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Original Article

Synthesis and *in vivo* evaluation of novel thiadiazoles as carbonic anhydrase inhibitors to attenuate inflammation-induced thermal hyperalgesia in irradiated Rats

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ABSTRACT

New series of thiadiazole derivatives (**2-8)** and thiadiazolo[3,2-a]pyrimidines (**15-17)** were synthesized from the starting compound **(1)** 5-amino-1,3,4-thiadiazole-2(3H)-thione. The structures of these compounds were confirmed by elemental analysis, IR, ^{1}H-NMR and ^{13}C-NMR spectral data. The compounds were tested against inflammation-induced hyperalgesia caused by intramuscular injection of carrageenan in rats. After administration of each of the thiadiazoles to irradiated inflamed rats; the inflammation-induced hyperalgesia, carbonic anhydrase activity, antioxidant effect and lactate dehydrogenase activity, were measured. To reveal the possible mechanism(s) of action of the most promising compounds (**3**, **9** and **10**); we tested their antioxidant, anti-inflammatory and the central analgesic effects and they showed promising results. These compounds reduced inflammation-induced hyperalgesia, probably by acting centrally and peripherally.

Keywords: thiadiazole, thiadiazolo[3,2-a]pyrimidine, Carbonic anhydrase inhibitors, Inflammation-induced hyperalgesia, Anti-inflammatory, Antioxidant.

INTRODUCTION

The majority of the current theories of pain states that the physiological pain arises from tissue damage (inflammatory pain), whereas the neuropathic pain results from changes in damaged nerves [1]. The inflammatory status has been reported to produce peripheral sensitization [2]; i.e. the nociceptive system during inflammation will be over-responding for a given stimulus; a status known as "inflammation-induced hyperalgesia". A variety of studies revealed that a single exposure to carrageenan; which is a classic agent for the induction of experimental inflammation and inflammatory pain that is relevant to clinical inflammatory pain states [3-5], was able to produce a prolonged hypersensitivity to subsequent exposure of hyperalgesic agents [6-8]. Some researchers have demonstrated that the inflammatory effect induced by carrageenan could be associated with free radicals production. Similarly, ionizing radiation has been shown to exaggerate the inflammatory responses and to enhance the release of inflammatory mediators in experimental animals [9-11] and humans [12-14]. Irradiation of biological tissues initiates a series of intracellular biochemical events, starting with the ionization of a single molecule and the consequent generation of ROS which play an important role in the inflammatory process [15,16]. Carbonic anhydrase (CA) (carbonic hydrolase; E.C. 4.2.1.1) catalyzes the reversible dehydration of HCO_3^- in solution. Several isoforms; carbonic anhydrases (CAs), of this zinc metalloenzyme have been described. They differ from one another in their kinetic properties, sensitivity to various inhibitors such as acetazolamide (ACTZ); a thiadiazole derivative, and subcellular localization [17,18]. CAs play an important role in diverse processes, such as physiological pH control, gas balance, calcification and photosynthesis [19,20]. The role of CAs in the regulation of pH within the interstitial space, where the nociceptor terminals are located, is not fully revealed [21]. During recent years there has been a large investigation on different classes of thiadiazole compounds, many of which were found to possess an extensive spectrum of pharmacological activities. In particular, derivatives of differently substituted 1,3,4-thiadiazole are known to exhibit antimicrobial [22] and anti-tuberculosis activities [23], as well as anticonvulsant [24], antidepressant [25] and anxiolitic agents [26]. In addition, a family of 1,3,4-thiadiazoles phosphodiesterase 7

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selective inhibitors was also reported in the study of Schenone *et.al.* [27]. Moreover, many reports indicate that compounds incorporating carboxamide derivatives possess both anti-inflammatory and analgesic activities [28-30]. Also, careful literature survey showed that compounds incorporating aryl acrylonitriles possess interesting analgesic activity [31,32]. On the other hand, pyrimidine derivatives and heterocyclic annelated pyrimidines have attracted a great deal of interest owing to their medicinal activities including anticancer [33-36], antiviral [37] and anti-inflammatory [38] ones. All these findings encouraged us to focus our attention on the 1,3,4-thiadiazole ring. Particularly, we reported the synthesis of two series of 5-(substituted)-caboxamide thiadiazole derivatives and thiadiazolo[3,2-a]pyrimidines derivatives from the previously reported starting compound 5-amino-1,3,4-thiadiazole-2(3H)-thione **(1)** [39]. Hence, this study was designed to explore the potential anti-hyperalgesic activity of the newly synthesized thiadiazole derivatives, as well as their possible mechanism(s) of action in irradiated inflamed animals.

MATERIAL AND METHODS

Chemicals: Acetazolamide (5-Acetamido-1,3,4-thiadiazole-2-sulfonamide) [ACTZ], Naloxone (dihydroxy-4-(propenyl) oxa-4-azapentacyclo octadecatrien-14-one) and Dimethyl sulfoxide [DMSO] as well as the other solvents and chemicals used in the synthesis and in-vivo study were all purchased from Sigma-Aldrich chemical company (St Louis, Missouri, USA).

Instruments: Melting points were uncorrected and were taken in an open capillary tube on a Stuart melting point apparatus (Stuart Scientific, Redhill, UK). The IR spectra of the compounds were recorded on ABB Bomem FT-IR spectrometer MB 104 with KBr pellets. ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker 300 NMR spectrometer operating at 400.13 and 100.77 MHz, respectively. Microanalyses were obtained with an Elemental analyses system GmbH VarioEL V300 element analyzer. The purity of the compounds was checked by TLC on pre-coated SiO_2 gel (HF254, 200 mesh) aluminium plates (E Merk) and visualized in UV chamber. IR, ^{1}H-NMR, ^{13}C-NMR and elemental analysis were consistent with the assigned structures and performed at the Microanalytical Laboratories of the Faculty of Science, Cairo University.

Animals: The experimental animals used in the present study were adult male Wistar albino rats weighing 130–150 g. The animals were purchased from the animal house of the National Research Center (Dokki, Cairo, Egypt). They were maintained on stock diet and kept under fixed appropriate conditions of housing and handling throughout the experimental period.

Chemistry:

*2-Cyano-N-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)acetamide (**2**):* A mixture of **1** [39] (1.21 g, 0.01 mol) and ethyl cyanoacetate (1.13 g, 0.01 mol) was fused at 220 °C for 3 h. The reaction mixture was concentrated, cooled then triturated with diethyl ether. The obtained compound was crystallized from ethanol to give 2.Yield%: 85, m.p. = 100-102 ˚C, IR cm^{-1}: 3193, 3177 (2NH), 2920, 2856 (CH aliph.), 2220 (CN), 1692 (C=O), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 3.3[s, 2H, CH_2], 7.2[s, 1H, NH, D_2O-exchangeable], 8.1[s, 1H, NH, D_2O-exchangeable].^{13}C-NMR (DMSO-d_6, ppm): 25.3(CH_2), 117.0(CN), 144.5(C-NH), 173.3(C=O), 181.2(C=S). Anal. Calcd. For $C_5H_4N_4OS_2$ (200): C, 29.99; H, 2.01; N, 27.98, Found: C, 30.21; H, 2.32; N, 28.10.

*4-Amino-2,3-dihydro-N-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-3-phenyl-2-thioxothiazole-5-carboxamide (**3**):* A mixture of **2** (0.5 g, 0.0025 mol), finely divided sulfur (0.08 g, 0.0025 mol) and triethylamine (3 drops) in absolute ethanol (30 mL) was stirred at room temperature for 30 min. Then, phenyl isothiocyanate (0.28 mL, 0.0025 mol) was gradually added and stirring was continued for 3h during which a crystalline product separated out. The separated solid was filtered, washed with ether, dried and cyrstallised from dimethyl formamide to give **3**.Yield%: 76, m.p. = >300 ˚C, IR cm^{-1}: 3230, 3178, 3156 (NH_2, 2NH), 3055 (CH arom.), 1692 (C=O), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 3.2[s, 2H, NH_2, D_2O-exchangeable], 6.5-7.2[m, 5H, Ar-H], 7.1[s, 1H, NH, D_2O-exchangeable], 8.1[s, 1H, NH, D_2O-exchangeable].^{13}C-NMR (DMSO-d_6, ppm): 78.1 (C-thiazole), 126.5, 125.2, 129.3, 134.2, 144.1(C-thiadiazole), 159.0(C-NH_2), 168.2 (C=O), 181.1(C=S, thiadiazole), 188.4 (C=S, thiazole). Anal. Calcd. For $C_{12}H_9N_5OS_4$ (367): C, 39.22; H, 2.47; N, 19.06, Found: C, 39.44; H, 2.31; N, 19.23.

*General procedure for the preparation of compounds (**4-6**):* A solution of **2** (0.0025 mol) and 4-fluoro, 4-chloro, or 4-bromobenzaldehyde (0.0025 mol) in dry dimethyl formamide (10 mL) was treated with 5 drops of 10% methanolic potassium hydroxide. The reaction mixture was refluxed for 2h and then the crude product that precipitated was filtered off and crystallized from dimethyl formamide to give compounds **4-6**, respectively.

*(2E)-2-Cyano-3-(4-fluorophenyl)-N-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)acrylamide (**4**):* Yield%: 95, m.p. = >300 ˚C, IR cm^{-1}: 3178, 3156 (2NH), 3065 (CH arom.), 2234 (CN), 1687 (C=O),

1235 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 6.9-7.5[m, 4H, Ar-H], 7.2[s, 1H, NH, D_2O-exchangeable], 8.1[s, 1H, NH, D_2O-exchangeable], 8.2 [s, 1H, CH].^{13}C-NMR (DMSO-d_6, ppm): 106.1 (C-CN), 115.3, 115.9 (CN), 128.2, 130.6, 144.3(C-NH), 153.2 (CH_2), 162.9, 168.4(C=O), 181.2(C=S). Anal. Calcd. For $C_{12}H_7FN_4OS_2$ (306): C, 47.05; H, 2.30; N, 18.29, Found: C, 47.37; H, 2.31; N, 19.93.

(2E)-3-(4-Chlorophenyl)-2-cyano-N-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)acrylamide (***5***)*:* Yield%: 91, m.p. = 286-289 ˚C, IR cm^{-1}: 3178, 3156 (2NH), 3065 (CH arom.), 2225 (CN), 1690 (C=O), 1235 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 7.2, 7.4[2d, 4H, Ar-H AB-system], 7.2[s, 1H, NH, D_2O-exchangeable], 8.1[s, 1H, NH, D_2O-exchangeable], 8.2 [s, 1H, CH].^{13}C-NMR (DMSO-d_6, ppm): 106.1 (C-CN), 115.9 (CN), 127.1, 128.5, 133.1, 133.5, 144.2(C-NH), 153.5 (CH_2), 169.1 (C=O), 181.0(C=S). Anal. Calcd. For $C_{12}H_7ClN_4OS_2$ (322): C, 44.65; H, 2.19; N, 17.36, Found: C, 44.21; H, 2.35; N, 17.66.

(2E)-3-(4-Bromophenyl)-2-cyano-N-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)acrylamide (***6***)*:* Yield%: 89, m.p. = 245-247 ˚C, IR cm^{-1}: 3178, 3156 (2NH), 3065 (CH arom.), 2222 (CN), 1695 (C=O), 1235 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 7.1, 7.5[2d, 4H, Ar-H AB-system], 7.2[s, 1H, NH, D_2O-exchangeable], 8.1[s, 1H, NH, D_2O-exchangeable], 8.2 [s, 1H, CH].^{13}C-NMR (DMSO-d_6, ppm): 106.1 (C-CN), 115.9 (CN), 122.1, 128.9, 131.6, 134.2, 144.3(C-NH), 153.5 (CH_2), 168.2 (C=O), 181.2(C=S). Anal. Calcd. For $C_{12}H_7BrN_4OS_2$ (367): C, 39.25; H, 1.92; N, 15.26, Found: C, 39.55; H, 2.21; N, 15.53.

General procedure for the preparation of compounds ***(7, 8)****:* To a solution of **2** (0.0025 mol) in absolute ethanol (30 mL), ammonium acetate (0.0025 mol) and cyclopentanone or cyclohexanone (0.0025 mol) were added. The reaction mixture was refluxed for 6h and left to cool to room temperature. The separated crystalline product was filtered off, dried and recrystallized from dimethyl formamide to give **7** and **8**, respectively.

2-Cyano-2-cyclopentylidene-N-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)acetamide (***7***)*:* Yield%: 75, m.p. = >300 ˚C, IR cm^{-1}: 3178, 3156 (2NH), 2978, 2812 (CH aliph.), 2225 (CN), 1689 (C=O), 1235 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 1.3[m, 4H, $2CH_2$], 2.1[t, 4H, $2CH_2$], 7.2[s, 1H, NH, D_2O-exchangeable], 8.1[s, 1H, NH, D_2O-exchangeable].^{13}C-NMR (DMSO-d_6, ppm): 28.7 and 32.9 (4C of cyclopentanone), 96.1 (C-CN), 115.9 (CN), 144.3(C-NH), 167.21 (C=O), 175.1 (C-cyclopentanone), 181.0(C=S). Anal. Calcd. For $C_{10}H_{10}N_4OS_2$ (266): C, 45.09; H, 3.78; N, 21.04, Found: C, 45.31; H, 3.56; N, 21.21.

2-Cyano-2-cyclohexylidene-N-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)acetamide (***8***)*:* Yield%: 78, m.p. = 280-282 ˚C, IR cm^{-1}: 3178, 3156 (2NH), 2967, 2862 (CH aliph.), 2225 (CN), 1689 (C=O), 1235 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 1.2-1.3[m, 6H, $3CH_2$], 1.9[t, 4H, $2CH_2$], 7.2[s, 1H, NH, D_2O-exchangeable], 8.1[s, 1H, NH, D_2O-exchangeable].^{13}C-NMR (DMSO-d_6, ppm): 27.1, 27.5 and 31.8 (5C of cyclohexanone), 95.1 (C-CN), 115.9 (CN), 144.1(C-NH), 168.1 (C=O), 181.0(C=S), 182.9 (C- cyclohexanone). Anal. Calcd. For $C_{11}H_{12}N_4OS_2$ (280): C, 47.12; H, 4.31; N, 19.98, Found: C, 47.31; H, 4.12; N, 19.68.

General procedure for the preparation of compounds ***(9-11):*** A mixture of **1** [39] and the appropriate active methylene (malononitrile, ethylcyanoacetate or diethylmalonate) (0.01 mol) in glacial acetic acid (30 mL) was refluxed for 12 h and refrigerated overnight. The separated solid was filtered, dried and recrystallized from ethanol to give **9**-**11**, respectively.

5-Amino-7-imino-3,7-dihydro-2H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-thione (***9***)*:* Yield%: 75, m.p. = 250-252 ˚C, IR cm^{-1}: 3350-3125 (br, NH_2, 2NH), 1235 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 2.2[s, 1H, NH, D_2O-exchangeable], 2.5[s, 2H, NH_2, D_2O-exchangeable], 4.0[s, 1H, CH], 8.1[s, 1H, NH, D_2O-exchangeable].^{13}C-NMR (DMSO-d_6, ppm): 87.9 (CH), 163.1 (C-NH2), 163.0 (C=N), 164.2 (C=NH), 203.0 (C=S) . Anal. Calcd. For $C_5H_5N_5S_2$ (199): C, 30.14; H, 2.53; N, 35.15, Found: C, 30.36; H, 2.12; N, 35.31.

5-Amino-7-thioxo-2H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-7(3H)-one (***10***)*:* Yield%: 70, m.p. = 231-233 ˚C, IR cm^{-1}: 3221- 3190 (NH_2, NH), 1680 (C=O), 1235 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 2.2[s, 2H, NH_2, D_2O-exchangeable], 5.4[s, 1H, CH], 8.1[S, 1H, NH, D_2O-exchangeable].^{13}C-NMR (DMSO-d_6, ppm): 73.9 (CH), 163.1 (C=N), 167.5 (C=O), 179.2 (C-NH_2), 203.0 (C=S). Anal. Calcd. For $C_5H_4N_4OS_2$ (200): C, 29.99; H, 2.01; N, 27.98, Found: C, 30.26; H, 2.31; N, 28.01.

2-Thioxo-2H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-5,7(3H, 6H)-dione (**11**): Yield%: 76, m.p. = 225-227 ˚C, IR cm^{-1}: 3235 (NH), 1691, 1680 (2C=O), 1235 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 2.2[s, 1H, NH, D_2O-exchangeable], 3.3[S, 2H, CH_2].^{13}C-NMR (DMSO-d_6, ppm): 56.2 (CH2), 163.2 (C=N), 165.1 (C=O), 171.2 (C=O), 203.0 (C=S). Anal. Calcd. For $C_5H_3N_3O_2S_2$ (201): C, 29.84; H, 1.50; N, 20.88, Found: C, 29.71; H, 1.62; N, 19.01.

4-[Diethyl-2-(aminomethylene)malonate]benzenesulfonamide ***(14):*** A mixture of sulfanilamide (0.01 mol), diethylmalonate (0.01 mol), triethylorthoformate (0.01 mol) and acetic acid (1 ml) in methanol (30 ml) was refluxed for 5 h. The reaction mixture was filtered and recrystallized from ethanol to give **14**. Yield%: 70, m.p. = 185-187 ˚C, IR cm^{-1}: 3289, 3212, 3190 (NH, NH_2), 3067 (CH arom.), 1701,

1690 (2C=O), 1312, 1167 (SO_2). ^{1}H-NMR (DMSO-d_6, ppm): 1.3[t, 6H, $2CH_3$], 2.2[s, 2H, NH_2, D_2O-exchangeable], 4.1[s, 1H, NH, D_2O-exchangeable], 4.3[q, 4H, $2CH_2$], 4.5[s, 1H, CH], 6.9, 7.5[2d, 4H, Ar-H, AB-system].^{13}C-NMR (DMSO-d_6, ppm): 14.2 ($2CH_3$), 61.5 ($2CH_2$), 92.4 (CH=C), 116.6, 128.1, 129.7, 147.6, 150.5 (2C=O), 165.2 (CH=C). Anal. Calcd. For $C_{14}H_{18}N_2O_6S$ (342): C, 49.11; H, 5.30; N, 8.18, Found: C, 49.34; H, 5.12; N, 8.29.

*General procedure for the preparation of compounds **(15-17)**:* A mixture of **1** [39] and compounds **12**, **13** or **14** (0.01 mol) in glacial acetic acid (30 mL) was refluxed for 12 h and refrigerated overnight. The separated solid was filtered, dried and recrystallized from ethanol to give **15-17**, respectively.

*4-(((5-Amino-7-imino-2-thioxo-3,7-dihydro-2H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-6-yl) methyl) amino) benzene sulfonamide (**15**):* Yield%: 65, m.p. = >300˚C, IR cm^{-1}: 3410- 3125 (br, $2NH_2$, 3NH), 3066 (CH arom.), 2945, 2812 (CH aliph.), 1367, 1123 (SO_2), 1235 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 2.2[s, 2H, NH_2, D_2O-exchangeable], 3.8[s, 2H, CH2], 4.1[S, 1H, NH, D_2O-exchangeable], 6.9, 7.5[2d, 4H, Ar-H, AB-system], 8.1[s, 4H, $2NH+SO_2NH_2$, D_2O-exchangeable].^{13}C-NMR (DMSO-d_6, ppm): 39.1 (C-CH_2), 81.0 (C-CH_2), 113.9, 128.1, 129.4, 150.3, 158.0 (C-NH_2), 163.1 (C=N), 164.0 (C=NH), 203.0 (C=S) . Anal. Calcd. For $C_{12}H_{13}N_7O_2S_3$ (383): C, 37.59; H, 3.42; N, 25.57, Found: C, 37.60; H, 3.54; N, 25.23.

*4-(((5-Amino-7-oxo-2-thioxo-3,7-dihydro-2H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-6-yl)methyl)amino) benzenesulfonamide (**16**):* Yield%: 68, m.p. = >300˚C, IR cm^{-1}: 3350- 3185 (br, $2NH_2$, 2NH), 3066 (CH arom.), 2912, 2822 (CH aliph.), 1690 (C=O), 1367, 1123 (SO_2), 1235 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 2.2[s, 2H, NH_2, D_2O-exchangeable], 3.5[s, 2H, CH_2], 4.3[s, 1H, NH, D_2O-exchangeable], 6.6, 7.4[2d, 4H, Ar-H, AB-system], 8.3[S, 3H, $NH+SO_2NH_2$, D_2O-exchangeable].^{13}C-NMR (DMSO-d_6, ppm): 44.2 (C-CH_2), 89.7 (C-CH_2), 113.9, 128.1, 129.0, 150.9, 163.5 (C=N), 169.2 (C=O), 171.1 (C-NH_2), 203.0 (C=S) . Anal. Calcd. For $C_{12}H_{12}N_6O_3S_3$ (384): C, 37.49; H, 3.15; N, 21.86, Found: C, 37.33; H, 3.24; N, 22.00.

*(E)-4-(((5,7-dioxo-2-thioxo-2H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-6(3H, 5H, 7H)-ylidene)methyl)amino) benzenesulfonamide (**17**):* Yield%: 76, m.p. = >300˚C, IR cm^{-1}: 3350, 3210, 3185 (NH_2, 2NH), 3066 (CH arom.), 1701, 1690 (2C=O), 1367, 1123 (SO_2), 1235 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 4.2[s, 1H, NH, D_2O-exchangeable], 6.9, 7.6[2d, 4H, Ar-H, AB-system], 8.1[s, 3H, $NH+SO_2NH_2$, D_2O-exchangeable], 8.8[s, 1H, CH].^{13}C-NMR (DMSO-d_6, ppm): 116.6, 118.6 (C=CH), 128.1, 129.7, 147.6, 156.9 (C=CH), 163.1 (C=N), 166.4 (C=O), 190.2 (C=O), 203.0 (C=S). Anal. Calcd. For $C_{12}H_9N_5O_4S_3$ (383): C, 37.59; H, 2.37; N, 18.27, Found: C, 37.21; H, 2.56; N, 18.45.

In vivo studies

Experimental design

***Antihyperalgesic, carbonic anhydrase inhibitory and antioxidant activities of the newly synthesized compounds**:* For the screening of the activities of the newly synthesized compounds rats were randomly divided into 19 experimental groups (6 rats each); as follows: Group 1: normal control, Group 2: normal irradiated group, Group 3: inflamed group, Group 4: irradiated inflamed group; Group 5: irradiated inflamed vehicle treated control that was injected i.p. with dimethyl sulfoxide [DMSO] (the vehicle of the tested agents; 2 ml/Kg), Group 6; irradiated inflamed group treated with ACTZ as a reference drug in a dose of 100 mg/Kg i.p. [21] dissolved in DMSO, Groups 7-19: irradiated inflamed groups each of which was treated with one of the newly synthesized compounds in a dose of 100 mg/Kg i.p., dissolved in DMSO.

Anti-inflammatory, antioxidant and central anti-hyperalgesic effects of the promising compounds: In order to study the possible mechanisms of action of the most promising compounds; rats were randomly assorted into 18 experimental groups (8 rats each); as follows:

- Groups 1-6 were treated as Groups 1-6 of the screening experiment (detailed above), while Groups 7-9 were irradiated inflamed groups each of which was treated with one of the compounds 2, 8 and 9. Blood samples were collected from these experimental groups and used for the determination of plasma total antioxidant capacity (TAC) and tumor necrosis factor-α (TNF-α) level.
- Groups 10-13 were treated as Groups 1-4 of the screening experiment (detailed above); their paw licking latency was determined by the hot plate method before and 2.5 h after the administration of naloxone in a dose of 8mg/Kg in dissolved in saline [40]. While the Groups 14-19 were irradiated inflamed groups, each of which was treated with either the vehicle, ACTZ or one of the compounds 2, 8 and 9; half an hour following naloxone administration.

Irradiation process: Whole-body γ-irradiation was performed at The National Centre for Radiation Research and Technology (NCRRT), Cairo, Egypt, using GAMMA-cell 40; Caesium-137 irradiation unit manufactured by the Atomic Energy of Canada Limited (Sheridan Science and Technology Park, Mississauga, Ontario, Canada). Radiation dose levels were delivered at a rate of 0.5 Gy/min. Rats

were whole body irradiated at a single acute dose level of 6 Gy [11].

Induction of inflammation: The hindpaw skin overlying the gastrocnemius muscle of the rats was shaved and cleaned with 70 % alcohol pads. Rats were injected with 100 µl of 3% carrageenan suspension into the left gastrocnemius muscle belly. Animals were returned to their cages and left for 24 h for the inflammation to develop. After 24 h, the circumference of the inflamed and the non-inflamed gastrocnemius muscles was measured over the skin using a measuring tape to confirm the development of inflammation [21].

Assessment of inflammation-induced hyperalgesia: Inflammation-induced heat hyperalgesia was quantified using a modified version of the hot plate apparatus after Espejo and Diego [41] consisting of a Plexi glass chamber with removable cover (IKA® C-MAG HS10, IKA-Werke, GmbH, Germany). The bottom of the chamber was heated to 55 ± 0.5 °C, cut-off time was set to 25 s to prevent damage to the skin. The rats were placed individually onto the hot plate and the latency time up to the first licking of one of the hind-paws was recorded. A decrease in withdrawal latency was interpreted as heat hyperalgesia for the purpose of this study. As for the irradiated inflamed groups; animals were irradiated 1 h before carrageenan injection. Administration of the vehicle/drug was carried out 24 h following carrageenan injection. Paw licking latency test was determined by the hot plate method 2 h after administration of the vehicle/drug or 26 h following carrageenan injection in non-treated groups. At the end of the experimental period, animals were sacrificed by decapitation; just after the paw licking latency test, then the blood was collected from the neck and divided into 2 portions, the first of which was collected in non-heparinized tubes, allowed to clot, and centrifuged for 15 min at 1,000 xg using Hettich Mikro 22R centrifuge (Andreas Hettich GmbH, Tuttlingen, Germany). The separated serum was then used for the determination of LDH activity. The second portion was collected in tubes containing EDTA (2mM), and was used in the preparation of erythrocytes haemolysate and plasma for the determination of CA activity and each of the levels of tumor necrosis factor-α (TNF-α), malondialdehyde (MDA) and total antioxidant capacity (TAC), respectively.

Determination of CA activity: The erythrocyte pellet obtained after separation of plasma (heparinized blood centrifuged at 1,000 xg for 15 min) was washed three times with cold 0.16 M KCl and the supernatant discarded. One volume of erythrocyte pellet was suspended in five volumes of ice water to give an erythrocyte haemolysate. CA activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (4-NPA) to 4-nitrophenylate ion over a period of 3 min at 25°C according to the method described by Verpoorte et al [42]. The enzymatic reaction, in a total of 3.0 ml, contained 1.4 ml 0.05M tris-SO_4 buffer (pH 7.4), 1 ml 3 mM 4-NPA, 0.5 ml H_2O, and 0.1 ml sample. A reference measurement was obtained by preparing the same mixture without sample. Activity was expressed as U/g hemoglobin. Hemoglobin concentration was determined using an automatic hematology analyzer HA-VET (CLINDIAG SYSTEMS, Pollare, Belgium).

Determination of the antioxidant activity: GSH levels was determined chemically using 5,5'-dithionitrobenzoic acid [43]. Lipid peroxidation was determined from MDA formation as an end product of the peroxidation of lipids. MDA reacts with thiobarbituric acid to generate a colored product that can be measured colorimetrically according to the method of Uchiyama and Mihara [44].

Determination of lactate dehydrogenase activity: Sera of rats were used for the determination of lactate dehydrogenase (LDH) activity using kinetic ultraviolet (UV) kit (LABKIT®, Barcelona, Spain), UV-Vis spectrophotometer [Double beam PC scanning spectrophotometer UVD-2950 (LABOMED INC., Los Angeles, CA, USA)].

Bioassays performed for the elucidation of the mechanism(s) of action of the promising derivatives among the evaluated compounds: The most promising compounds were further tested to elucidate their possible mechanism of action as anti-hyperalgesic agents by measuring plasma TNF-α level and TAC. Furthermore, we evaluated their anti-hyperalgesic activity following administration of the opioid receptor blocker naloxone; aiming to examine their potential central anti-hyperalgesic activity.

Measurement of plasma levels of tumor necrosis factor-α and total antioxidant capacity: Plasma levels of tumor necrosis factor-α **(**TNF-α) were determined using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Quantikine® R&D systems, Gmbh, Viesbaden, Germany) according to the manufacturer's instruction. TNF-*α* was determined from a standard curve for the combination of these cytokines. The concentrations were expressed as pg/ml. Plasma TAC was determined using commercially available colorimetric kits; and the assay was carried on according to the manufacturer's instructions

(Biodiagnostics®, Dokki, Cairo, Egypt). The concentrations were expressed as mmol/L.

Assessment of the central anti-hyperalgesic effects: Thirty minutes before the administration of the vehicle/drug, the animals were injected with naloxone, a specific blocker of opioid μ-receptors. Naloxone was administred in a solution containing 1mg naloxone in 0.5 ml of saline [40]. Two hours after the administration of the vehicle/drug, the rats were placed individually onto the hot plate and the latency time (in seconds) up to the first licking of one of the hind-paws was recorded.

Statistical analysis: The data were analyzed using one-way analysis of variance (ANOVA). Comparisons among groups were carried out using Tukey-Kramer's multiple comparisons test. The significance level was tested at $p<0.05$.

RESULTS AND DISCUSSION

Chemistry: Considering *5-amino-3H-[1,3,4]thiadiazole-2-thione* **(1)** system as scaffold, we synthesized new derivatives. The synthesis of our target compounds **(2-8), (9-11)** and **(15-17)** is outlined in schemes 1 and 2; respectively. The starting compound **(1)**, was prepared according to the method reported by Cho et al. [39], depending on the reaction between thiosemicarbazide and carbon disulfide in alkaline medium followed by acidification. *2-Cyano-N-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)acetamide* (**2**) was synthesized by reaction of compound **(1)** with ethylcyanoacetate under fusion condition according to the reported method [45], the structure was confirmed by microanalytical and spectral data, its IR spectrum showed bands for carbonitrile and aliphatic CH along with the disappearance of the forked band for NH_2, while, it's ^{1}H-NMR spectrum showed a singlet at 3.3 ppm corresponding to CH_2, on the other hand, three signals appeared at 25.3, 117.0, 173.3 in the ^{13}C-NMR corresponding to CH_2, CN, C=O, respectively (scheme 1). Whereas, reacting **(2)** with sulfur and phenyl isothiocyanate, following the reaction conditions reported for the preparation of related compounds [46] afforded *4-amino-2,3-dihydro-N-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)-3-phenyl-2-thioxothiazole-5-carboxamide* (**3**). The structure of compound **(3)** was confirmed by IR and ^{1}H and ^{13}C- NMR spectra. The IR spectra proved as useful in tracing the disappearance of the CN stretching absorption of the parent compound **(2)**. The ^{1}H-NMR spectrum of compound **(3)** displayed the disappearance of CH_2 absorption of the precursor **(2)** and the presence of the expected protons signals of the aromatic ring together with other singlet assigned to NH_2 at 3.2 ppm which is exchangeable with D_2O. While, ^{13}C-NMR showed two signals assigned for the thiazole ring at 78.1 and 188.4 ppm together with an observed shift in the value of C=O which appeared at 168.2 ppm as a result of substitution by the thiazole ring (scheme 1).

It is reported that compounds containing a halogenated substituent were highly active analgesic and antiinflammatory agents [47, 48]. Therefore, *(2E)-2-cyano-3-(4-substituted-phenyl)-N-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)acrylamide* **(4-6)** were prepared via the treatment of **(2)** with 4-fluoro/chloro/bromo-benzaldehyde in dimethylformamide following reported reaction conditions [49]. The ^{1}H-NMR spectra showed significant absorption bands at 8.2 ppm corresponding to CH proton of compounds **(4-6)** (scheme 1).

In addition, *2-cyano-2-substituted-N-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)acetamide* **(7, 8)** were prepared by reacting **(2)** with cyclopentanone or cyclohexanone in the presence of ammonium acetate. The ^{1}H-NMR spectra were consistent with the proposed structures and were proved by the appearance of stretching bands for CH aliphatic in the IR spectra, while, ^{1}H-NMR spectra showed triplet and doublet signals at 1.3 and 2.1 assigned for the protons of cyclopentanone in **(7)** and a multiplet in the range of 1.2-1.3 ppm and a doublet at 1.9 ppm assigned for the protons of cyclohexanone in **(8)**. On the other hand, their ^{13}C-NMR spectra revealed characteristic signals at 28.7, 32.9, 175.1 ppm assigned for the carbons of cyclopentanone in **(7)** and at 27.1, 27.5, 31.8, 182.9 ppm assigned for the carbons of cyclohexanone in **(8)** (scheme 1).

New thiadiazolo[3,2-a]pyrimidine derivatives **(9-11)** and **(15-17)** were prepared through the reaction of compound **(1)** with active methylenes containing compounds such as malononitrile, ethylcyanoacetate or diethylmalonate in refluxing glacial acetic acid to provide the corresponding thiadiazolo[3,2-a]pyrimidines **(9-11)**, the mechanism of formation of compound **(9)** is outlined in (scheme 3) through the nucleophilic attack of the amino group in the thiadiazole ring on the carbon of one the two carbonitrile groups followed by intramolecular cyclization between the NH of thiadiazole and the other CN group to form compound **(9)** and this mechanism is also applicable in the formation of compounds **(10, 11, 15-17)**.

The key reagents **(12, 13)** were synthesized and reported in previous work [34], this encouraged us to synthesize novel sulfanilamide derivative **(14)** by the same reported method [50] through reaction of sulfanilamide with diethylmalonate,

triethylorthoformate and refluxed in methanol containing catalytic amount of acetic acid. The synthesized compounds **(12-14)** were then reacted with compound **(1)** in refluxing glacial acetic acid following the reaction condition [51] to give the corresponding thiadiazolo[3,2-a]pyrimidines **(15-17)** and the aim of these derivatives is to explore the addition of sulfonamide moiety on the activity (scheme 2). The structures of compounds **(9-11, 15-17)** were proved by microanalytical and spectral data and were consistent with the proposed structures.

In vivo studies: The newly synthesized thiadiazole-derivatives were examined for their anti-hyperalgesic, carbonic anhydrase inhibitory and antioxidant activities using a model of inflammation-induced hyperalgesia in irradiated rats. Inflammation was induced by the injection of carrageenan into the gastrocnemius muscle following an acute exposure to γ-radiation at a dose level of 6 Gy. The antihyperalgesic effect of each of the newly synthesized thiadiazoles (compounds **2-11**, **15-17**) was investigated and quantified by hot plate hyperalgesia method. In addition, these compounds were also tested for their CA inhibitory effects (CA activity), antioxidant activity [malondialdehyde (MDA) and reduced glutathione (GSH) levels] and their effects on lactate dehydrogenase (LDH) activity (as a non-specific marker for cellular damage). The most promising candidates were then examined for their anti-inflammatory, antioxidant and possible central analgesic effects in order to reveal their probable mechanism(s) of action. The results demonstrated in Table 1 indicate that the induction of inflammation in non-irradiated rats (carrageenan-induced inflammation) resulted in a significant increase in hyperalgesia (a decrease in the hind-paw licking latency in the hot plate test) that was accompanied by a rise in plasma MDA level (a marker of lipid peroxidation) and serum LDH activity. Similar results were observed in the study of Hargreaves et al. [52]; where a dose dependent increase in hyperalgesia was induced by carrageenan paw inflammation. The mechanism of inflammation-induced pain has been extensively studied, the inflammatory mediators have been reported to activate local pain receptors and nerve terminals and to produce hypersensitivity in the area of injury. Activity of such mediators results in the excitation of pain receptors in the skin, ligaments, muscle, nerves and joints. Excitation of these pain receptors stimulates the specialized nerves that carry pain impulses to the spinal cord and brain. In addition, subsequent to tissue injury, the expression of sodium channels in nerve fibers is altered significantly thus leading to abnormal excitability in the sensory neurons. Furthermore, the nerve impulses arriving in the spinal cord stimulate the release of the inflammatory protein Substance-P [53]. As for the neuropathic pain, tissue damage induces the release of peripheral chemicals which sensitize the sensory nerve endings resulting in excitability changes within the nerve itself [54].

On the other hand, gamma (γ)-irradiation of rats prior to the induction of inflammation resulted in statistically significant changes in some of the investigated parameters except for the inflammation-induced hyperalgesia. However, this observation is contradictory to that obtained by Kereškenyiova and Šmajda, [40]; who reported a radiation-induced analgesia in rats mediated through the induction of endogenous opioid release in the central nervous system (CNS). The effects of ionizing radiation on the opioid mechanism were studied early after the discovery of endogenous opioids in the 1970s. Teitelbaum et al. [55] observed that the exposure to radiation led to behavioral and physiological responses that resemble those observed after administration of the exogenous opiate, morphine. In addition, beta-endorphin levels were found to be elevated in irradiated mice [56, 57]. A supporting observation was, also, reported by Teskey and Kavaliers [58] who showed that exposure of CF-1 mice to doses of ionizing radiation as low as 2.5 Gy caused an increase in their nociceptive thresholds in the hot-plate test, indicating analgesia.

Additionally, an elevated CA and LDH activities, the decreased GSH level and the increased MDA level were recorded in inflamed irradiated rats as compared to inflamed non-irradiated ones. These effects could be attributed to the increased free radicals production by each of irradiation, inflammation and their combination. Free radicals, prostaglandin and nitric oxide (NO) were reported to increase for 6 h following carrageenan injection [59]. The edema effect was raised to maximum at the third hour [60] and it was accompanied by MDA production which was attributed to the free-radical attack on the plasma membrane polyunsaturated fatty acids [61]. Thus, the inflammatory effect would result in the accumulation of MDA [62]. At present, more than 50% of cancer patients undergo radiotherapy as a part of their treatment [63] and are in need of pain treatment adjuvant therapy. Nevertheless, γ-irradiation was reported to increase the inflammatory response in more than a model of experimentally-induced inflammation, in a dose-dependent manner [64]. Another supporting observation was reported in the study of Yanardag et al. [65]; who showed an increase in the serum activity of LDH following γ-irradiation indicating

cellular damage. Hui et al. [66] showed that lipid peroxidation of biological membranes contributes significantly to the development of radiation induced cell injury. In addition, the high serum levels of LDH, induced by irradiation were reported by Mac William and Bhakthan [67], who showed that ionizing radiation instigates the alterations in dynamic permeability of membranes allowing leakage of biologically active material out of the injured cell.

Each of inflammation and γ-radiation; in the present study, induced a state of oxidative stress as indicated by the rise in plasma MDA levels. The mechanism of inflammation-induced oxidative stress has been explored in earlier studies [68, 69] who reported that TNF-α reduced the levels of intracellular GSH, an important physiological antioxidant, by a mechanism involving intracellular oxidative stress. In addition, TNF-α and ILs were also reported to induce expression of the inducible form of nitric oxide synthase and to enhance mitochondrial generation of reactive oxygen species (ROS) [69, 70]. Ionizing radiation is a well known inducer of free radicals formation with the consequent lipid peroxidation which can be initiated also by hydrogen abstraction from lipid molecules by lipid radiolytic products [71-73]. It was argued that the oxidant/antioxidant imbalance due to oxidative stress is the main cause of the excessive formation of peroxides. The significant acceleration of lipid peroxidation measured as MDA content has been previously attributed to peroxidation of the membrane unsaturated fatty acids due to free radicals propagation [74]. These free radicals might be the cause of the increased activity of serum LDH; observed herein, due to the cellular destruction by the increased production of free radicals with the subsequent release of the intracellular LDH.

From the results demonstrated in Table 1, we can conclude that, considering the 1,3,4-thiadiazole series (**2**-**8**), all the tested compounds showed antioxidant activity this was observed by a significant decrease in the levels of MDA (to a range of 9-30% of the irradiated inflamed group value) and consequently the LDH activity (to a range of 3-12% of the irradiated inflamed group value) together with an observed increase in the level of GSH in the groups treated with compounds **2** and **3**. Also, these two compounds decreased the inflammation-induced hyperalgesic state as indicated by the increase in the paw licking latency observed in this two groups; amounting to 3 and 5 folds the value recorded for the irradiated inflamed group, respectively. This may be attributed to the introduction of cyanoacetamide group in **2** and a thiazole ring in **3** and these results proved the efficacy of this cyclization reaction especially with the significant decrease in CA activity observed for compound **3,** which amounted to 20% of the value observed in the irradiated inflamed group. Consequently, these findings prompted us to explore their anti-inflammatory, antioxidant and possible central effect to predict its mechanism of action. In addition, compound **5** bearing chloro-benzyl group attached to the cyanoacetamide showed significant decrease in CA activity to a value of 25% of that recorded for the irradiated inflamed group.

Considering the thiadiazolo [3,2-a] pyrimidine series (**9-11, 15-17**), all the tested compounds showed antioxidant activities indicated by a significant decrease in the levels of MDA (to a range of 10-35% of the irradiated inflamed group value) and a consequent decrease in LDH activity (to a range of 3-11% of the irradiated inflamed group value), while, they showed no effect on the levels of GSH. On the other hand, a significant decrease in hyperalgasia was observed for compounds **9**, **10** and **15** indicated by an increase in the paw licking latency to about 5 folds when compared to the inflamed irradiated group. While, a potent inhibition in CA activity was observed for compounds **9**, **10**, **11**, **16** and **17** (amounting to 13, 20, 15, 9 and 7% of the irradiated inflamed group value, respectively) this may be attributed to the introduction of a pyrimidine ring fused to thiadiazole and addition of a moiety of sulfonamide in compounds **16** and **17** which is known to possess high CA inhibition activity [75].

Saturated carbon dioxide (CO_2) solution is a source of protons and was reported to activate a distinct subpopulation of mechano/heat-sensitive "polymodal" C-units in rat skin-nerve preparation [76], which were inhibited by the application of the CA inhibitor ACTZ. Similary, CAs generate protons in the tissues from CO_2, resulting in increased primary afferent fiber activity. In humans, referred pain experienced after abdominal laparoscopic surgery under CO_2 insufflation is reduced by systemic administration of ACTZ [77], supporting a role for CAs in nociception. However, ACTZ also inhibits ion channels [78, 79] that are known to be involved in pain transmission. Thus, ACTZ could have effects peripherally and/or centrally to reduce hyperalgesia by acting on CAs and/or ion channels [21]. Taking into consideration the fact that muscle injury increases the levels of CA [80], a hypothesis was proposed by Radhakrishnan and Sluka [21] that the inhibition of CAs in the muscle reduces the generation of protons in the extracellular space and reduces inflammation-induced hyperalgesia. In their study, although ACTZ reversed the inflammation-induced

heat hyperalgesia, the tissue pH did not change during inflammation. This observation was contrary to their original hypothesis that ACTZ reduces inflammatory hyperalgesia by raising the reduced pH in inflamed muscle; they also tested the central effects of ACTZ by administering ACTZ spinally, which potently reduced the inflammation-induced hyperalgesia, confirming a role of CA in pain processing in the central nervous system, as ACTZ is known to cross the blood brain barrier [81]. Interestingly, ACTZ was previously reported to possess both anti-inflammatory [82] and antioxidant [83] activities.

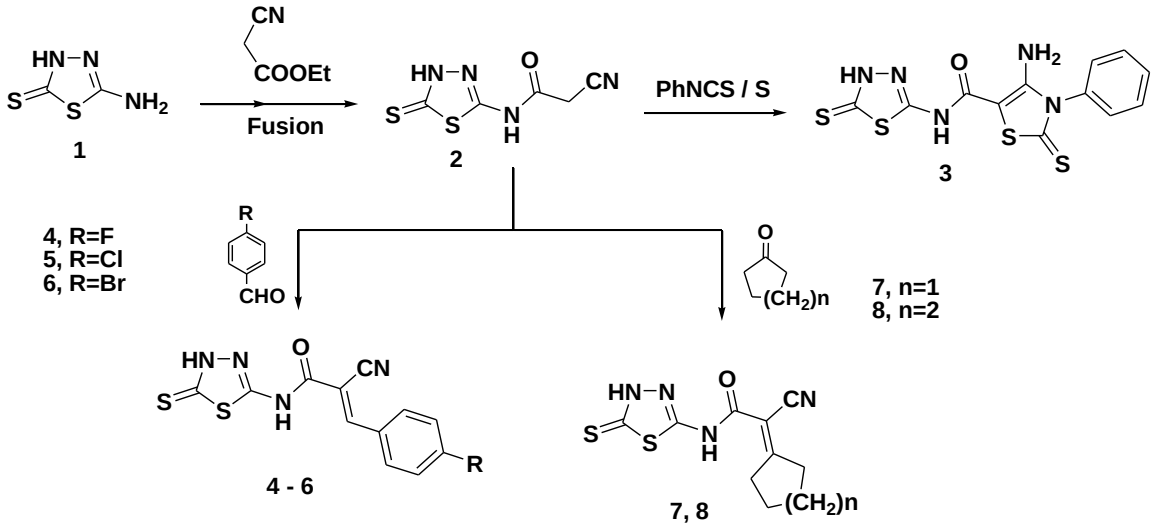
The anti-hyperalgesic effects of some of the newly synthesized thiadiazole derivatives; which were observed in our study, could be attributed to their CA inhibitory activity; where the levels of CAs were reported to increase during tissue damage and inflammation [80], which could increase the proton formation in the site of inflammation. Most isoforms of CAs are found intracellularly, but at least one form (CA IV) is found on the sarcolemma of muscles [84] and might be involved in pain sensation mechanism. Furthermore, CAs are found in medium to large dorsal root ganglia [85], suggesting that CAs are found on peripheral terminals of primary afferents [21]. Nevertheless, the observation that some of the tested compounds had anti-hyperalgesic but not CA inhibitory effects inspired us to propose other possible mechanisms of anti-hyperalgesia and try to explore them.

Generally, all the tested compounds showed high antioxidant activity possibly due to the thione group in position 2 on the thiadiazole ring which was previously reported to have free radical scavenging activity [33]. Whereas, among the tested newly synthesized derivatives, compounds **3**, **9** and **10** were found to be the most promising candidates that might be useful in the treatment of inflammation-induced hyperalgesia. In an attempt to further reveal their mechanism(s) of peripheral action, plasma TNF-α level and TAC were assayed in rats treated with each of these three compounds. The results obtained in these groups (Figure 1) revealed a possible anti-inflammatory and antioxidant effects for these compounds as indicated by the significant decrement in the plasma levels of TNF-α (compounds **9** and **10**) to about 75% of the irradiated inflamed group value, as well as the normalization of plasma TAC (compounds **3**, **9** and **10**) as compared to the irradiated inflamed group. Such an observation suggests that the anti-hyperalgesic effects of these compounds might be, at least in part, mediated by their anti-inflammatory and/or antioxidant effects. In addition, each of the three compounds was administered to rats following the injection of naloxone (a blocker of the endogenous opioid μ-receptors). In these groups, administration of naloxone resulted in a significant attenuation of the anti-hyperalgesic effects of these compounds (Table 2) amounting to 72, 64 and 73% of the values recorded in the corresponding non-naloxone treated groups, respectively; suggesting a possibility of a central analgesic action exerted by each of these compounds.

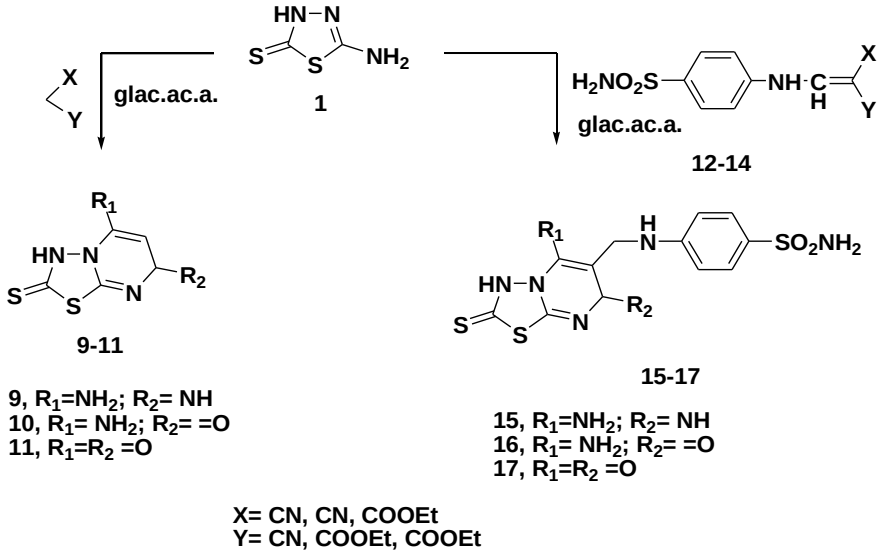
It worth mentioning that ACTZ administration, in the current study, produced an anti-hyperalgesic (a four-fold rise in the paw licking latency) and an antioxidant (a decrease in plasma MDA to 5% and an increase in TAC to 115%) effects as compared to the irradiated inflamed group. Interestingly, the anti-inflammatory and antioxidant effects offered by the compounds tested for their mechanism could be compared to those previously reported to be possessed by ACTZ as an anti-inflammatory [82] and antioxidant [83]. In addition, in the latter study, ACTZ was able to attenuate MDA formation and to increase the activities of the antioxidant enzymes SOD, CAT and GPx in rat kidney, an action that was attributed, at least in part, to its being a sulphur containing drug [83].

CONCLUSION

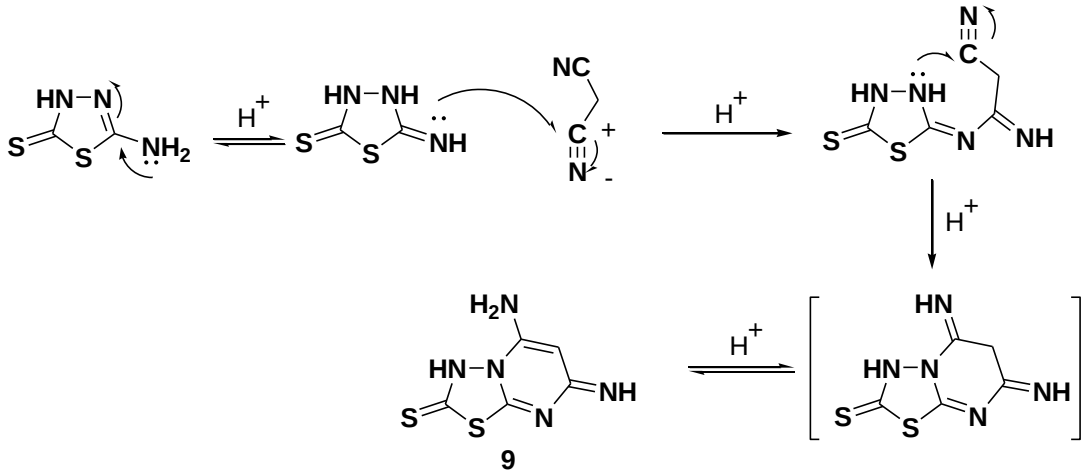
In conclusion, we reported here a simple and convenient route for the synthesis of some new 1,3,4-thiadiazole **(2-8)** and thiadiazolo[3,2-a]pyrimidine **(9-11, 15-17)** derivatives. The results showed that compounds **(3, 9 and 10)** were able to reduce inflammation-induced heat hyperalgesia (in the tested doses), probably by acting both centrally and peripherally (as an antioxidant and/or anti-inflammatory). Further investigations on different probable mechanisms of action and dose-response studies would be helpful in identifying the specific site(s) of action and optimum doses of such thiadiazole derivatives. These investigations would be crucial in discovering more selective CA inhibitors useful in alleviating hyperalgesia induced by inflammation.



Scheme 1. Synthetic Pathways for the synthesis of compounds **(2-8)**



Scheme 2. Synthetic Pathways for the synthesis of compounds **(9-17)**



Scheme 3. Proposed mechanism for the synthesis of compound **(9)**

Table (1): Effect of ACTZ and each of the test compounds on paw licking latency, erythrocytes CA activity, blood GSH level, plasma MDA level, serum LDH activity in irradiated rats subjected to intramuscular carrageenan injection.

Parameter Groups	Paw licking latency (sec)	Erythrocytes CA activity (U/g Hgb)	Blood GSH level (mg/ml)	Plasma MDA level (nmol/ml)	Serum LDH activity (U/L)
Normal	10.67 ± 1.28	47.48 ± 7.60	36.18 ± 1.85	3.61 ± 0.70	36.91 ± 8.98
Irradiated	7.5 ± 0.96	40.22 ± 4.80	30.12 ± 2.56 a	10.91 ± 1.62 a	86.87 ± 7.63 a
Inflamed	2.57 ± 0.29 a	36.71 ± 5.26	19.62 ± 1.58	21.94 ± 3.32 ac	142.20 ± 28.55 a
Irradiated Inflamed	2.00 ± 0.31 a	51.95 ± 7.88 b	9.41 ± 0.71 ab	40.96 ± 2.45 ab	220.0 ± 71.99 ab
Irradiated Inflamed + Vehicle	2.06 ± 0.29 a	49.22 ± 6.71	10.22 ± 0.97 a	38.63 ± 3.41 a	203.6 ± 17.25 a
Irradiated Inflamed + ACTZ	9.00 ± 1.14 c	11.70 ± 0.87 c	27.17 ± 3.11	2.30 ± 0.20 c	18.45 ± 2.46 c
Irradiated Inflamed + **2**	11.5 ± 2.53 c	49.30 ± 12.43	33.12 ± 6.35 c	3.88 ± 0.22 c	10.88 ± 2.90 c
Irradiated Inflamed + **3**	9.75 ± 0.85 c	9.67 ± 3.39 c	36.91 ± 8.56 c	8.68 ± 1.04 c	9.88 ± 1.90 c
Irradiated Inflamed + **4**	4.5 ± 1.04 a	21.39 ± 8.66	12.18 ± 3.07 a	6.08 ± 1.08 c	28.01 ± 5.63 c
Irradiated Inflamed + **5**	4.75 ± 1.25	3.78 ± 0.63 ac	24.52 ± 9.36	10.2 ± 0.64 c	11.53 ± 1.64 c
Irradiated Inflamed + **6**	7.00 ± 0.40	26.33 ± 7.00	9.91 ± 2.86 a	11.12 ± 0.23 c	11.83 ± 3.15 c
Irradiated Inflamed + **7**	6.50 ± 1.19	18.40 ± 6.50	5.79 ± 0.78 a	13.32 ± 0.28 ac	6.59 ± 0.70 c
Irradiated Inflamed + **8**	5.50 ± 1.84	22.72 ± 13.76	4.61 ± 0.86 a	12.92 ± 0.33 ac	8.23 ± 1.64 c
Irradiated Inflamed + **9**	10.00 ± 0.91 c	6.79 ± 1.52 ac	7.89 ± 0.91 a	14.64 ± 0.73 ac	6.59 ± 0.90 c
Irradiated Inflamed + **10**	9.50 ± 0.50 c	10.36 ± 4.59 c	14.29 ± 4.42	5.72 ± 0.96 c	19.77 ± 8.07c
Irradiated Inflamed + **11**	4.75 ± 1.10	8.11 ± 3.95 ac	15.72 ± 6.73	6.12 ± 0.84 c	21.75 ± 9.32 c
Irradiated Inflamed + **15**	9.75 ± 1.84 c	35.08 ± 15.78	17.39 ± 5.14	4.64 ± 0.84 c	24.72 ± 10.20 c
Irradiated Inflamed + **16**	6.75 ± 1.03	4.59 ± 0.47 ac	31.27 ± 6.01	5.08 ± 1.65 c	6.63 ± 0.80 c
Irradiated Inflamed + **17**	7.75 ± 1.79	3.57 ± 0.62 ac	17.06 ± 7.20	6.64 ±1.45 c	21.42 ± 10.89 c

Values are represented as means ± Standard Error (SE), (n=6). Letters designate significant difference from normal group (a), inflamed group (b) and irradiated inflamed group (c), $p<0.05$.

Table (2): Effect of administration of naloxone (8 mg/kg) prior to injection of ACTZ, compounds **3**, **9** and **10** and their vehicle (DMSO) on in irradiated rats subjected to intramuscular carrageenan injection.

Parameter Group	Paw licking latency (sec)	Paw licking latency after naloxone injection(sec)
Normal	6.95 ± 0.96	7.3 ± 0.52
Irradiated	5.88 ± 0.61	6.78 ± 0.96
Inflamed	3.62 ± 0.47	3.87 ± 0.37
Irradiated Inflamed	3.02 ± 0.21	4.22 ± 0.31
Irradiated Inflamed + Vehicle	3.49 ± 0.47	3.16 ± 0.41
Irradiated Inflamed + ACTZ	10.33 ± 1.18 *	4.51 ± 0.47
Irradiated Inflamed + **3**	11.18 ± 0.46 *	3.13 ± 0.28
Irradiated Inflamed + **9**	9.20 ± 0.55 *	3.26 ± 0.27
Irradiated Inflamed + **10**	9.19 ± 0.52 *	2.49 ± 0.25

Values are represented as means ± SE, (n=8). * designate significant difference from the corresponding naloxone treated group) at $p<0.05$.

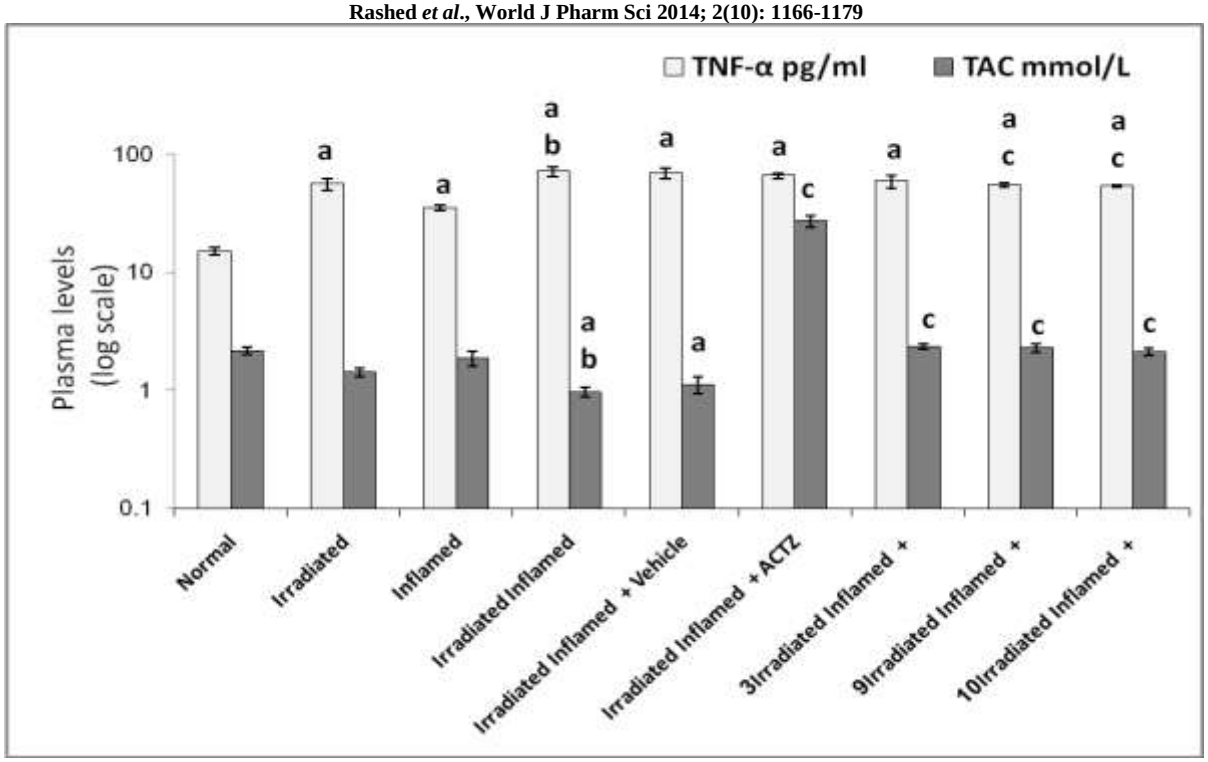


Figure (1): Effect of ACTZ, compounds **3**, **9** and **10** and their vehicle (DMSO) on plasma tumor necrosis factor-α (TNF-α) level and total antioxidant capacity (TAC) in irradiated rats subjected to intramuscular carrageenan injection. Values are represented as means ± Standard Error (SE), (n=6). Letters designate significant difference from normal group (a), inflamed group (b) and irradiated inflamed group (c), $p<0.05$.

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