 (29), 91.7 (58).

Preparation of di-cyclohexyl 1,4-dihydro-2,6-dimethyl-4- (2- (2-chlorophenyl) thiazol-4-yl) -3,5-pyridine dicarboxylate (6g): Compound of 6g was prepared similar to 6a, m.p. 149-151°C, yield: 33. ^{1}H NMR ($CDCl_3$): δ 1.07 (m, 20H, cyclohexyl), 2.28 (s, 6H, CH_3), 4.83 (m, 2H, CO_2CH-cyclohexyl), 5.32 (s, 1H, H_4), 6.27 (bs, 1H, NH), 7.05 (s, 1H, H_5-thiazole), 7.20-7.50 (m, 3H, aromatic), 9.01-8.13 (m, 1H, aromatic), IR (KBr): ν 3325 (NH), 1691 (C = O), MS m/z (%) 555 (14), 502 (21), 435 (29), 401 (43), 376 (59), 308 (64), 300 (21), 252 (14), 196 (100), 150 (29), 90 (100).

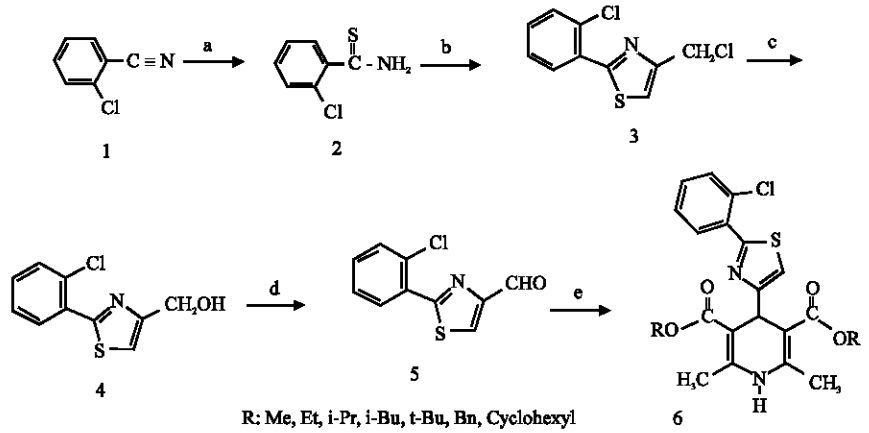
Semen samples and sperm preparation: An andrological outpatient clinic supplied the required semen samples (H. R. Sadeghipour Roodsari, Noor Laboratory Keshavarz Blvd Karegar. Ten healthy donors with informed consent after 48-72 h of abstinence supplied the required semen samples via masturbation that were collected into plastic containers. The samples complied with WHO required sample specification and guideline and processed immediately upon liquefaction. After liquefaction at 37°C for 30 min, the spermatozoa were separated from seminal plasma by two consecutive washing (300 g, 5 min) with PBS (phosphate-buffer saline, pH 7.4: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 in distilled water). The semen samples were allowed to swim-up for 1 h at 37°C in 1 mL of Ham's F-10 medium containing 10% HSA (human serum albumin) and subsequently the supernatant were carefully aspirated. The sperm concentration was adjusted to 1 million per millilitre for subsequent steps. Nifedipine, a calcium channel blocker and seven synthesized dihydropyridine compounds (6a-g) were dissolved in DMSO that were used as solvent medium. To evaluate the activity of compounds, 95 μL of motile sperm samples (1 million per mL) were incubated with 5 μL of synthesized compounds (6a-g) (1×10^{-2} M) to obtain the final concentrations of 5×10^{-4} M in volume of 100 μL. Nifedipine and DMSO were used as standard and blank respectively. All incubations were performed at 37°C in presence of 5% CO_2. Sperm motility were measured during incubation process after 1 and 2 h of contact time.

Human sperm motility studies: The percentage of motile sperms were assessed by visual inspection of 100 sperms. The degree of sperm movement for every encountered sperm was graded as a, b, c or d and defined as follows: a: rapid progressive motility; b: slow progressive motility; c: non-progressive motility and d: immotility (WHO, 1999). Total motility (a + b + c) was reported. All samples used in this study had values of immotile spermatozoa (grade d) lower than 20% of the total value. The count of spermatozoa were carried out by ×400 multiplied microscope (Zeiss).

Statistical analysis: Data was analysed using a computer software pakage (SPSS ver 11.5 Chicago, Ill.). Paried t-Test statistical analysis was provided within the pakage. The data was shown as mean±SD and the p-value <0.5 considered as significant.

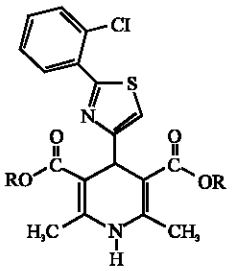
RESULTS AND DISCUSSION

Chemistry: Treatment of 2-chlorothiobenzamide (2) and 1,3-dichloroacetone render compound (3). That when refluxed in acidic media led to the 2 hydroxy methyl derivatives of thiazol (4). Oxidation of hydroxymethyl group by MnO_2 in dichloromethane resulted aldehyde (5). The compounds (6a-g) were prepared according to the conventional procedures for synthesis of dihydropyridine compounds (Scheme 1).



Scheme 1: Reagents: (a) SH_2, TEA/pyridine, (b) 1,3-dichloroacetone, (c) H_2SO_4/H_2O, (d) MnO_2/CH_2Cl_2 and (e) CH_3COCH_2COOR, ammonium acetate/MeOH

Table 1: Physical properties and *in vitro* sperm motility studies of 6a-g



Compound	R	m.p. (°C)	Yield (%)	Motility (%) after 1 h±CV	Motility (%) after 2 h±CV
6a	Methyl	194-195	48	82	52
6b	Ethyl	143-145	48	78	65
6c	i-Propyl	145-146	47	77	68
6d	i-Butyl	101-102	33	79	65
6e	t-Butyl	163-165	22	73	64
6f	Benzyl	117-118	28	74	64
6g	Cyclohexyl	149-151	33	78	63
Nifedipine				0	0

***In vitro* study:** Nifedipine were mentioned previously as the agent responsible for failure of an IVF trial case due to sperm immobilizing activity (Benoff *et al*., 1994). In this study, the sperm motility activity inhibition of nifedipine were used as a reference to evaluate the potential motility activity inhibition of seven candidate compounds. Table 1 shows the results obtained after determination of total sperm motility in the presence of the seven compounds and Nifedipine after 1 and 2 h of incubation period.

Nifedipine reduced motility completely both after 1 and 2 h of incubation. Sperm motility reduced value for candidate compounds (6a-g) shows a gradual increase proportional to the size elongation of alkyl ester group. As a result the methyl ester of the test compounds with 50% of activity in comparison to nifedipine (in 2 h group) is the most active compound ($p<0.05$). Though the other members of the synthetic compounds did not statistically show a significant activity differences in comparison to DMSO. Therefore, the results could be attributed to the point that reducing the size of ester group (R_1) is critical in the activity of the compounds. Increase in the size from methyl to other lipophilic compounds led to the reduction of the activity.

ACKNOWLEDGMENTS

This research was supported by grants from INSF (Iran National Science Foundation) and the Research Council of Tehran University of Medical Sciences.

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