RSC Advances

PAPER

View Article Online
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Cite this: *RSC Adv.*, 2024, **14**, 995

Design, synthesis, pharmacological evaluation, and *in silico* studies of the activity of novel spiro pyrrolo [3,4-*d*]pyrimidine derivatives†

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In the present study, spiro compounds are shown to have distinctive characteristics because of their interesting conformations and their structural impacts on biological systems. A new family of functionalized spiro pyrrolo[3,4-*d*]pyrimidines is prepared *via* the one-pot condensation reaction of amino cyclohexane derivatives with benzaldehyde to prepare fused azaspiroundecanedione and azaspirodecenone/thione derivatives. A series of synthesized spiro compounds were scanned against DPPH and evaluated for their ability to inhibit COX-1 and COX-2. All compounds exhibit significant antiinflammatory activity, and they inhibited both COX-1 and COX-2 enzymes with a selectivity index higher than celecoxib as a reference drug. The most powerful and selective COX-2 inhibitor compounds were **11** and **6**, with selectivity indices of 175 and 129.21 in comparison to 31.52 of the standard celecoxib. However, candidate **14** showed a very promising antiinflammatory activity with an IC_{50} of 6.00, while celecoxib had an IC_{50} of 14.50. Our findings are promising in the area of medicinal chemistry for further optimization of the newly designed and synthesized compounds regarding the discussed structure–activity relationship study (SAR), in order to obtain a superior antioxidant lead compound in the near future. All chemical structures of the novel synthesized candidates were unequivocally elucidated and confirmed utilizing spectroscopic and elemental investigations.

Received 17th October 2023
Accepted 9th December 2023

DOI: 10.1039/d3ra07078f

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Introduction

The pyrimidine ring is an azaaromatic scaffold that can be found in many molecules of biological or pharmaceutical interest. Multicomponent reactions (MCRs) are interesting types of reaction due to the mixing of three or more reactants in one-pot and the generation of one product, and they are economically useful and environmentally secure compared to multi-step methods. Spiro heterocycles have significant uses in the pharmaceutical industry.[1] The spiro heterocyclic structure is a distinguishing property of various natural and synthetic compounds with extraordinary biological activity.[2–4] The potential application of spiro heterocycles in pharmaceutical chemistry has been widely reported due to their good pharmacological characteristics[5–11] and geochemistry.[12–14] In addition, they have different uses as photocurrent resources[15] and asymmetric catalysts.[16] Recently, piperidines have been among the most crucial synthetic building blocks for drug development and are essential to the pharmaceutical sector.[17] Together with alkaloids, their derivatives are found in more than twenty different families of drugs. Numerous reviews on distinct piperidine synthesis techniques,[18–21] their functionalization,[22] and their pharmacological applications[23–25] have been published recently. Moreover, the bile pigments bilirubin and biliverdin, as well as vitamin B12, include analogs of one of the heterocycles, pyrrole, which is not naturally generated.[26–28] Pharmaceutical effects of pyrrole-ring-containing medications include antipsychotic,[29] antiinflammatory,[30,31] analgesic, antidepressant,[32] antimicrobial,[33] anticonvulsant,[34] antineoplastic,[35,36] *etc.* More active compounds have been produced by combining different pharmacological agents with a pyrrole and pyrrolidine ring structure.[37–39] There has been growing interest in the biological effects of pyrrole and its derivatives.[40,41] Antioxidant activity has been demonstrated for pyrroles and their derivatives, which include an active hydrogen atom (N–H).[42–44] Pyrimidines and pyridine-related compounds, on the other hand, are an important family of heterocycles due to their numerous chemical and biological uses. They are frequently used in the fields of medicine and material science.[45–47] Their pharmacological effects include those that are antiinflammatory,[48] antipyretic,[49] antihypotensive,[50] anticonvulsant,[51]

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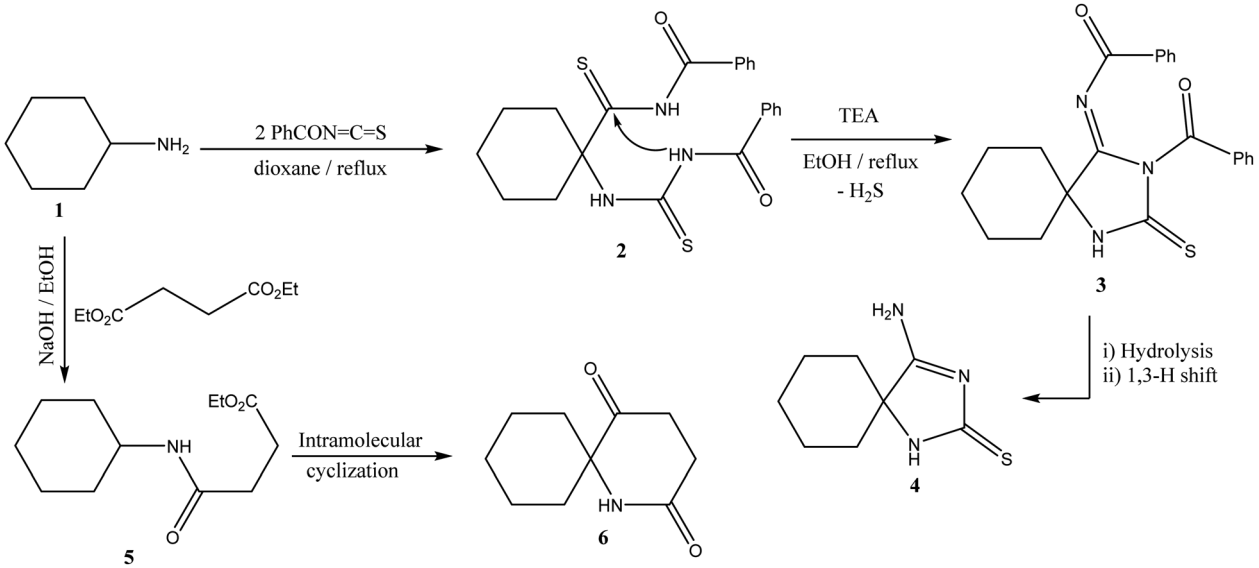
† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d3ra07078f

antiviral,[52] antibacterial,[53] and antidiabetic.[54] We previously developed and produced some novel antiinflammatory and antioxidant molecules.[55] One of the best-known computational tools applied in the field of medicinal chemistry is molecular docking, which is one of the most effective techniques to exhibit the architectures expected to have a high impact on specific proteins.[56] Consequently, it will be possible to recognize which specific structural modifications are required to achieve outstanding efficiency in a simple and low-cost way.[57–60] Fortunately, our candidates exhibited significant antiinflammatory inhibitory activity. These candidates also exhibited considerable antioxidant activity compared to ascorbic acid. The main objective of our study was to continue our efforts in this approach by synthesizing and characterizing a new set of spiro heterocyclic compounds and estimating their energies, which is crucial for both theoretical investigations and chemical reactivity.

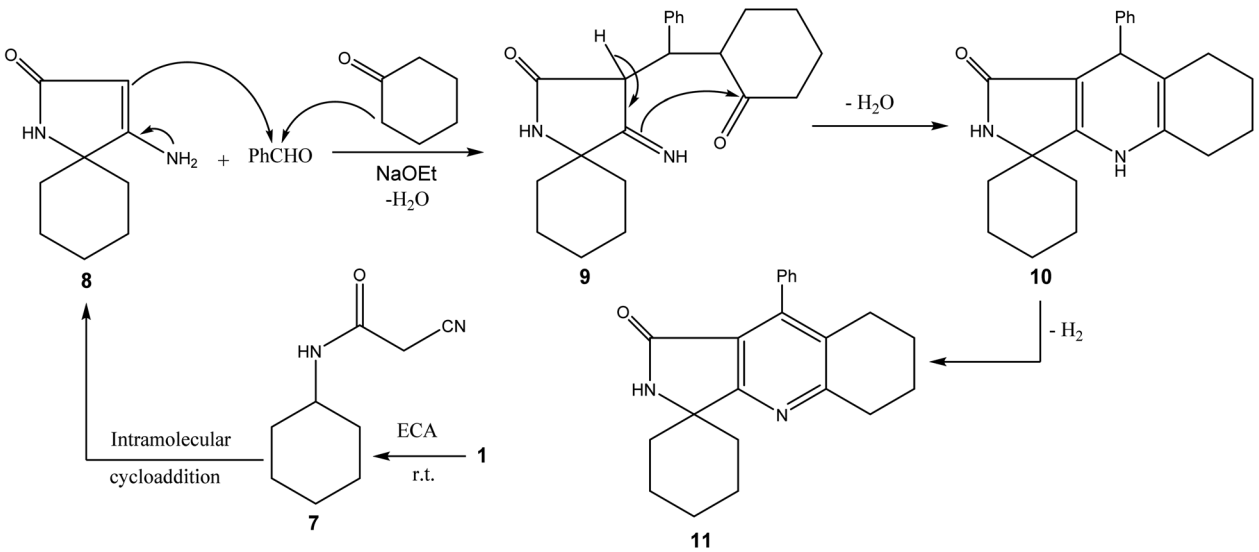
Results and discussion

Chemistry

Our strategy to design and synthesize novel heterocyclic moieties involves the nucleophilic addition of the amino group of cyclohexyl amine to the heteroallene carbon of benzoyl isothiocyanate through aza Michael reaction producing acyl thiourea **3** that undergoes cyclization through the evolution of H_2S and basic hydrolysis forming spiro pyrazole **4**. The IR spectrum of **4** shows C=S absorption at 1248 cm^{-1}. The downfield singlet signals located at 8.1 and 8.08 ppm were attributed to NH and NH_2, respectively. Base-mediated cyclo-condensation of



Scheme 1 Synthetic route of spiro azines **4** and **6**.



Scheme 2 Synthesis of polycyclic azine **11**.

compound **1** and diethyl succinate resulted in pyridine cyclization affording spiro derivative **6**. The NH downfield exchangeable singlet signal for NH was observed at 8.15 ppm, and elemental analysis data have been discussed for a representative (Scheme 1).

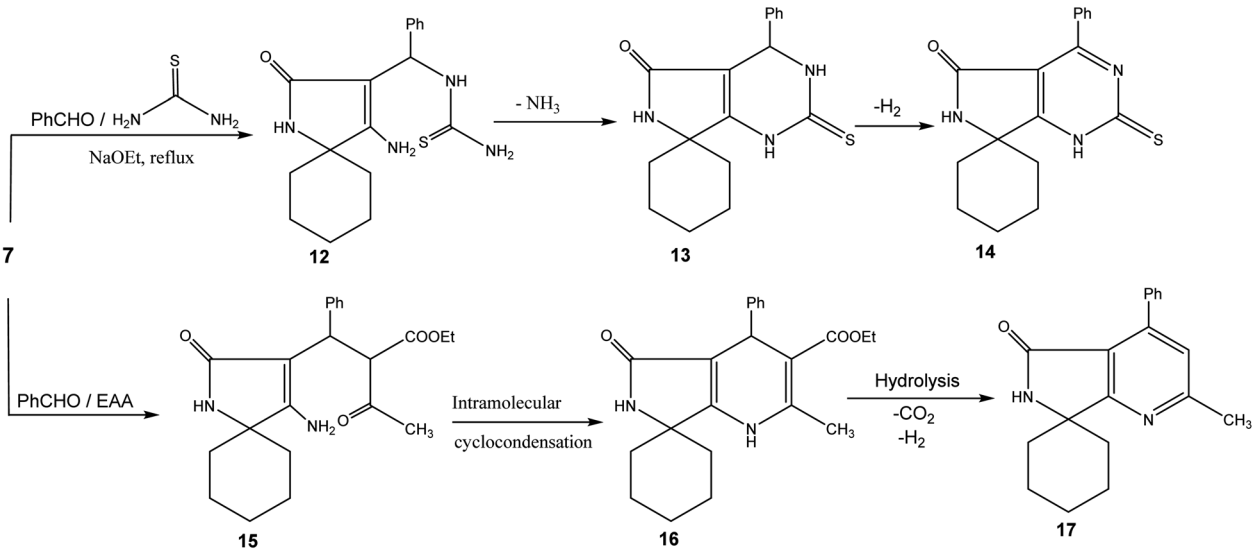
Compound **1** was cyclized with ethyl cyanoacetate at room temperature to give 4-amino-1-azaspiro[4.5]dec-3-en-2-one **8**.[1] The latter compound underwent cyclization with cyclohexanone and benzaldehyde through 1,4 additions to give a polycyclic derivative **11**. The carbonyl absorption band of **11** was exhibited at 1636 cm^{-1}. The exchangeable NH signal was detected at 9.16 ppm, and the carbonyl carbon signal was shown at 198.26 ppm (Scheme 2).

Reacting spiro compound **8** with equivalent amounts of benzaldehyde and thiourea provided pyrimidine derivative **14**. NH, C=O, and C=S absorption bands of **14** were shown at 3349, 1670, and 1251 cm^{-1}, respectively. The ^{1}H NMR spectrum of **14** showed two NH signals at 8.25 and 8.23 ppm.

Cyclic enamine **8** was allowed to react with a mixture of ethyl acetoacetate and benzaldehyde in a basic medium providing non-isolable Michael adduct **15** that goes through cyclodehydration and hydrolysis, followed by evolution of CO_2 and subsequent aromatization. The NH and C=O peaks were shown at 3348 and 1668 cm^{-1}, respectively. The NH singlet signal was located at 8.25 ppm and the carbonyl carbon signal was detected at 160.32 ppm (Scheme 3).

In silico studies

Molecular docking studies. Docking studies are used to predict the effectiveness of newly synthesized compounds. These theoretical studies (molecular docking) emphasize the bioactivities of our candidates. Molecular Operating Environment (MOE) 2015 software was used to assess our candidates. Due to the structural similarity of our candidates with indomethacin and celecoxib, we performed docking studies for the active sites of proteins COX-1 (PDB: 3KK6) and COX-2 (PDB:



Scheme 3 Synthesis of pyrimidine and pyridine derivatives **14** and **17**.

Table 1 The binding scores, receptor interactions, distances, and energies of our promising candidates **4**, **6**, **11**, **14**, and **17** compared to indomethacin as a reference standard inside the binding pocket of cyclooxygenase-2

			Interaction				
Ligand	Docking score	RMSD	Ligand	Receptor	Type of interaction	Distance (Å)	E (kcal mol^{-1})
4	−5.01	1.2	S12	Val523	H-acceptor	3.79	−1.1
			S12	Gly526	H-acceptor	3.66	−0.9
6	−5.5	1.4	O13	Arg120	H-acceptor	3.19	−3.7
11	−5.03	1.3	6-Ring	Arg120	Pi-cation	4.39	−1.2
14	−6.5	0.78	S3	Arg120	H-acceptor	3.81	−0.8
17	−5.5	1.3	O16	Arg120	H-acceptor	3.17	−0.8
			O16	Tyr355	H-acceptor	2.60	−0.9
Indomethacin (docked)	−8.8	1.2	O7	Arg120	H-acceptor	2.84	−3.9
			O7	Tyr355	H-acceptor	2.85	−3.5
			O25	Ser530	H-acceptor	2.92	−1.2
			O6	Arg120	Ionic	2.86	−5.5
			O7	Arg120	Ionic	2.84	−5.6

Table 2 Predicted two and three-dimensional binding interactions of **4**, **6**, **11**, **14**, and **17** visualized in the cyclooxygenase-2 active site (PDB: 4COX) compared to the predicted binding orientations of indomethacin; ligands are shown in green

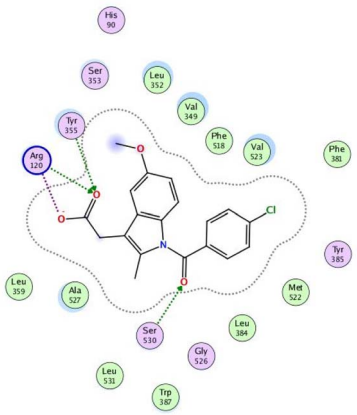
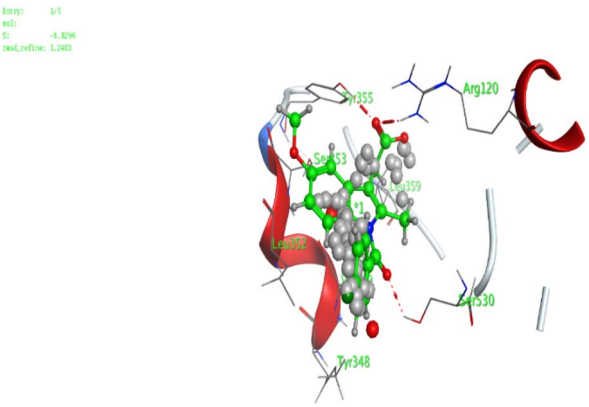
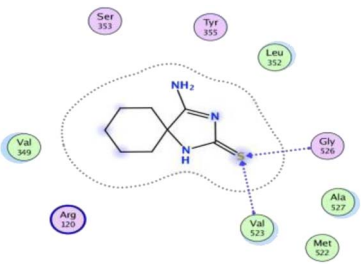
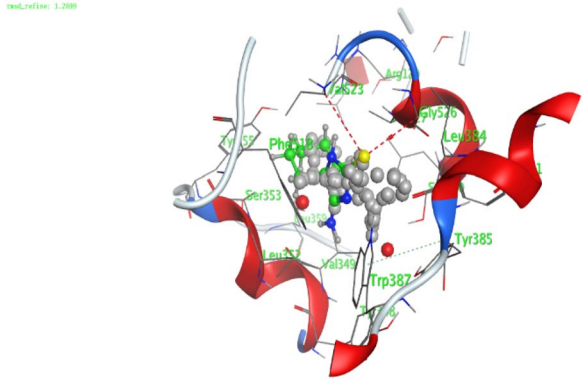
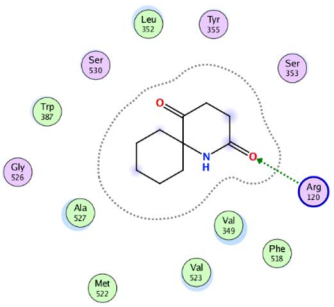
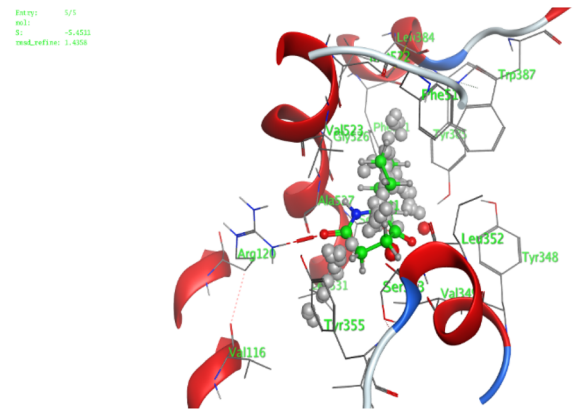
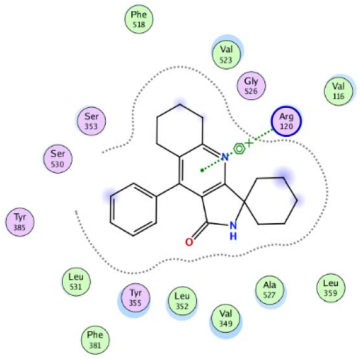
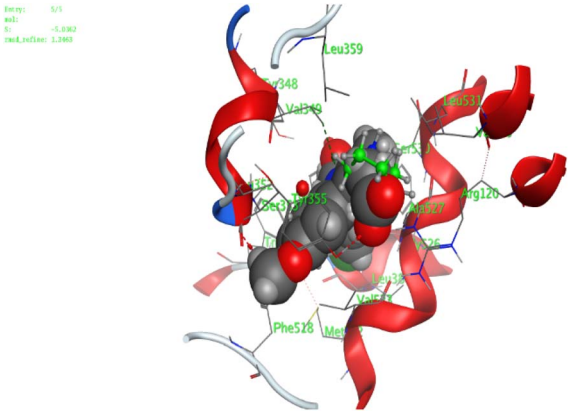
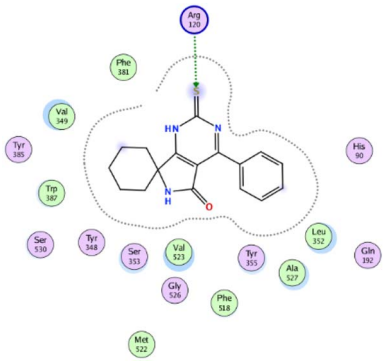
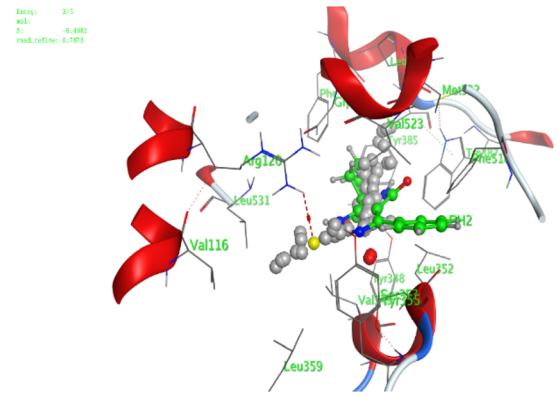
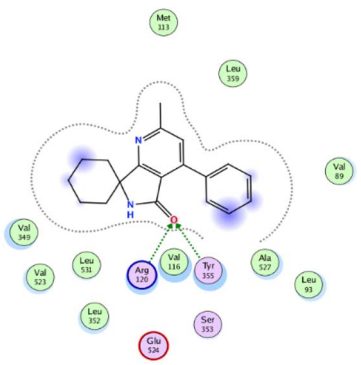
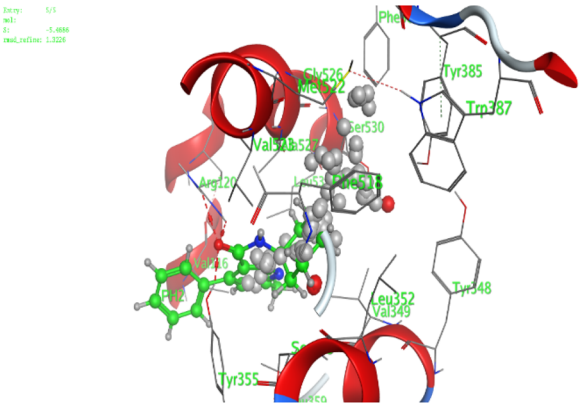
Ligand	2D receptor interaction	3D receptor interaction
Indomethacin		
Compound **4**		
Compound **6**		

Table 2 (*Contd.*)

Ligand	2D receptor interaction	3D receptor interaction
Compound **11**		
Compound **14**		
Compound **17**		

4COX).[61,62] Therefore, the crystal structure of cyclooxygenase-2 enzyme in a complex with indomethacin (PDB: 4COX) and the crystal structure of cyclooxygenase-1 in a complex with Celecoxib (PDB: 3KK6) were downloaded from the protein data bank. All proteins were prepared by removing water molecules and 3D protonation, then fixing the potential energies. Table 1 reveals the docking studies results of compounds **4**, **6**, **11**, **14**, and **16** with the receptors of the largest pocket of chain A of cyclooxygenase-2 enzyme 4COX. Indomethacin as a reference compound was re-docked in the prepared largest pocket to bind with Arg120, Tyr355, and Ser530 *via* three hydrogen bonds at 2.84, 2.85, and 2.92 Å with binding score −8.8 kcal mol^{-1}. Our candidates exhibit significant binding scores by comparing with indomethacin. Compounds **14** and **17** show superb antiinflammatory activities due to their high stabilization inside the active site of the COX-2 enzyme with considerable binding scores (−6.5 and −5.5 kcal mol^{-1}, respectively). These candidates were bound with crucial amino acids Arg120 and Tyr355 *via* hydrogen bonding, as shown in Table 2. Compounds **4**, **6**, and **11** exhibit moderate inhibition values for the COX-2 enzyme with notable binding scores of -5.01, −5.5, and −5.03 kcal mol^{-1}, respectively, as shown in Tables 1 and 2.

Table 3 The binding scores, receptor interactions, distances, and energies of our promising candidates **4**, **6**, **11**, **14**, and **17** compared to celecoxib as a reference standard inside the binding pocket of cyclooxygenase-1

			Interaction				
Ligand	Docking score	RMSD	Ligand	Receptor	Type of interaction	Distance (Å)	E (kcal mol^{-1})
4	−5.07	0.74	N11	Met 522	H-donor	3.56	−0.8
			S12	Ser 353	H-acceptor	3.49	−1.7
6	−4.6	1.55	O12	Arg120	H-acceptor	2.94	−1.2
11	−6.26	0.77	O19	Ile 523	H-acceptor	3.16	−0.7
14	−6.78	1.02	N9	Ser 530	H-donor	2.81	−2.0
			S3	Ser 353	H-acceptor	3.63	−1.2
17	−6.9	1.9	6-Ring	Ser 353	Pi–H	4.43	−1.2
Celecoxib (docked)	−7.7	0.8	N3	Leu 352	H-donor	2.81	−7.0
			O2	His 90	H-acceptor	2.88	−2.8
			5-Ring	Ser 353	Pi–H	4.35	−0.9

In the current work, our candidates exhibit very promising activities against the COX-1 enzyme compared with celecoxib as an isolated reference ligand from the original protein pocket, as shown in Table 3. Celecoxib was stabilized inside cyclooxygenase-1 with binding energy −7.7 kcal mol^{-1} through the formation of three H-bonds with the amino acid residues Leu352, His90, and Ser353. Compounds **14** and **17** show very promising inhibition potential of the COX-1 enzyme with high binding scores (−6.78 and −6.9 kcal mol^{-1}, respectively). Candidate **11** reveals moderate inhibition of COX-1 through interaction with amino acid residue Ile 523 inside the prepared protein pocket with binding energy −6.26 kcal mol^{-1}, while candidates **4** and **6** show modest inhibition activities, as shown in Tables 3 and 4.

In vitro antiinflammatory activity

The human red blood cell (RBC) membrane stabilization assay is a method used to evaluate the ability of a substance to prevent damage to the RBC membrane. The assay is based on the principle that certain substances can stabilize the RBC membrane and prevent hemolysis, or the breakdown of RBCs subjected to stressors. The extent of hemolysis is measured by spectrophotometry or by other methods such as the release of hemoglobin. The degree of RBC membrane stabilization can be quantified by calculating the percentage of hemolysis inhibition.

The human RBC membrane stabilization assay is commonly used to evaluate the antiinflammatory and antioxidant activity of natural products, such as plant extracts, and synthetic compounds. The assay can also be used to assess the potential toxicity of drugs and chemicals to RBC membranes. All the tested compounds showed *in vitro* antiinflammatory activity upon using human red blood cell hemolysis and membrane stabilization assay. As shown in Table 5, of the newly synthesized azaspiro compounds, compound **4** exhibited potent hemolytic inhibition of 89.9% in comparison to 90% of that of the standard indomethacin, when all of the tested compounds were used at a concentration of 200 μg mL^{-1}. Other cyclohexane pyrrolo compounds showed hemolysis inhibitions varying from 61.3% to 79.2% when used at the same concentration of 200 μg mL^{-1}. On the other hand, previous studies have reported that some azaspiro compounds had higher efficacy and selectivity than polyunsaturated fatty acids in alleviating inflammatory symptoms in prototypical autoimmune diseases.[63]

GPCRs are very important to prevent colorectal cancer progression, as it is essential to maintain the integrity of the mucosal barriers.[64] Azaspiro compounds were evaluated biologically, and tested as potential antitumor agents.[65] They affect different cell cycle stages, and granular actin diffusion through the cytoplasm was observed using confocal microscopy. Other compounds containing azaspiro rings exhibited a potent kinase inhibitor activity.[66]

These compounds can be used to stop metastasis and the progression of tumors. Tyrosine kinase inhibitors were found to reduce proinflammatory cytokines such as TNF-alpha and IL-1 in addition to reducing the expression of cyclooxygenase 2 and inducible nitric oxide synthase (INOS).[67] It was also found that compounds containing the cyclohexane group attenuated the cardiac injury induced by CCl_4 in Wistar rats. They inhibited lipooxygenase and decreased inflammatory markers like TNF-α and IL-6.[68] Additionally, new cyclohexane fused spiroselenuranes showed potent antioxidant activity.[69] It has been reported that some azaspiro compounds had antioxidant and antielastase activity,[70] as it was found that human neutrophil elastase modulates cytokine and growth factor expression. The antiinflammatory activity of the newly synthesized compounds was also evaluated for COX-1/COX-2 inhibitory activities using an ovine COX-1/human recombinant COX-2 assay kit, and celecoxib was used as a reference standard drug as shown in Table 6. The results revealed that all the tested compounds showed a potent antiinflammatory activity and they inhibited both COX-2 and COX-1 enzymes, and with a selectivity index higher than that of celecoxib as a reference drug. The most powerful and selective COX-2 inhibitor compounds were **11** and **6** with selectivity indices of 175 and 129.21 in comparison to 31.52 of the standard celecoxib.

In vitro antioxidant activity

Free radicals and reactive oxygen species are associated with aging, inflammation, cancer, atherosclerosis, and

Table 4 Predicted two and three-dimensional binding interactions of **4**, **6**, **11**, **14**, and **17** visualized in the cyclooxygenase-1 active site (PDB: 3KK6) compared to the predicted binding orientation of celecoxib; ligands are shown in green

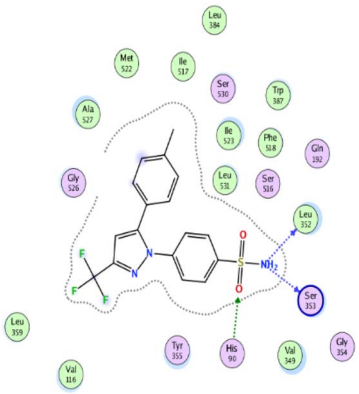
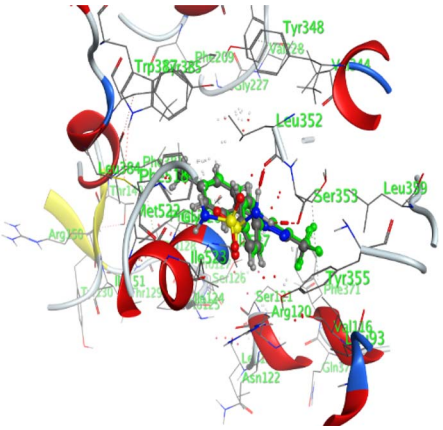
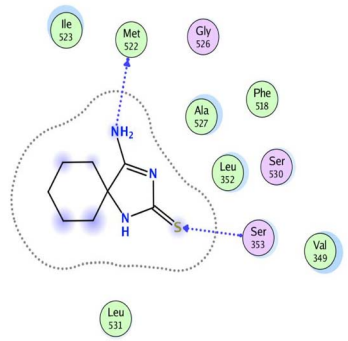
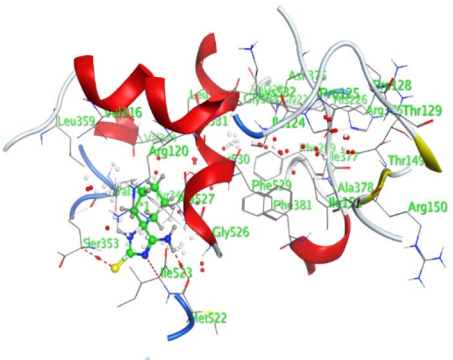
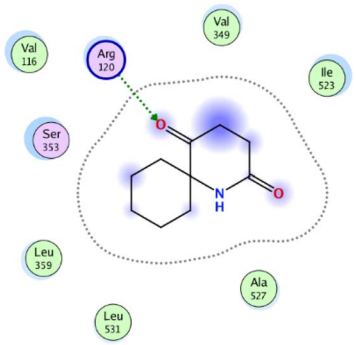
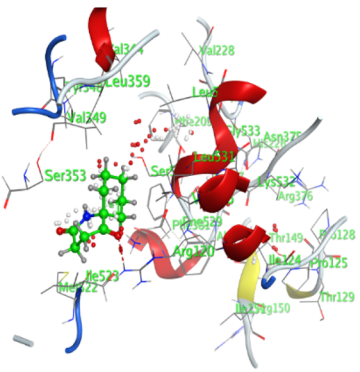
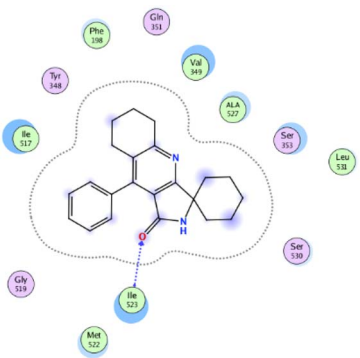
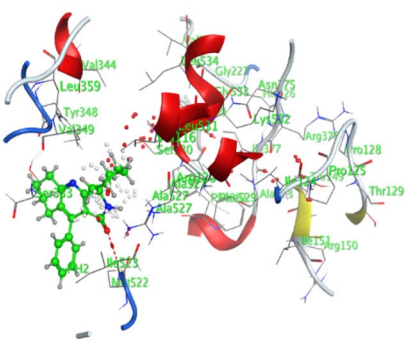
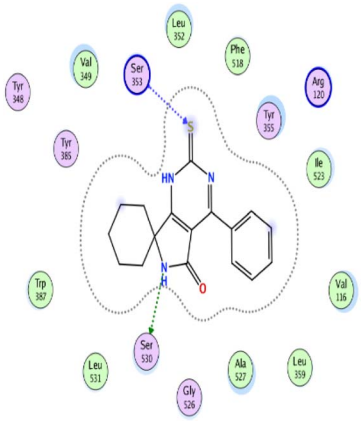
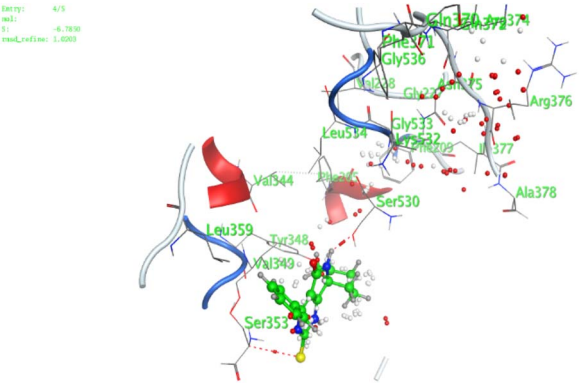
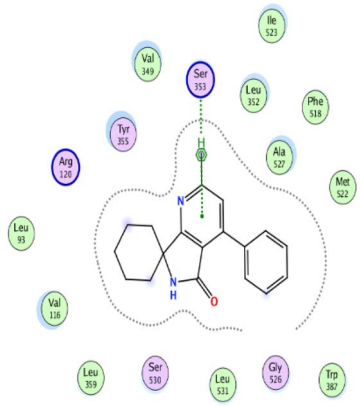
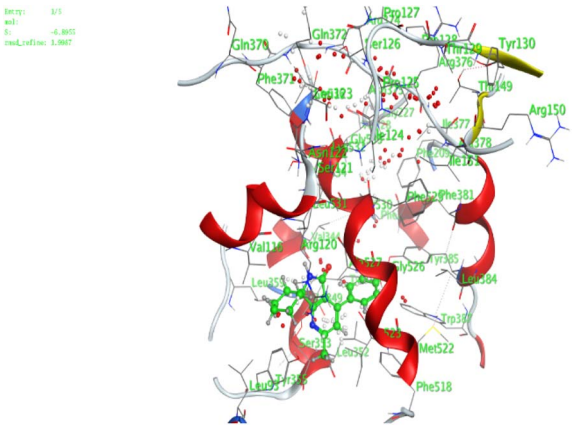
Ligand	2D receptor interaction	3D receptor interaction
Celecoxib		
Compound **4**		
Compound **6**		
Compound **11**		

Table 4 (*Contd.*)

Ligand	2D receptor interaction	3D receptor interaction
Compound **14**		
Compound **17**		

neurodegenerative disorders. Antioxidants play an important role to prevent and or stop the progression of such conditions.[71] The antioxidant activity of the newly synthesized compounds was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. This assay principle is based on electron transfer, where the DPPH radical, a stable free radical, is reduced by an antioxidant to form a non-radical form. The degree of discoloration of the DPPH radical solution is measured spectrophotometrically at 517 nm, and the decrease in absorbance indicates the scavenging activity of the antioxidant.

Table 5 Hemolysis inhibition (%) for the synthesized compounds at different concentrations

Sample concentration (μg mL^{-1})	100	200	400	600	800	1000
Comp. **4**	88.4	89.9	92.8	94.6	96.0	97.8
Comp. **6**	58.2	61.3	69.0	73.2	78.2	84.7
Comp. **11**	76.1	79.2	107.8	86.4	91.1	96.0
Comp. **14**	67.6	74.4	78.8	82.6	85.1	94.0
Comp. **17**	69.2	74.6	79.4	82.7	85.4	90.1
Standard (indomethacin)	88.0	90.1	93.5	95.3	97.3	98.9

The antioxidant activity of a compound is expressed as the percentage of DPPH radical scavenging, and the IC_{50} value, which is the concentration of the antioxidant required for scavenging of 50% of the DPPH radicals as a measure of the antioxidant capacity. It was found that all the tested compounds had different antioxidant activities (at a concentration of 1000 μg mL^{-1}) to that of ascorbic acid as a standard drug (Table 7). As per Jun *et al.* and referenced textbooks,[72,73] antioxidant effectiveness is categorized based on IC_{50} values. An IC_{50} below 50 μg mL^{-1} denotes highly potent antioxidant activity, while activity is labeled as strong within the range of 50–100 μg mL^{-1}, moderate at 101–250 μg mL^{-1}, and weak at 250–500 μg mL^{-1}, and the compound is deemed inactive at an IC_{50} of greater than 500 μg mL^{-1}. The obtained results revealed that compound 11 exhibited a strong antioxidant activity while compound 6 had a moderate activity with IC_{50} values of 33.0 and 94.04 μg mL^{-1} in comparison to 4.08 μg mL^{-1} of the standard ascorbic acid, and DPPH scavenging percentages of 86.1%, 74.4% and 97%, respectively. Compounds 14 and 4 demonstrated the lowest antioxidant activity, with IC_{50} values of 222.9 and 263.3 μg mL^{-1}, respectively, along with DPPH scavenging percentages of 66.5% and 61.6%, respectively.

Table 6 *In vitro* antiinflammatory activity for COX-1 and COX-2

Sample number	IC_{50}[a] (μM) COX-1	IC_{50}[a] (μM) COX-2	IC_{50}[b] (μM) COX-1/COX-2
4	7.50	0.135	55.56
6	11.50	0.089	129.21
11	10.50	0.060	175.00
14	6.00	0.080	75.00
17	8.00	0.099	80.81
Celecoxib	14.50	0.0460	31.52

[a] Values are means of three determinations acquired using an ovine COX-1/COX-2 assay kit (catalog no. 560131; Cayman Chemicals Inc. Ann Arbour, MI, USA). Celecoxib was used as a reference drug. [b] *In vitro* COX-2 selectivity index (IC50 of COX-1/COX-2).

Table 7 Antioxidant assay for the tested compounds

Compound	IC_{50}	DPPH scavenging%
Comp. **4**	263.3	61.1
Comp. **6**	94.04	74.4
Comp. **11**	33.0	86.1
Comp. **14**	222.9	66.5
Standard (ascorbic acid)	4.08	97

Materials and methods

Synthesis

***N*-((1-(Benzoylcarbamothioyl)cyclohexyl)carbamothioyl)benzamide (2).** A mixture of cyclohexyl amine (0.025 mol) and benzoyl isothiocyanate (0.025 mol) in dioxane (15 mL) was heated under reflux for 30 min., then stirred for 12 hours and the formed precipitate was filtered off, dried, and recrystallized from dilute ethanol to give compound **3** as a yellowish white powder.

M.P. 130 °C, yield: 96%. IR (KBr) ν_{max}: 3220, 3299 (NH), 2935 (CH aliphatic), 1675 (C=O), 1233 (C=S) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.23 (s, 1H, NH), 9.85 (s, 1H, NH), 9.56 (s, 1H, NH), 7.48–7.93 (m, 10H, Ph-H), 2.2 (m, 10H, cyclohexane-H). Anal. Calcd for $C_{22}H_{23}N_3O_2S_2$ (425.57): C, 62.09; H, 5.45; N, 9.87; S, 15.07; found: C, 62.14; H, 5.42; N, 9.85; S, 15.04.

4-Amino-1,3-diazaspiro[4.5]dec-3-ene-2-thione (4). A mixture of compound **2** (0.025 mol) and TEA (4 drops) in 15 mL of ethanol was heated under reflux for 2 hours. The precipitate formed after concentrating the reaction mixture and acidification with acetic acid was filtered off, dried, and recrystallized from dilute ethanol to give compound **6** as a white powder.

M.P.: 110 °C, yield: 93%. IR (KBr) ν_{max}: 3279 (NH), 2933 (CH aliphatic), 1248 (C=S) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.10 (s, 1H, NH), 8.08 (s, 2H, NH_2), 2.2 (m, 10H, Ph-H), 1.27–1.75 (m, 10H, cyclohexane-H). Anal. Calcd for $C_8H_{13}N_3S$ (183.27): C, 52.43; H, 7.15; N, 22.93; S, 17.50; found: C, 52.38; H, 7.18; N, 22.90; S, 17.55.

1-Azaspiro[5.5]undecane-2,5-dione (6). A mixture of cyclohexylamine (0.02 mol), diethyl succinate (0.02 mol), and NaOH (0.02 mol) in ethanol (15 mL) was heated under reflux for 5 hours. The precipitate formed after concentrating the reaction mixture and acidification with HCl was filtered off, dried, and recrystallized from ethanol to give compound **16** as white crystals.

M.P.: 190 °C, yield: 65%. IR (KBr) ν_{max}: 2937 (CH aliphatic), 1665 (C=O) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.15 (s, 1H, exchangeable with D_2O, NH), 2.41–2.50 (m, 4H, $2CH_2$, pyridine-H) 1.18–1.92 (m, 10H, cyclohexane-H). Anal. Calcd for $C_{10}H_{15}NO_2$ (181.23): C, 66.27; H, 8.34; N, 7.73; found: C, 66.32; H, 8.29; N, 7.77.

9′-Phenyl-5′,6′,7′,8′-tetrahydrospiro[cyclohexane-1,3′-pyrrolo[3,4-*b*]quinolin]-1′(2′*H*)-one (11). A mixture of enaminone **8** (0.02 mol), cyclohexanone (0.02 mol), benzaldehyde (0.02 mol), and sod. ethoxide (0.02) in ethanol (15 mL) was stirred for 2 hours and then heated under reflux for 2 hours. The precipitate formed after concentrating the reaction mixture and acidification with HCl was filtered off, dried, and recrystallized from EtOH/AcOH to give compound **21** as a yellowish-white powder.

M.P.: 90 °C, yield: 75%. IR (KBr) ν_{max}: 2927 (CH aliphatic), 1636 (C=O) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.16 (s, 1H, exchangeable with D_2O, NH), 7.07–7.39 (m, 5H, Ph-H) 1.02–3.28 (m, 18H, cyclohexane-H). ^{13}C NMR (400 MHz, DMSO-d_6) δ (ppm): 26.97, 118.11, 118.29, 123.57, 124.28, 127.83, 128.37, 129.33, 129.49, 130.53, 130.83, 131.52, 135.05, 144.31, 152.51, 198.26. Anal. Calcd for $C_{22}H_{24}N_2O$ (332.44): C, 79.48; H, 7.28; N, 8.43; found: C, 79.55; H, 7.21; N, 8.47.

4′-Phenyl-2′-thioxo-1′,2′-dihydrospiro[cyclohexane-1,7′-pyrrolo[3,4-*d*]pyrimidin]-5′(6′*H*)-one (14). A mixture of enaminone **8** (0.01 mol), benzaldehyde (0.01 mol), thiourea (0.01 mol), and sod. ethoxide (0.01) in ethanol (15 mL) was heated under reflux for 4 hours. The precipitate formed after concentrating the reaction mixture and acidification with HCl was filtered off, dried, and recrystallized from dilute ethanol to give compound **14** as a white powder.

M.P.: 130 °C, yield: 90%. 3349 (NH), 2920 (CH aliphatic), 1670 (C=O), 1251 (C=S) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.25 (s, 1H, NH), 8.23 (s, 1H, NH), 7.56–8.10 (m, 5H, Ph-H) 1.0–1.80 (m, 10H, cyclohexane-H). ^{13}C NMR (400 MHz, DMSO-d_6) δ (ppm): 24.77, 25.14, 32.00, 49.02, 107.13, 116.42, 129.21, 129.88, 132.03, 132.16, 149.91, 160.35. Anal. Calcd for $C_{17}H_{17}N_3OS$ (311.40): C, 65.57; H, 5.50; N, 13.49; S, 10.30; found: C, 65.61; H, 5.46; N, 13.47; S, 10.26.

2′-Methyl-4′-phenylspiro[cyclohexane-1,7′-pyrrolo[3,4-*b*]pyridin]-5′(6′*H*)-one (17). A mixture of enaminone **8** (0.01 mol), ethyl acetoacetate (0.01), benzaldehyde (0.01 mol), and TEA (4 drops) in dioxane (20 mL) was heated under reflux for 3 hours.

The precipitate formed after concentrating the reaction mixture and acidification with acetic acid was filtered off, dried, and recrystallized from ethanol to give compound **17** as a white powder.

M.P.: 125 °C, yield: 95%. 3348 (NH), 2919 (CH aliphatic), 1668 (C=O), 1603 (C=N) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.25 (s, 1H, NH), 7.27–8.10 (m, 6H, Ph-H + pyridine-H), 3.66 (s, 3H, CH_3), 1.13–1.81 (m, 10H, cyclohexane-H). ^{13}C NMR (400 MHz, DMSO-d_6) δ (ppm): 24.76, 25.13, 31.98, 49.00, 107.12, 116.40, 129.18, 129.86, 132.02, 132.13, 149.89, 160.32. Anal. Calcd for $C_{19}H_{20}N_2O$ (292.37): C, 78.05; H, 6.89; N, 9.58; found: C, 78.12; H, 6.84; N, 9.63.

In vitro antiinflammatory activity

Using human RBC hemolysis and membrane stabilization assay

Preparation of erythrocyte suspension. Fresh whole blood (3 mL) collected from healthy volunteers into heparinized tubes was centrifuged at 3000 rpm for 10 min. A volume of normal saline equivalent to that of the supernatant was used to dissolve the red blood pellets. The volume of the dissolved red blood pellets obtained was measured and reconstituted as a 40% v/v suspension with isotonic buffer solution (10 mM sodium phosphate buffer, pH 7.4). The buffer solution contained 0.2 g of NaH_2PO_4, 1.15 g of NaH_2PO_4, and 9 g of NaCl in 1 liter of distilled water. The reconstituted red blood cells (resuspended supernatant) were used.[72]

Hypotonicity-induced hemolysis. Samples used in this test were dissolved in distilled water (hypotonic solution). The hypotonic solution (5 mL) containing graded doses of the sample (100, 200, 400, 600, 800, and 1000 μg mL) was put into duplicate pairs (per dose) of centrifuge tubes. Isotonic solution (5 mL) containing graded doses of the sample (100–1000 μg mL^{-1}) was also put into duplicate pairs (per dose) of centrifuge tubes. Control tubes contained 5 mL of the vehicle (distilled water) and 5 mL of 200 μg mL^{-1} of indomethacin. Erythrocyte suspension (0.1 mL) was added to each of the tubes and mixed gently. The mixtures were incubated for 1 hour at room temperature (37 °C), and afterwards, centrifuged for 3 min at 1300 g. Absorbance (OD) of the hemoglobin content of the supernatant was estimated at 540 nm using a Spectronic (Milton Roy) spectrophotometer. The percentage of hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water to be 100%. The percent inhibition of hemolysis by the extract was calculated thus:

$$\%\ \text{Inhibition of hemolysis} = 1 - ((\text{OD2} - \text{OD1})/(\text{OD3} - \text{OD1})) \times 100$$

where OD1 = absorbance of test in an isotonic solution, OD2 = absorbance of test in a hypotonic solution, OD3 = absorbance of control in a hypotonic solution.

In vitro inhibitory effect of COX-1 and COX-2

All the synthesized compounds are evaluated for their ability to inhibit COX-1 and COX-2 using an ovine COX-1/COX-2 inhibitor screening assay kit (catalog no. 560131; Cayman Chemicals Inc. Ann Arbour, MI, USA) that utilizes the peroxidase component of COX. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine (TMPD), which is produced during the reduction of PGG2 to PGH2, at 590 nm.[38] The results are listed in Table 6 as the compound's concentration causing 50% enzyme inhibition (IC_{50}) and they are the means of three determinations. In addition, the selectivity index (SI values), which was defined as IC_{50} (COX-1)/IC_{50} (COX-2), was calculated for the tested compounds. Additionally, the IC_{50} values of celecoxib (the reference standard) on COX-1 and COX-2 were determined.[73]

In vitro antioxidant activity by DPPH scavenging%

Free radical scavenging activities of different newly synthesized compounds were measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH).[74] In brief, a 0.1 mM solution of DPPH in ethanol was prepared. This solution (1 mL) was added to 3 mL of different samples in ethanol at a concentration of 1000 μg mL^{-1}. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min, then the absorbance was measured at 517 nm by using a spectrophotometer (UV-VIS Milton Roy). The reference standard compound being used was ascorbic acid and the experiment was done in triplicate. The IC_{50} value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using the log dose inhibition curve. A lower absorbance of the reaction mixture indicated higher free radical activity. The percent DPPH scavenging effect was calculated by using the following equation:

$$\text{DPPH scavenging effect (\%) or percent inhibition} = A_0 - A_1/A_0 \times 100.$$

where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of a test or standard sample.

Molecular docking studies

The molecular docking program, Molecular Operating Environment (MOE) version 2015 software, can directly simulate the interaction of ligands with enzymes cyclooxygenase-1 and cyclooxygenase-2. The X-ray crystallographic structures of the cyclooxygenase-2 enzyme (PDB ID: 4COX) and cyclooxygenase-1 enzyme (PDB: 3KK6) were downloaded from the protein data bank.[75,76] Celecoxib and indomethacin, as reference anti-inflammatory agents, and all candidates were drawn using ChemDraw, copied into the MOE program window, modified to take on 3D shapes, and corrected for partial charges, and their energies were minimized to the lowest possible level. Proteins (PDB code: 4COX and 3KK6) were prepared by removing water molecules and adding 3D hydrogen atoms. Finally, before proceeding with the docking process, we chose the most suitable pocket protein by subjecting it to the site finder option on the MOE 2015 program.

Conclusion

The authors have developed a novel, rapid and efficient protocol for the synthesis of novel spiro derivatives. Out of our belief in the biological efficiency of spiro hetero moieties, we aimed to synthesize fused azaspiroundecanedione and azaspirodecenone/thione derivatives. Fortunately, the most powerful and selective COX-2 inhibitor compounds were **11** and **6**, with selectivity indices of 175 and 129.21, in comparison to 31.52 of the standard celecoxib. However, candidate **14** showed a very promising antiinflammatory activity with an IC_{50} of 6.00, while celecoxib had an IC_{50} of 14.50. Candidate **11** exhibited a potent antioxidant activity of 86%, while ascorbic acid as a standard reference inhibits oxidation by 97%. Interestingly, most of our newly synthesized candidates give significant yields (over 90%), such as candidates **2**, **3**, **14**, and **17**. Compounds **14** and **17** exhibit significant inhibition activities against enzymes COX-1 and COX-2. *In silico* studies showed that candidates **14** and **17** inhibit COX-1 with significant docking scores of −6.78 and −6.9 kcal mol^{-1}, respectively, compared to −7.7 kcal mol^{-1} for celecoxib. These are promising lead compounds, which *via* structural modification lead to the design and synthesis of novel powerful antioxidants. Furthermore, the mechanism of action for the new compounds was proposed to involve cytochrome *c* peroxidase inhibitors *via* molecular docking, and compared to ascorbic acid as a reference standard.

Consent for publication

All authors consent to the publication.

Ethics approval

This article does not include any studies with human participants or animals performed by any of the authors.

Data availability

All data generated or analyzed during this work are available from the corresponding author on request.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

The authors extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through the large group Research Project under grant number (RGP2/413/44).

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