Journal of Enzyme Inhibition and Medicinal Chemistry

http://informahealthcare.com/enz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, 2014; 29(1): 109–117
© 2014 Informa UK Ltd. DOI: 10.3109/14756366.2012.755623

informa healthcare

Synthesis and biological evaluation of modified purine homo-*N*-nucleosides containing pyrazole or 2-pyrazoline moiety

Andreas Thalassitis[1], Anna-Maria Katsori[2], Konstantinos Dimas[3], Dimitra J. Hadjipavlou-Litina[2], Fokion Pyleris[1], Nikolaos Sakellaridis[3], and Konstantinos E. Litinas[1]

[1]Laboratory of Organic Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece, [2]Department of Pharmaceutical Chemistry, School of Pharmacy, Aristotle University of Thessaloniki, Thessaloniki, Greece, and [3]Department of Pharmacology, Faculty of Medicine, Thessaly University, Biopolis Larissa, Greece

Abstract

9-Substituted (pyrazol-5-yl)methyl- or (2-pyrazolin-5-yl)methyl-9H-purines were synthesized from 9-allyl-6-chloro-9H-purine through the 1,3-dipolar cycloaddition reaction with nitrile imines, prepared *in situ* from the corresponding hydrazone and NBS/Et_3N under MW or from hydrazinoylchloride and Et_3N under reflux. The coupling of new 6-chloropurines with amines in H_2O under microwaves resulted quantitatively to modified pyrazol-5-yl- or 2-pyrazolin-5-yl adenine homo-*N*-nucleosides. The new compounds were tested *in vitro* for their ability to: (i) interact with 1,1-diphenyl-2-picryl-hydrazyl (DPPH), (ii) inhibit lipid peroxidation, (iii) inhibit the activity of soybean lipoxygenase, (iv) inhibit *in vitro* thrombin and for (v) their antiproliferative and cytotoxic activity. Pyrazolines were found to be more potent *in vitro*. Compound **7a** exhibited satisfactory combined antioxidant and anti-lipid peroxidation activity, inhibition of lipoxygenase (89%) and thrombin inhibitory ability, whereas compound **7b** exhibited high lipoxygenase inhibitory activity in combination to significant anti-thrombin activity. No compound exhibited a significant cytotoxic activity, while all showed moderate antiproliferative activity.

Keywords

9-Allylpurines, 1,3-dipolar cycloaddition reaction, homo-*N*-nucleosides, lipid peroxidation inhibitors, nitrile imines, pyrazole, pyrazoline, thrombin inhibitors

History

Received 19 November 2012
Revised 1 December 2012
Accepted 1 December 2012
Published online 23 January 2013

Introduction

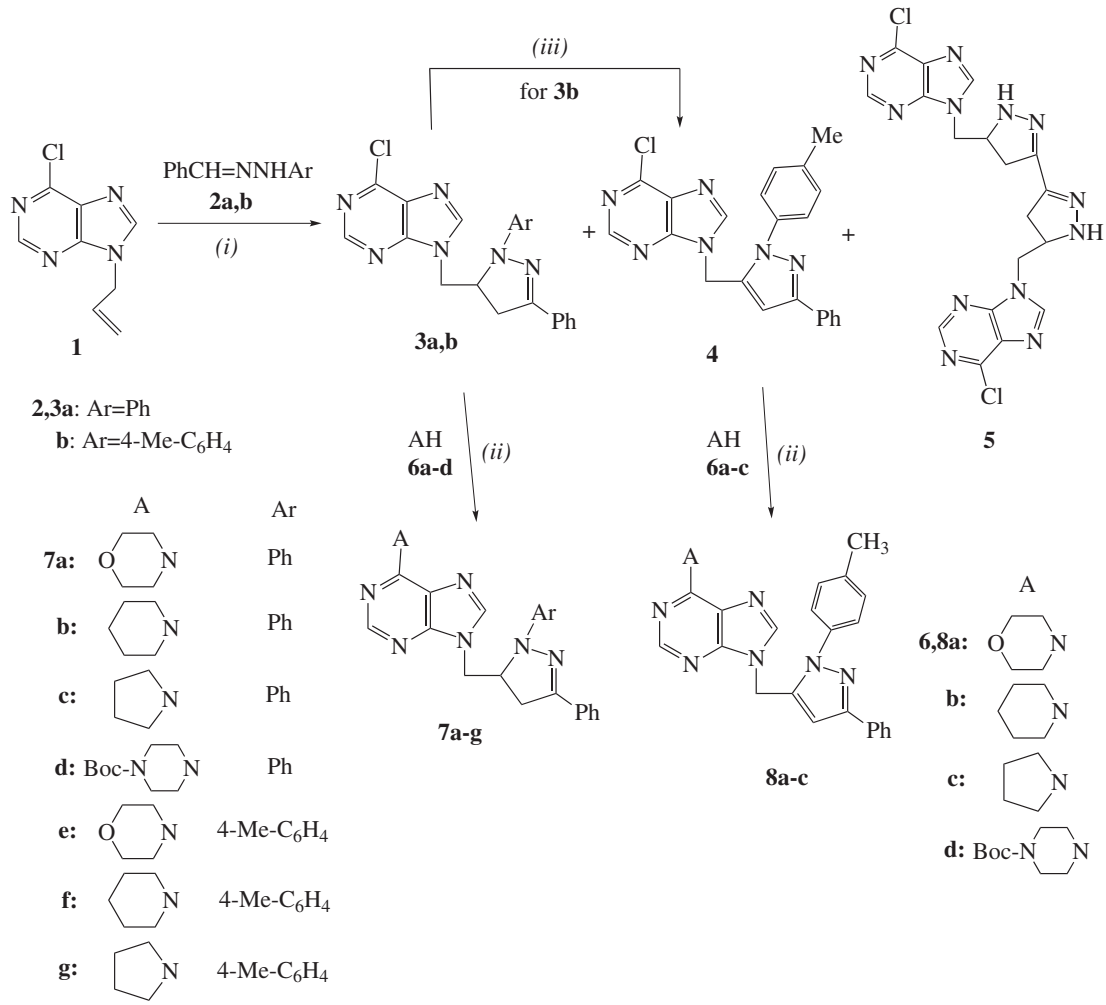
Nucleosides represent a class of compounds that possess very interesting biological activities[1,2]. The adenosine generated at inflamed site is receiving increasing interest as an endogenous anti-inflammatory agent and presents potential pharmacological uses as anti-inflammatory agent[3,4]. The homo-*N*-nucleosides with a CH_2-group between adenine and carbocyclic or heterocyclic ring possess higher conformational flexibility[5,6] to combine with the bases of DNA/RNA by a lowering of the electrostatic repulsion[6].

Oxidation is an important process which produces free radicals in living systems. Persistently high levels of reactive oxygen species (ROS) are believed to produce pathological conditions. ROS, like superoxide radical anion, hydrogen peroxide and hydroxyl radical, are produced during the inflammation process by phagocytic leukocytes at the inflamed site and they are involved in the biosynthesis of prostaglandins and in the cycloxygenase (COX)- and lipoxygenase (LO)-mediated conversion of arachidonic acid into proinflammatory intermediates[7,8]. Initial studies of the effects of adenosine on human neutrophiles[9] indicated that adenosine inhibits stimulated O_2^- or H_2O_2 generation. From our recent research[10], it was found that some new homo-*N*-nucleosides, derived from the 1,3-dipolar cycloaddition reactions of mesityl nitrile oxide with 9-allyl derivatives of 6-chloropurine, 6-piperidinylpurine, 6-morpholinylpurine, 6-pyrrolidinyl purine and 6-*N*,*N*-dibenzoyladenine inhibited thrombin and lipid peroxidation. The majority of these compounds showed significant lipoxygenase inhibitory activity too. Most of the LO inhibitors are antioxidants or free radical scavengers, since lipoxygenation occurs via a carbon-centered radical[11].

It is generally accepted that there is a close association between cancer and chronic inflammation[12–14]. Epidemiological studies have also shown that chronic inflammation preexists in some types of cancer. It is therefore evident that the use of multi-target ligands, that interact with multiple targets, could be valuable for the treatment of the abovementioned pathophysiological conditions[15]. It has already been proven for a number of commercially available nonsteroidal anti-inflammatory drugs (NSAIDs), e.g. aspirin, that they possess a combination of these properties.

Pyrazoline[16–18] and pyrazole[19] derivatives are important biological agents. In particular, pyrazoline derivatives have antimicrobial, anticancer, antiviral, anti-inflammatory[20], antidepressant[21], anticonvulsant[22], antiamoebic[23] activity. The pyrazole derivatives present[19] analgesic, hypoglycemic, antibacterial, anti-inflammatory, insecticidal, anti-influenza virus[24], antimicrobial[25] activity. Pyrazolines are prepared mainly by cyclization of chalcones with hydrazines[16,20,23], from 1,3-dipolar cycloaddition reactions of nitrile imines to alkenes[26] by heating[27] or under microwaves[28] or from the cyclocondensation of hydrazines with 1,3-dihalides under MW irradiation[29]. Pyrazoles are synthesized

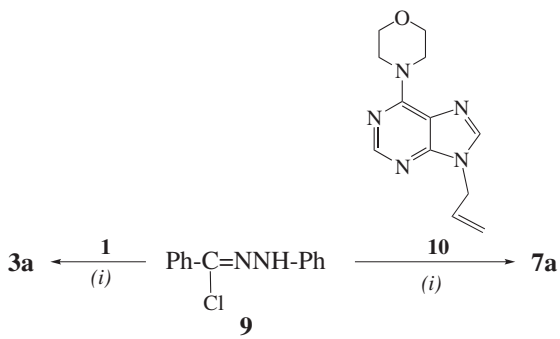
Address for correspondence: Konstantinos E. Litinas, Laboratory of Organic Chemistry, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece. Tel: +302310997864. Fax: +302310997679. E-mail: klitinas@chem.auth.gr



Scheme 1. Reagents and conditions: (i) **2**, NBS, benzene, stirring 15 min r.t.; **1**, Et_3N, MW, 80 °C, 80 min. (ii) H_2O, MW, 100 °C, 1 min. (iii) DDQ, dry toluene, reflux, 48 h.

mainly by the reaction of hydrazines with β-difunctional compounds[30], from tosylhydrazones of α,β-unsaturated ketones under MW irradiation[31] and by 1,3-dipolar cycloaddition reactions of tosylhydrazone salts[32,33] or nitrile imines[25] with alkynes.

In continuation to our work in the field of modified homo-*N*-nucleosides, we present here the reactions of 9-allyl-6-chloropurine with nitrile imines under MW irradiation and the replacement of chlorine by amines in the above products (Schemes 1 and 2). The biological evaluation of the new 2-pyrazolines and pyrazoles as lipoxygenase and lipid peroxidation inhibitors, cytotoxic and antiproliferative agents and simultaneously as thrombin inhibitors is also studied.



Scheme 2. Reagents and conditions: (i) $CHCl_3$, Et_3N, reflux, 7 h.

Materials and methods

Melting points were determined on a Kofler hot-stage apparatus (Arthur H. Thomas, Co, Philadelphia, PA) and are uncorrected. IR spectra were obtained with a Perkin-Elmer (Waltham, MA) 1310 spectrophotometer as Nujol mulls. NMR spectra were recorded on a Bruker AM 300 (Bruker A.G., Karlsruhe, Germany) (300 MHz and 75 MHz for ^1H and ^{13}C, respectively) using $CDCl_3$ as solvent and TMS as an internal standard. *J* values are reported in Hz. Mass spectra were determined on a Shimadzu (Manchester, UK) LCMS-2010 EV system under electrospray ionization (ESI) conditions. Microanalyses were performed on a Perkin-Elmer 2400-II Element analyzer. For *in vitro* determination a UV-Vis Perkin-Elmer Lambda Spectrophotometer was used. The absorbance for cytotoxic activity assay was measured at 530 nm, in an EL-311 BIOTEK microelisa reader (BioTek, Winooski, VT). The MW experiments were performed in a Biotage (Initiator 2.0) scientific MW oven. All the reagents used were commercially available by Merck A.G. (Darmstadt, Germany). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), nordihydroguaiaretic acid (NDGA) were purchased from the Aldrich Chemical Co. (Milwaukee, WI. Soybean lipoxygenase, linoleic acid sodium salt, nicotinamido-adenine-dinucleotide (NADH), nitrotetrazolium blue (NBT), porcine heme and indomethacin were obtained from Sigma Chemical, Co. (St. Louis, MO). Silica gel No. 60, Merck A.G. was

used for column chromatography. A549 (non-small cell lung cancer), PC3 (prostate cancer), MB435 (melanoma), CAKI and SN12C (renal cancer) were obtained from the National Cancer Institute, NIH (Bethesda, MD).

Synthesis

General procedure for 1,3-dipolar cycloaddition reaction with nitrile imines (from hydrazones) under MW irradiation

A mixture of phenylhydrazone (**2a**) (0.441 g, 2.25 mmol) and NBS (0.4 g, 2.25 mmol) in benzene (15 mL) was stirred in an MW vial at r. t. for 15 min. The purine (**1**) (0.146 g, 0.75 mmol) and Et_3N (0.225 g, 0.31 mL, 2.25 mmol) were then added and the mixture was irradiated under MW at 80 °C for 80 min. The resulted solution after cooling was evaporated and separated by column chromatography [ethyl acetate:hexane (1:4)] to give after the elution of unreacted hydrazone (**2a**) and the product (**3a**) (0.155 g, 53% yield). The not consumed starting purine (**1**) (63 mg, 43%) was eluted next.

6-Chloro-9-[(1,3-diphenyl-4,5-dihydro-1H-pyrazol-5-yl)methyl]-9H-purine (3a). Beige solid, m.p. 168–170 °C (DCM); IR (Nujol): 3050, 1630, 1585 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 3.26 (dd, 1H, $J_1 = 4.2$ Hz, $J_2 = 17.2$ Hz), 3.43 (dd, 1H, $J_1 = 11.1$ Hz, $J_2 = 17.2$ Hz), 4.43 (dd, 1H, $J_1 = 5.7$ Hz, $J_2 = 14.4$ Hz), 4.67 (dd, 1H, $J_1 = 3.0$ Hz, $J_2 = 14.4$ Hz), 4.97–5.05 (m, 1H), 6.93 (t, 1H, $J = 7.2$ Hz), 7.24–7.38 (m, 7H), 7.45–7.50 (m, 2H), 7.84 (s, 1H), 8.71 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 36.7, 44.8, 58.5, 113.4, 120.2, 125.5, 126.3, 128.5, 129.1, 129.7, 131.6, 143.6, 145.7, 147.8, 151.3, 152.0, 152.2; MS (ESI): 389/391 $[M+H]^{+\cdot}$, 411/413 $[M+Na]^+$; 427/429 $[M+K]^+$. Anal. Calcd for $C_{21}H_{17}ClN_6$: C, 64.31; H, 2.65; N, 20.35. Found: C, 64.05; H, 2.68; N, 20.55.

6-Chloro-9-{[1-(4-methylphenyl)-3-phenyl-4,5-dihydro-1H-pyrazol-5-yl]methyl}-9H-purine (3b). (from the reaction of 4-methyphenylhydrazone (**2b**) after MW irradiation for 90 min; eluted first) (48% yield). Beige solid, m.p. 76–78 °C(DCM-hexane), IR (Nujol): 3040, 1580, 1545, 1495 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 2.31 (s, 3H), 3.18 (dd, 1H, $J_1 = 4.5$ Hz, $J_2 = 17.2$ Hz), 3.35 (dd, 1H, $J_1 = 11.1$ Hz, $J_2 = 17.2$ Hz), 4.37 (dd, 1H, $J_1 = 5.7$ Hz, $J_2 = 14.4$ Hz), 4.61 (dd, 1H, $J_1 = 3.1$ Hz, $J_2 = 14.4$ Hz), 4.85–4.98 (m, 1H), 7.10–7.14 (m, 4H), 7.24–7.31 (m, 3H), 7.44–7.49 (m, 2H), 7.84 (s, 1H), 8.69 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 20.5, 36.7, 44.9, 58.9, 113.7, 125.5, 125.7, 128.4, 128.7, 128.9, 129.7, 131.7, 130.2, 141.5, 145.9, 147.4, 151.9, 152.2; MS (ESI): 403/405 $[M+H]^{+\cdot}$, 425/427 $[M+Na]^+$. Anal. Calcd for $C_{22}H_{19}ClN_6$: C, 65.59; H, 4.75; N, 20.86. Found: C, 65.51; H, 4.40; N, 20.68.

6-Chloro-9-{[1-(4-methylphenyl)-3-phenyl-1H-pyrazol-5-yl] methyl}-9H-purine (4). (from the above reaction; eluted after compound **3b**) (3% yield). Light brown solid, m.p. 79–81 °C (dec.), IR (Nujol): 3030, 1590, 1560, 1510 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 2.40 (s, 3H), 5.52 (s, 2H), 6.73 (s, 1H), 7.20–7.56 (m, 7H), 7.78–7.82 (m, 2H), 7.79 (s, 1H), 8.72 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 21.1, 39.0, 105.4, 125.6, 125.7, 125.9, 128.3, 128.4, 128.6, 128.7, 130.2, 137.3, 139.5, 144.3, 152.0, 152.1; MS (ESI): 401/403 $[M+H]^{+\cdot}$439/441 $[M+K]^+$. Anal. Calcd for $C_{22}H_{17}ClN_6$: C, 65.92; H, 4.27; N, 20.96. Found: C, 65.83; H, 4.37; N, 20.74.

5,5′-Bis[(6-chloro-9H-purin-9-yl)methyl]-4,4′,5,5′-tetrahydro-1H,1′H-3,3′-bipyrazole (5). (from the above reaction; eluted after compound **4**) (6% yield). Oil, IR (Nujol): 3230, 3060, 3020, 1585, 1560, 1490 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 3.73 (dd, 1H, $J_1 = 8.6$ Hz, $J_2 = 11.1$ Hz), 3.88 (dd, 1H, $J_1 = 4.1$ Hz, $J_2 = 11.1$ Hz), 4.59 (dd, 1H, $J_1 = 8.3$ Hz, $J_2 = 14.4$ Hz), 4.67–4.78 (m, 1H), 5.03 (dd, 1H, $J_1 = 3.8$ Hz, $J_2 = 14.4$ Hz), 8.25 (s, 1H), 8.77 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 33.0, 47.5, 49.2, 125.5, 125.7, 128.7, 130.2, 145.4, 152.3; MS (ESI): 471/473/475 $[M+H]^+$. Anal. Calcd for $C_{18}H_{16}Cl_2N_{12}$: C, 45.87; H, 3.42; N, 35.66. Found: C, 45.98; H, 3.57; N, 35.41. The unchanged starting material (41%) was eluted as the last one.

General procedure for 1,3-dipolar cycloaddition reaction with diphenylnitrile imine (from hydrazonoyl chloride) under reflux

In a mixture of purine (**1**) (65 mg, 0.336 mmol) and hydrazonoyl chloride (**9**) (0.125 g, 0.544 mmol) in $CHCl_3$ (7 mL) Et_3N (58 mg, 0.08 mL, 0.58 mmol) was added and refluxed for 7 h. After cooling and evaporation, the residue was separated by column chromatography [ethyl acetate/hexane (1:2)] to give from the faster moving band the derivative (**3a**) (67 mg, 51% yield) followed by the unreacted purine (**1**) (23 mg, 36%).

9-[(1,3-Diphenyl-4,5-dihydro-1H-pyrazol-5-yl)methyl]-6-morpolinyl-9H-purine (7a). (from the reaction of allylpurine (**10**)) (37% yield). Orange solid, m.p. 129–131 °C (DCM), IR (Nujol): 3070, 1640, 1580, 1495 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 3.25 (dd, 1H, $J_1 = 4.9$ Hz, $J_2 = 17.3$ Hz), 3.36 (dd, 1H, $J_1 = 10.8$ Hz, $J_2 = 17.3$ Hz), 3.77 (t, 4H, $J = 4.9$ Hz), 4.19–4.28 (m, 4H), 4.30 (dd, 1H, $J_1 = 5.7$ Hz, $J_2 = 14.3$ Hz), 4.61 (dd, 1H, $J_1 = 3.3$ Hz, $J_2 = 14.3$ Hz), 4.93–5.04 (m, 1H), 6.91 (tt, 1H, $J_1 = 1.4$ Hz, $J_2 = 7.0$ Hz), 7.25–7.38 (m, 7H), 7.50 (s, 1H), 7.53 (dd, 2H, $J_1 = 1.7$ Hz, $J_2 = 7.9$ Hz), 8.36 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 36.3, 44.0, 45.7, 58.7, 67.0, 113.4, 119.8, 119.9, 125.6, 128.4, 128.8, 129.6, 132.1, 139.1, 143.8, 147.8, 151.3, 152.2, 153.8; MS (ESI): 440 $[M+H]^{+\cdot}$, 462 $[M+Na]^+$; 478 $[M+K]^+$. Anal. Calcd for $C_{25}H_{25}N_7O$: C, 68.32; H, 5.73; N, 22.31. Found: C, 68.34; H, 5.80; N, 22.09. The unreacted purine (**10**) (61%) eluted next.

Oxidation of 6-chloro-9-{[1-(4-methylphenyl)-3-phenyl-4,5-dihydro-1H-pyrazol-5-yl]methyl}-9H-purine (3b) to 6-chloro-9-{[1-(4-methylphenyl)-3-phenyl-1H-pyrazol-5-yl]methyl}-9H-purine (4). A solution of compound **3b** (50 mg, 0.12 mmol) in dry toluene (5 mL) was treated with DDQ (39 mg, 0.17 mmol) under reflux for 48 h [after 24 h more DDQ (10 mg, 0.044 mmol, total 49 mg, 0.214 mmol) was added]. The solvent was evaporated and the residue was separated by column chromatography [ethyl acetate:hexane (1:2)] to give compound **4** (47 mg, 94% yield).

General procedure for the amination of 6-chloropurine derivatives under MW irradiation

In an MW vial the chloropurine (**3a**) (20 mg, 0.051 mmol) was added in H_2O (1 mL) along with morpholine (**6a**) (0.009 mL, 9 mg, 0.103 mmol) and the mixture was irradiated at 100 °C for 1 min. Extraction with DCM (3 × 20 mL), drying with anhydrous Na_2SO_4 and evaporation of the solvent resulted to 9-[(1,3-Diphenyl-4,5-dihydro-1H-pyrazol-5-yl)methyl]-6-morpolinyl-9H-purine (**7a**) (94% yield).

9-[(1,3-Diphenyl-4,5-dihydro-1H-pyrazol-5-yl)methyl]-6-piperidinyl-9H-purine (7b). (97% yield). Orange solid, m.p. 68–70 °C (DCM), IR (Nujol): 3050, 1635, 1570, 1495 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.58–1.80 (m, 6H), 3.25 (dd, 1H, $J_1 = 4.9$ Hz, $J_2 = 17.3$ Hz), 3.36 (dd, 1H, $J_1 = 10.8$ Hz, $J_2 = 17.3$ Hz), 4.10–4.24 (m, 4H), 4.27 (dd, 1H, $J_1 = 5.9$ Hz, $J_2 = 14.3$ Hz), 4.60 (dd, 1H, $J_1 = 3.4$ Hz, $J_2 = 14.3$ Hz), 4.93–5.05 (m, 1H), 6.90 (t, 1H, $J = 6.9$ Hz), 7.24–7.43 (m, 7H), 7.51 (s, 1H), 7.55 (dd, 2H, $J_1 = 1.7$ Hz, $J_2 = 7.7$ Hz), 8.34 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 24.7, 26.1, 36.4, 44.1, 46.6, 58.8, 113.5, 119.7, 119.9, 125.7, 128.5, 128.8, 129.6, 132.2, 138.6, 143.9, 147.9, 150.6, 151.1, 152.0; MS (ESI): 438 $[M+H]^+$, 460 $[M+Na]^+$; 476 $[M+K]^+$. Anal. Calcd for $C_{26}H_{27}N_7$: C, 71.37; H, 6.22; N, 22.41. Found: C, 71.32; H, 6.35; N, 22.16.

9-[(1,3-Diphenyl-4,5-dihydro-1H-pyrazol-5-yl)methyl]-6-pyrrolidinyl-9H-purine (7c). (97% yield). Yellow solid, m.p. 98–100 °C (DCM), IR (Nujol): 3060, 1640, 1585 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.90–2.11 (m, 4H), 3.24 (dd, 1H, $J_1 = 5.0$ Hz, $J_2 = 17.3$ Hz), 3.35 (dd, 1H, $J_1 = 10.8$ Hz, $J_2 = 17.3$ Hz), 3.60–4.23 (m, 4H), 4.27 (dd, 1H, $J_1 = 6.1$ Hz, $J_2 = 14.3$ Hz), 4.60 (dd, 1H, $J_1 = 3.3$ Hz, $J_2 = 14.3$ Hz), 4.92–5.04 (m, 1H), 6.90 (tt, 1H, $J_1 = 1.7$ Hz, $J_2 = 6.7$ Hz), 7.24–7.39 (m, 7H), 7.50 (s, 1H), 7.55 (dd, 2H, $J_1 = 1.8$ Hz, $J_2 = 7.8$ Hz), 8.36 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 29.7, 36.4, 44.0, 48.1, 58.8, 113.5, 119.8, 120.1, 125.7, 128.4, 128.7, 129.6, 132.2, 139.3, 143.9, 147.8, 150.6, 152.6, 154.3; MS (ESI): 424 $[M+H]^+$, 446 $[M+Na]^+$; 462 $[M+K]^+$. Anal. Calcd for $C_{25}H_{25}N_7$: C, 70.90; H, 5.95; N, 23.15. Found: C, 70.71; H, 6.12; N, 22.93.

***tert*-Butyl 4-{9-[(1,3-diphenyl-4,5-dihydro-1H-pyrazol-5-yl)methyl]-9H-purin-6-yl}piperazine-1-carboxylate (7d).** (87% yield). Beige solid, m.p. 130–132 °C (DCM), IR (Nujol): 3040, 1665, 1640, 1585 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.49 (s, 9H), 3.27 (dd, 1H, $J_1 = 5.7$ Hz, $J_2 = 17.1$ Hz), 3.38 (dd, 1H, $J_1 = 10.8$ Hz, $J_2 = 17.1$ Hz), 3.49 (t, 1H, $J = 4.8$ Hz), 4.15–4.26 (m, 4H), 4.30 (dd, 1H, $J_1 = 5.6$ Hz, $J_2 = 14.3$ Hz), 4.60 (dd, 1H, $J_1 = 2.9$ Hz, $J_2 = 14.3$ Hz), 4.93–5.03 (m, 1H), 6.90 (t, 1H, $J = 6.8$ Hz), 7.23–7.39 (m, 7H), 7.50 (s, 1H), 7.52 (dd, 2H, $J_1 = 1.7$ Hz, $J_2 = 7.9$ Hz), 8.35 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 29.7, 36.4, 43.9, 44.0, 45.0, 58.7, 79.7, 113.4, 119.8, 125.6, 126.3, 128.4, 128.8, 129.6, 132.1, 139.1, 143.8, 147.8, 151.4, 152.4, 153.9, 154.8; MS (ESI): 539 $[M+H]^{+\cdot}$, 561 $[M+Na]^+$; 577 $[M+K]^+$. Anal. Calcd for $C_{30}H_{34}N_8O_2$: C, 66.89; H, 6.36; N, 20.80. Found: C, 67.11; H, 6.12; N, 20.96.

9-{[1-(4-Methylphenyl)-3-phenyl-4,5-dihydro-1H-pyrazol-5-yl]methyl}-6-morpholinyl-9H-purine (7e). (95% yield). Beige solid, m.p. 167–169 °C (DCM-hexane), IR (Nujol): 3040, 1580, 1545 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 3.20 (dd, 1H, $J_1 = 5.1$ Hz, $J_2 = 17.2$ Hz), 3.31 (dd, 1H, $J_1 = 10.8$ Hz, $J_2 = 17.2$ Hz), 3.76 (t, 4H, $J = 4.8$ Hz), 4.22 (t, 4H, $J = 4.8$ Hz), 4.36 (dd, 1H, $J_1 = 5.6$ Hz, $J_2 = 14.3$ Hz), 4.57 (dd, 1H, $J_1 = 3.3$ Hz, $J_2 = 14.3$ Hz), 4.85–4.97 (m, 1H), 7.12 (d, 2H, $J = 8.7$ Hz), 7.17 (d, 2H, $J = 8.7$ Hz), 7.24–7.32 (m, 3H), 7.49 (s, 1H), 7.48–7.54 (m, 2H), 8.34 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 20.5, 36.4, 44.0, 45.7, 59.2, 67.0, 113.7, 119.8, 125.6, 128.4, 128.6, 129.3, 130.1, 132.3, 139.1, 141.8, 147.4, 151.5, 152.4, 153.9; MS (ESI): 454 $[M+H]^{+\cdot}$, 476 $[M+Na]^+$. Anal. Calcd for $C_{26}H_{27}N_7O$: C, 68.85; H, 6.00; N, 21.62. Found: C, 68.56; H, 5.72; N, 21.85.

9-{[1-(4-Methylphenyl)-3-phenyl-4,5-dihydro-1H-pyrazol-5-yl]methyl}-6-piperidinyl-9H-purine (7f). (96% yield). Beige solid m.p. 144–146 °C (DCM-hexane), IR (Nujol): 3040, 1570, 1540 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.55–1.77 (m, 6H), 3.20 (dd, 1H, $J_1 = 5.2$ Hz, $J_2 = 17.2$ Hz), 3.31 (dd, 1H, $J_1 = 10.8$ Hz, $J_2 = 17.2$ Hz), 4.11–4.22 (m, 4H), 4.23 (dd, 1H, $J_1 = 4.9$ Hz, $J_2 = 14.2$ Hz), 4.56 (dd, 1H, $J_1 = 3.3$ Hz, $J_2 = 14.2$ Hz), 4.85–4.97 (m, 1H), 7.13 (d, 2H, $J = 8.5$ Hz), 7.18 (d, 2H, $J = 8.5$ Hz), 7.23–7.34 (m, 3H), 7.49 (s, 1H), 7.53 (dd, 1H, $J_1 = 1.5$ Hz, $J_2 = 7.8$ Hz), 8.32 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 20.5, 24.8, 26.0, 36.4, 44.5, 46.4, 59.1, 113.7, 119.7, 125.6, 128.4, 128.6, 129.2, 130.0, 132.3, 138.5, 141.9, 147.4, 151.3, 152.6, 153.9; MS (ESI): 452 $[M+H]^+$, 474 $[M+Na]^+$; 490 $[M+K]^+$. Anal. Calcd for $C_{27}H_{29}N_7$: C, 71.81; H, 6.47; N, 21.71. Found: C, 72.05; H, 6.34; N, 21.62.

9-{[1-(4-Methylphenyl)-3-phenyl-4,5-dihydro-1H-pyrazol-5-yl]methyl}-6-pyrrolidinyl-9H-purine (7 g). (98% yield). Beige solid m.p. 169–171 °C (DCM-hexane), IR (Nujol): 3020, 1580, 1550 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.90–2.10 (m, 4H), 3.19 (dd, 1H, $J_1 = 5.2$ Hz, $J_2 = 17.2$ Hz), 3.31 (dd, 1H, $J_1 = 10.9$ Hz, $J_2 = 17.2$ Hz), 3.60–4.15 (m, 4H), 4.24 (dd, 1H, $J_1 = 5.9$ Hz, $J_2 = 14.3$ Hz), 4.56 (dd, 1H, $J_1 = 3.3$ Hz, $J_2 = 14.3$ Hz), 4.86–4.97 (m, 1H), 7.14 (d, 2H, $J = 8.6$ Hz), 7.18 (d, 2H, $J = 8.6$ Hz), 7.23–7.36 (m, 3H), 7.49 (s, 1H), 7.53 (dd, 1H, $J_1 = 1.6$ Hz, $J_2 = 7.8$ Hz), 8.34 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 20.5, 29.7, 36.4, 43.9, 47.9, 59.2, 113.8, 120.1, 125.6, 128.4, 128.5, 129.2, 130.0, 132.4, 139.2, 141.9, 147.4, 150.6, 152.9, 153.1; MS (ESI): 438 $[M+H]^{+\cdot}$, 460 $[M+Na]^+$. Anal. Calcd for $C_{26}H_{27}N_7$: C, 71.37; H, 6.22; N, 22.41. Found: C, 71.66; H, 5.94; N, 22.32.

9-{[1-(4-Methylphenyl)-3-phenyl-1H-pyrazol-5-yl] methyl}-6-morpholinyl-9H-purine (8a). (94% yield). Light brown solid, m.p. 68–70 °C (DCM-hexane), IR (Nujol): 3020, 1595, 1520 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 2.41 (s, 3H), 3.82 (t, 2H, $J = 4.8$ Hz), 4.28 (t, 2H, $J = 4.8$ Hz), 5.41 (s, 2H), 6.61 (s, 1H), 7.24–7.47 (m, 7H), 7.49 (s, 1H), 7.79 (dd, 2H, $J_1 = 1.5$ Hz, $J_2 = 6.8$ Hz), 8.34 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 21.2, 43.5, 45.7, 67.1, 105.0, 113.8, 125.5, 125.8, 128.6, 130.0, 132.7, 136.5, 137.8, 138.6, 138.9, 139.0, 141.0, 152.0, 152.5, 152.6; MS (ESI): 452 $[M+H]^{+\cdot}$, 474 $[M+Na]^+$. Anal. Calcd for $C_{26}H_{25}N_7O$: C, 69.16; H, 5.58; N, 21.71. Found: C, 69.41; H, 5.83; N, 21.48.

9-{[1-(4-Methylphenyl)-3-phenyl-1H-pyrazol-5-yl]methyl}-6-piperidinyl-9H-purine (8b) (95% yield). Light brown solid, m.p. 68–70 °C (DCM-hexane), IR (Nujol): 3040, 1580, 1560, 1495 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.60–1.79 (m, 6H), 2.43 (s, 3H), 4.14–4.31 (m, 4H), 5.40 (s, 2H), 6.61 (s, 1H), 7.22–7.43 (m, 7H), 7.48 (s, 1H), 7.79 (dd, 2H, $J_1 = 1.6$ Hz, $J_2 = 8.4$ Hz), 8.31 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 21.2, 24.6, 26.2, 38.6, 46.4, 105.0, 113.8, 125.5, 125.8, 125.9, 128.1, 128.6, 130.1, 136.6, 137.2, 138.7, 139.1, 141.3, 152.0, 152.6, 152.8; MS (ESI): 450 $[M+H]^+$. Anal. Calcd for $C_{27}H_{27}N_7$: C, 72.14; H, 6.05; N, 21.81. Found: C, 72.11; H, 6.29; N, 21.54.

9-{[1-(4-Methylphenyl)-3-phenyl-1H-pyrazol-5-yl]methyl}-6-pyrrolidinyl-9H-purine (8c) (97% yield). Light brown solid, m.p. 68–70 °C (DCM-hexane), IR (Nujol): 3020, 1580, 1510 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.94–2.11 (m, 4H), 2.41 (s, 3H),), 3.70–4.11 (m, 4H), 5.39 (s, 2H), 6.59 (s, 1H), 7.24–7.47 (m, 7H), 7.48 (s, 1H), 7.79 (d, 2H, $J = 7.0$ Hz), 8.33 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 21.2, 38.6, 48.2, 49.2, 105.0, 114.0, 125.5, 125.8, 126.0, 128.1, 128.6, 130.1, 136.6, 138.0, 138.8, 139.0, 141.0, 151.9, 153.1, 153.2; MS (ESI): 436 $[M+H]^+$. Anal. Calcd for $C_{26}H_{25}N_7$: C, 71.70; H, 5.79; N, 22.51. Found: C, 71.71; H, 5.50; N, 22.60.

Biological assay

In vitro *experiments*

In the *in vitro* assays each experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10% of the mean.

Determination of the reducing activity of the stable radical DPPH

To an ethanolic solution of DPPH (0.05 mM) in absolute ethanol an equal volume of the compounds dissolved in DMSO was added[34]. The mixture was shaken vigorously and allowed to stand for 20 min or 60 min; absorbance at 517 nm was determined spectrophotometrically and the percentage of activity was calculated. All tests were undertaken on three replicates and the results were averaged (Table 1).

Soybean lipoxygenase inhibition study in vitro

The tested compounds dissolved in DMSO were incubated at room temperature with sodium linoleate (0.1 mL) and 0.2 mL of enzyme solution ($1/9 \times 10^{-4}$ w/v in saline)[34]. The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor.

Table 1. Interaction-reducing activity with DPPH (RA%); inhibition of lipid peroxidation (AAPH%); *in vitro* inhibition of soybean lipoxygenase (LO); *in vitro* inhibition of thrombin (% Thr).

Compounds	Clog P[39]	RA% 0.05 mM 20 min ± SD	AAPH% 0.1 mM ± SD	LO (%) 0.1 mM ± SD	Thr (%) 0.1 mM ± SD
3a	4.28	19 ± 0.1	94 ± 2.1	97 ± 1.9	18 ± 0.1
3b	4.78	20 ± 0.8	58 ± 1.7	34 ± 1.0	nt
4	4.42	62 ± 2.1	5 ± 0.3	44 ± 0.8	nt
5	na	66 ± 1.5	82 ± 1.9	92 ± 3.8	nt
7a	3.80	22 ± 1.1	100 ± 1.5	89 ± 2.4	75 ± 2.6
7b	5.19	24 ± 1.0	72 ± 2.0	80 ± 1.8	73 ± 1.8
7c	4.63	13 ± 0.6	73 ± 1.4	37 ± 0.9	19 ± 0.1
7d	5.30	17 ± 0.1	54 ± 1.0	74 ± 1.6	48 ± 0.6
7e	4.30	43 ± 1.2	5.8 ± 0.1	30 ± 1.1	nt
7f	5.68	20 ± 1.5	91.5 ± 2.0	41 ± 0.8	nt
7g	5.13	43 ± 0.9	49 ± 1.8	12 ± 0.08	nt
8a	3.94	74 ± 1.6	99 ± 2.2	No	nt
8b	5.32	79 ± 2.1	96 ± 2.0	15 ± 0.1	nt
8c	4.77	44 ± 1.2	87 ± 1.5	40 ± 1.3	nt
NDGA	–	81 ± 1.7	–	84 ± 2.1	nt
Trolox	–	–	64 ± 3	–	
NAPAP	–	–	–	–	100 ± 4

na: not available; nt: not tested; ±SD ≤ 10%. NDGA: nordihydroguaiaretic acid; NAPAP: N^{α}-(2-naphthyl-sulfonyl-glycyl)-D,L-*p*-amidinophenylalanyl-piperidine; each experiment was performed at least in triplicate.

Inhibition of linoleic acid lipid peroxidation

Production of conjugated diene hydroperoxide by oxidation of linoleic acid in an aqueous dispersion is monitored at 234 nm[34]. AAPH is used as a free radical initiator. This assay can be used to follow oxidative changes and to understand the contribution of each tested compound.

Azo compounds generating free radicals through spontaneous thermal decomposition are useful for *in vitro* studies of free radical production. The water soluble azo compound AAPH has been extensively used as a clean and controllable source of thermally produced alkylperoxyl free radicals. Ten microliters of the 16 mM linoleic acid dispersion was added to the UV cuvette containing 0.93 mL of 0.05 M phosphate buffer, pH 7.4 prethermostated at 37 °C. The oxidation reaction was initiated at 37 °C under air by the addition of 50 µL of 40 mM AAPH solution. Oxidation was carried out in the presence of aliquots (10 µL) in the assay without antioxidant, lipid oxidation was measured in the presence of the same level of DMSO. The rate of oxidation at 37 °C was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides.

Inhibition of thrombin

As a substrate, tosyl-Gly-Pro-Arg-pNA was used at 1 mM final concentration[35]. Compounds were dissolved at a final concentration of 100 µM in a Tris-buffer (0.05 M Tris, 0.154 M NaCl, ethanol 5%, pH 8.0). Three minutes after the addition of bovine thrombin (2.5 unit/mg), the reaction was ended by adding 0.1 mL acetic acid 50%. The absorption of the released *p*-nitroaniline was measured at 405 nm. NAPAP: N^{α}-(2-naphthyl-sulfonyl-glycyl)-D,L-*p*-amidinophenylalanyl-piperidine) was used as a reference compound.

In vitro *cytotoxic and antiproliferative activity of the compounds*

A549 (Non-small cell lung cancer), PC3 (prostate cancer), MB435 (melanoma), CAKI and SN12C (renal cancer) were adapted to propagate in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, 2 mM L-glutamine and antibiotics. The cultures were grown in a 37 °C humidified incubator with 5% CO_2-atmosphere

In vitro cytotoxic activity of all congeners was determined by the SRB assay[36]. Cell viability was assessed at the beginning of each experiment by the trypan blue dye exclusion method, and was always greater than 97%. For the SRB assay, cells were seeded into 96-well plates in 100 µL of medium at a density of 5000–15 000 cells per well, depending on the cell line, and subsequently, the plates were incubated at standard conditions for 24 h to allow the cells to resume exponential growth prior to addition of the compounds. In order to measure the starting cell population, cells in one plate were fixed *in situ* with TCA 50% (w/v) followed by SRB staining as described[37]. To determine cytotoxic activity, all compounds were dissolved in DMSO and then directly added in the cultures at four 10-fold dilutions (from 100 to 0.1 µM) and incubation continued for an additional period of 48 h. The assay was terminated by the addition of cold TCA 50% (w/v) followed by SRB staining and the absorbance was measured at 530 nm, in an EL-311 BIOTEK microelisa reader (BioTek, Winooski, VT), in order to determine the three parameters GI_{50}, TGI and LC_{50}[38].

Determination of lipophilicity as clog *P*

Lipophilicity was theoretically calculated as clog *P* values in *n*-octanol buffer by CLOGP Programme of Biobyte Corp[39].

Results and discussion

Synthesis

The reaction of 9-allyl-6-chloropurine[10] (**1**) with the diphenylnitrile imine, generated *in situ* from the hydrazone (**2a**) with NBS in the presence of Et_3N, in benzene[40] under MW irradiation at 80 °C for 80 min resulted in 1,3-dipolar cycloaddition product (**3a**) (53%) (Scheme 1) with 43% of the starting purine remaining unchanged. The regioselectivity of this reaction for the formation of 5-substituted 2-pyrazoline is quite similar to that expected from the literature[27]. The same product (**3a**) (51%) is isolated also from the reaction of purine (**1**) with the diphenylnitrile imine prepared *in situ* from the treatment of hydrazonoyl chloride[41] (**9**) with Et_3N under reflux (Scheme 2), while 36% of the starting purine remained unchanged.

The amination of compound **3a** (Scheme 1) with morpholine (**6a**), piperidine (**6b**), pyrrolidine (**6c**) or N-Boc-piperazine (**6d**) under microwave (MW) irradiation at 100°C in H_2O for 1 min afforded almost quantitatively the adenine derivatives (**7a–d**). This procedure seems to be very efficient and eco friendly without the use of any other reagent except H_2O. The morpholinyl-substituted

compound **7a** (37%) is isolated also [accompanied by the unreacted purine (**10**) (61%)] from the 1,3-dipolar cycloaddition reaction of 9-allyl-6-morpholin-4-yl-9H-purine[10] (**10**) with the diphenylnitrile imine, received *in situ* under reflux from the hydrazonoyl chloride (**9**) (Scheme 2), after treatment with Et_3N.

The reaction of 9-allylpurine (**1**) with the nitrile imine, generated *in situ* from the *p*-tolylhydrazone (**2b**) with NBS in the presence of Et_3N, in benzene under microwaves at 80 °C for 90 min gave the pyrazoline derivative (**3b**) (48% yield) followed by its oxidation product the pyrazole derivative (**4**) (3%), the bipyrazoline derivative (**5**) (6%) and the unchanged starting material (41%). The derivative (**5**) shows the expected molecular ion in MS spectrum (471/473/475 $[M + H]^+$), the expected pattern for 2-pyrazolin-5-yl methylene group in the ^{1}H-NMR spectrum [3.73 (dd, 1H, $J_1 = 8.6$ Hz, $J_2 = 11.1$ Hz), 3.88 (dd, 1H, $J_1 = 4.1$ Hz, $J_2 = 11.1$ Hz), 4.59 (dd, 1H, $J_1 = 8.3$ Hz, $J_2 = 14.4$ Hz), 4.67–4.78 (m, 1H), 5.03 (dd, 1H, $J_1 = 3.8$ Hz, $J_2 = 14.4$ Hz)], while there are no other aromatic protons except the protons of the purine skeleton. There is also absorption for N–H group in the IR spectrum (3230 cm^{-1}). Oxidation of pyrazoline (**3b**) with 2,3-dichloro-4,5-dicyano-*o*-benzoquinone (DDQ) in toluene under reflux produced pyrazole (**4**) in 94% yield. Derivative **3b** after one month exposure to the air oxidized to pyrazole (**4**).

The aminations of pyrazolines (**3b**) and pyrazoles (**4**) (Scheme 1) with morpholine (**6a**), piperidine (**6b**) or pyrrolidine (**6c**) under microwave irradiation at 100 °C in H_2O for only 1 min afforded almost quantitatively the homo-*N*-nucleosides containing pyrazoline (**7e–g**) and pyrazole (**8a–c**) moieties, respectively.

Biological studies

Oxidation is an important process, which produces reactive oxygen species ROS. Antioxidants are defined as substances that even at low concentration significantly delay or prevent oxidation of easy oxidizable substrates and there is an increased interest of using antioxidants for medical purposes in recent years. Free radicals are formed in both physiological and pathological conditions in mammalian tissues. The uncontrolled production of free radicals is considered as an important factor in the tissue damage induced by several pathophysiologies. It is well known that free radicals play an important role in the inflammatory process. Lipid peroxidation mediated by free radicals is considered to be the major mechanism of cell membrane destruction and cell damage inducing alterations of ion transport and inhibition of metabolic processes[42]. Phospholipids containing polyunsaturated fatty acids are predominantly susceptible to peroxidation. ROS are produced during the inflammation process by phagocytic leukocytes at the inflamed site and they are involved in the biosynthesis of prostaglandins and in the cycloxygenase- and lipoxygenase-mediated conversion of arachidonic acid into proinflammatory intermediates[7,8]. LO products are regulators of platelet $[Ca^{2+}]$ mobilization and aggregation in response to some agonists. LO inhibitors may work in part by modifying platelet cyclic AMP metabolism.

Taking into account the multifactorial character of oxidative stress and inflammation, we evaluated the *in vitro* antioxidant activity of the synthesized molecules using two different antioxidant assays: (a) interaction with the stable free radical DPPH and (b) interaction with the water-soluble azo compound AAPH.

Both require a spectrophotometric measurement and a certain reaction time in order to obtain reproducible results[43]. In its oxidized form, the DPPH radical has an absorbance maximum centered at about 517 nm[44]. The DPPH method is described as a simple, rapid and convenient method independent of sample polarity for screening many samples for radical scavenging activity[45]. These advantages made the DPPH method interesting for the testing of our compounds.

The use of the free radical reactions initiator AAPH is recommended as more appropriate for measuring radical-scavenging activity *in vitro*, because the activity of the peroxyl radicals produced by the action of AAPH shows a greater similarity to cellular activities such as lipid peroxidation[46].

The interaction/reducing activity (RA) of the examined compounds with the stable free radical DPPH is shown in Table 1. This interaction, indicating their radical scavenging ability in an iron-free system, was measured at 50 μM (20 min). The new compounds **3a–b**, **7a–d**, **7f** present mild reducing activities (13–24%) after 20 min, whereas **7e**, **7g**, **8c** present 43–44% and compounds **4**, **5**, **8a** and **8b** (62–79%). The presence of a pyrazole ring (compound **4**) leads to a higher interaction value, compared to the corresponding pyrazoline analogue (**4** = 62%, whereas **3b** = 20%). Compound **5** shows similar antioxidant activity with **4**. Comparing **7a** to **8a** and **7b** to **8b**, it seems that the pyrazoles (**8a–8b**) are more potent that the corresponding pyrazolines (**7a–7b**).

In the DPPH assay, the dominant chemical reaction involved is the reduction of the DPPH radical by an electron transfer (ET) from the antioxidant. Particularly effective such antioxidants are the phenoxide anions from phenolic compounds like catechol and derivatives, such as nordihydroguaiaretic acid (NDGA). Herein, the most potent antioxidants **8a** and **8b** did not contain such structural characteristics. Their interaction values did present big differences although in **8a**, substituent A is a morpholinyl group and in **8b** A is a piperidinyl moiety. In general, the replacement of Cl by an amine (a substituent) did not lead the antioxidant activity in an increase.

Compounds with antioxidant properties are expected to offer protection in inflammation and thrombosis and to lead to effective drugs, in comparison to the well known antioxidant agent NDGA.

In our studies AAPH was used as a free radical initiator to follow oxidative changes of linoleic acid to the conjugated diene hydroperoxide[34,46]. Azo compounds generating free radicals through spontaneous thermal decomposition are useful for free radical production studies *in vitro*. All compounds showed significant inhibition of lipid peroxidation 49–100 (Table 1) compared to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), used as a standard (64%). No differences are observed between compounds 7**b** (piperidinyl) and **7c** (pyrrodinyl) indicating that the magnitude of the ring (5- or 6-membered) does not influence significantly the inhibition. However, **7d** presents a decrease (54%) possibly due to the presence of the bulky *Boc* group, whereas **7a**, the morpholinyl-substituted molecule, highly inhibits lipid peroxidation 100%. Between the two **3a**, **3b** (6-Cl substituted pyrazolines), **3a** seems to be more potent inhibitor lipid peroxidation. The presence of Ar = 4-Me–C_6H_4– group decreases the biological response. Minor changes are observed within the inhibitory activities of **3a–7a** with the replacement of the 6-Cl group by the morpholinyl moiety. On the contrary, significant high differences are observed between the antioxidant and inhibitory values of **3b–7e**.

It is worthy to note that small changes in Ar substituent are followed by significant decrease in the anti-lipid peroxidation behavior, e.g. **7a–7e, 7b–7f, 7c–7g**. Comparing pyrazolines **7e–g** to pyrazoles **8a–c**, big differences are observed within the % inhibition values of **7e** and **8a, 7g** and **8c**.

Since lipophilicity is a significant physicochemical property determining distribution, bioavailability, metabolic activity and elimination, we tried to theoretically calculate clog *P* values[39] as an expression of lipophilicity. Perusal of these values did not give any evidence of influence on the above determined antioxidant properties.

Table 2. *In vitro* results for cytotoxic and antiproliferative activity.

		Compounds								
CELL LINES	Parameters	**7a**	**7d**	**7b**	**7c**	**4**	**7g**	**8b**	**8a**	**8c**
A549	*GI50*	55.2	54.7	59.0	50.4	36.8	44.6	51.0	55.4	63.5
	TGI	>100	>100	>100	99.4	65.4	>100	>100	>100	>100
	LC50	>100	>100	>100	>100	94.1	>100	>100	>100	>100
PC3	*GI50*	43.3	25.7	41.3	35.2	25.8	28.7	40.5	43.2	52.2
	TGI	83.2	91.8	86.9	80.7	55.2	82.5	92.5	95.9	99.4
	LC50	>100	>100	>100	>100	84.7	>100	>100	>100	>100
MB435	*GI50*	64.4	75.2	52.1	52.0	39.3	64.4	80.6	71.2	75.6
	TGI	>100	>100	92.9	92.2	66.8	>100	>100	>100	>100
	LC50	>100	>100	>100	>100	94.3	>100	>100	>100	>100
CAKI	*GI50*	74.8	103.8	70.7	58.9	37.6	104.7	103.7	>100	>100
	TGI	>100	>100	>100	>100	67.0	>100	>100	>100	>100
	LC50	>100	>100	>100	>100	96.4	>100	>100	>100	>100
SN12C	*GI50*	56.3	55.2	51.3	35.2	36.1	78.7	61.6	66.3	64.9
	TGI	104.0	>100	90.4	81.0	64.0	>100	>100	>100	>100
	LC50	>100	>100	>100	>100	91.9	>100	>100	>100	>100

The *in vitro* anticancer activity of compounds was determined using the SRB antiproliferative assay as instructed by the NCI. The nine compounds were tested against a panel of five human cancer cell lines derived from four different types of solid tumors. Three parameters growth inhibiting activity (GI_{50}), cytostatic activity (TGI) and cytotoxic activity (LC_{50}) were evaluated for each cell line and each compound tested. Results represent mean of three experiments each one run in triplicates with a C.V. $\leq$ 15%.

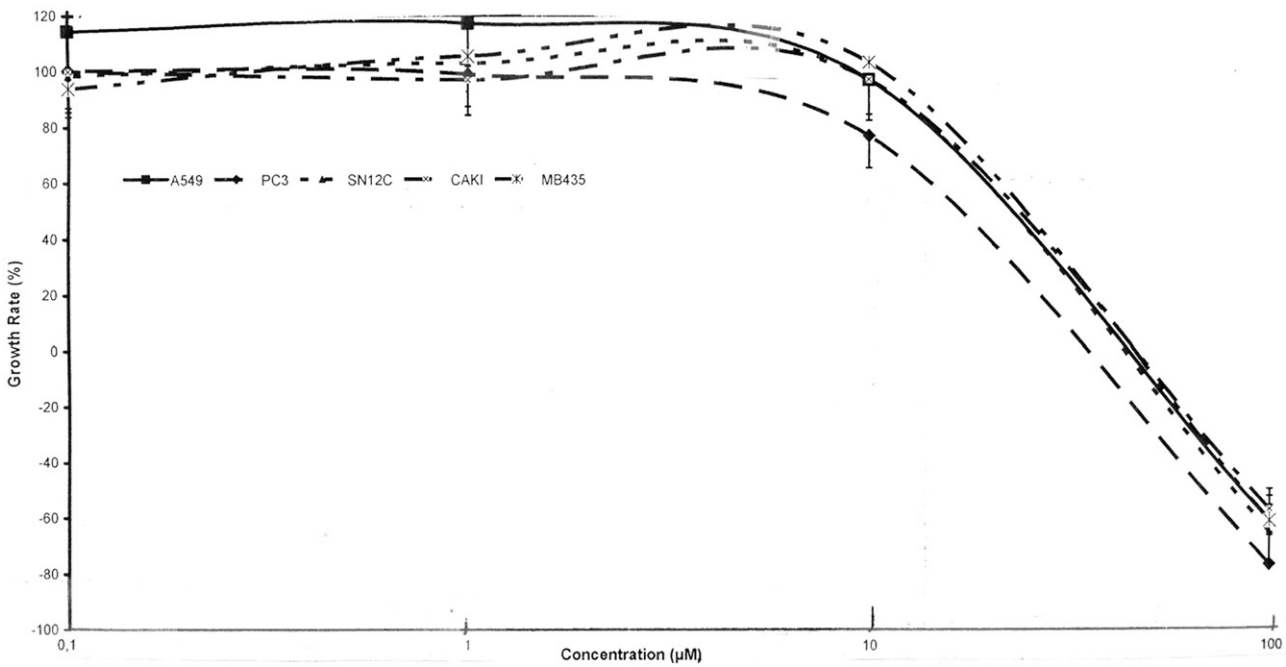


Figure 1. The growth curves of cells treated with the compound **4** which was found to be the most active. Cells were exposed to various concentrations of the agent for 48 h and the growth rates were calculated using the SRB method (see Materials and methods section). Points represent the mean of three independent experiments each one run in triplicates ± SD.

Compounds were further evaluated for inhibition of soybean lipoxygenase (LO) by the UV absorbance based enzyme assay[10]. All the tested derivatives inhibit soybean LO with the exception of **8a**, which under the experimental conditions did not present any inhibition, as well as **7g** and **8b**, which showed limited inhibition. Compounds **3b**, **4**, **7c**, **7e**, **8c** follow with close inhibition values (30–44%). Slight differences are observed among the most potent derivatives **5**, **7a**, **7b** and **7**d. Compound **5** presents the higher activity at 92% as well as compound **3a** (97%). Compound **3a** seems to be more potent inhibitor of LO and of lipid peroxidation. No differentiation was observed between the piperidinyl-(**7b**) and *N-Boc*-piperazinyl (**7d**) substituted derivatives.

Lipophilicity is referred[47–49] as an important physichochemical property for lipoxygenase inhibition. However, in this data set lipophilicity does not seem to affect the LO inhibition.

It is now widely accepted that activation of the coagulation cascade, with initiation of thrombin and fibrin deposition is a consequence of inflammation. Once thought to be completely different processes, the boundaries between inflammation and coagulation are now nearly indistinguishable[50]. Serineprotease thrombin, can elicit many inflammatory responses in microvascular endothelium. Its multiple role in thrombosis makes thrombin an important target for the therapeutic agents designed for thrombus prevention[51]. LO inhibitors reduce platelet aggregation induced by thrombin and U46619 and modify release of Ca^{2+} from intracellular stores[52]. The development of selective, orally active, low molecular weight synthetic thrombin inhibitors have

been an intense research focus in the search for new anticoagulants.

Since compounds **3a, 7a–d**, highly inhibited the soybean lipoxygenase, we decided to evaluate their ability to inhibit thrombin[35]. Compounds **7a–b** are both the most potent and equipotent inhibitors (73% and 75%, Table 1), followed by **7d.** Compound **3a** as well as derivative **7c** presented limited activity. The presence of a piperidinyl or of a morpholinyl ring leads to similar biological responses (75% and 73%). The replacement of a Cl atom (electron acceptor group, compound **3a**) by a morpholinyl group (**7a**) is followed by an increase in inhibitory activity. Herein, the role of lipophilicity is well documented. Thus, lower lipophilicity is followed by higher biological response. However, this concept does not support the **7a**, **7b** findings.

The compounds **7a-d, 7g**, **4** and **8a–c** were tested for *in vitro* antiproliferative activity against five human cancer cell lines representing four different types of human cancers. No compound exhibited a significant cytotoxic activity as this is represented by the LC_{50} parameter (Table 2). All the compounds tested demonstrated moderate antiproliferative activity against all cell lines at the high μM range (Table 2 and Figure 1). Compound **4** was found to be slightly more potent in terms of antiproliferative activity demonstrating the lowest GI_{50} (Table 2) against PC3 cells ($GI_{50} \sim 26\,\mu M$). As the compound shares the same chemical structure with compounds **8a–c**, it is possible that the difference may be due to the different A substituent, i.e. Cl at compound **4**, suggesting a critical role for this C-atom at the antiproliferative activity of these compounds.

Conclusion

The pyrazoline derivatives are prepared through the 1,3-dipolar cycloaddition reaction of nitrile imines to 9-allyl-6-chloropurine under MW irradiation in moderate yields. The pyrazoline skeleton is oxidized by DDQ to the corresponding pyrazole in high yield. Both 2-pyrazoline and pyrazole derivatives are transformed to 2-pyrazoline or pyrazole adenine derivatives by treatment with amines under an eco friendly MW irradiation in water. The main structural difference is located to substituent A.

Our studies confirm that the presence of a pyrazolinyl substituted purine is an important structural scaffold for the antioxidant, anti-LO and anti-thrombin activity. This provides an impetus for designing new biological active agents using the *N*-substituted 6-chloropurine scaffold as the starting point. The presence of the substituted 9-chloropurine seems to be highly implicated in the antiproliferative response.

The suggested structural variations could affect both efficacy and their tolerability partly due to differences in their physicochemical properties, which determine their distribution in the body and their ability to pass through and to enter into the interior membranes. These compounds may prove useful for treating a variety of inflammatory and coronary artery diseases and may lead to the development of new drugs.

Acknowledgements

We would like to thank Dr A. Leo and Biobyte for free access to the C-QSAR program.

Declaration of interest

This research has been co-financed by the European Union (European Social Fund – ESF) and Greek national funds through the Operational Program ''Education and Lifelong Learning'' of the National Strategic Reference Framework (NSRF) – Research Funding Program: Heracleitus II (A. Thalassitis) Investing in knowledge society through the European Social Fund.

The authors report no conflicts of interest.

References

1. De Clercq E. Toward improved anti-HIV chemotherapy: therapeutic strategies for intervention with HIV infections. J Med Chem 1995;38:2491–517.
2. De Clercq E. New developments in anti-HIV chemotherapy. Curr Med Chem 2001;8:1543–72.
3. Serhan CN, Ward PA. Molecular and cellular basis of inflammation. In: Cronstein BN, ed. Current inflammation research. Ch. 12. Totowa, NJ: Humana Press Series; 1999: 259.
4. Cronstein BN, Kramer SB, Weissmann G, Hirschhorn R. Adenosine: a physiological modulator of superoxide anion generation by human neutrophils. J Exp Med 1983;158:1160–77.
5. Herdewijn P. Conformationally restricted carbohydrate-modified nucleic acids and antisense technology. Biochim Biophys Acta 1999;1489:167–79.
6. Chiacchio U, Genovese F, Iannazzo D, et al. Diastereoselective synthesis of homo-N,O-nucleosides. Tetrahedron 2004;60:441–8.
7. Garrido G, Gonzalez D, Delporte C, et al. Analgesic and anti-inflammatory effects of *Mangifera indica* L. extract (Vimang). Phytother Res 2001;15:18–21.
8. Weber V, Coudert P, Rubat C, et al. Novel 4,5-diaryl-3-hydroxy-2(5*h*)-furanones as anti-oxidants and anti-inflammatory agents. Bioorg Med Chem 2002;10:1647–58.
9. Crimmins MT. New developments in the enantioselective synthesis of cyclopentyl carbocyclic nucleosides. Tetrahedron 1998;54:9229–72.
10. Thalassitis A, Hadjipavlou-Litina DJ, Litinas KE, Miltiadou P. Synthesis of modified homo-N-nucleosides from the reactions of mesityl nitrile oxide with 9-allylpurines and their influence on lipid peroxidation and thrombin inhibition. Bioorg Med Chem Lett 2009;19:6433–6.
11. Muller K. 5-Lipoxygenase and 12-lipoxygenase: attractive targets for the development of novel antipsoriatic drugs. Arch Pharm (Weinheim) 1994;327:3–19.
12. Colotta F, Allavena P, Sica A, et al. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis 2009;30:1073–81.
13. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008;454:436–44.
14. Aggarwal BB. Inflammation, a silent killer in cancer is not so silent! Curr Opin Pharmacol 2009;9:347–50.
15. Kashfi K, Enna SJ, Michael W. Anti-inflammatory agents as cancer therapeutics. Adv Pharmacol 2009;57:31–89.
16. Kumar S, Bawa S, Drabu S, et al. Recent patents on anti-infective drugs. Drug Discovery 2009;4:154–63.
17. Johnson M, Younglove B, Lee L, et al. Design, synthesis, and biological testing of pyrazoline derivatives of combretastatin-A4. Bioorg Med Chem Lett 2007;17:5897–901.
18. Oezdemir Z, Kandilci HB, Guemuesel B, et al. Synthesis and studies on antidepressant and anticonvulsant activities of some 3-(2-furyl)-pyrazoline derivatives. Eur J Med Chem 2007;42:373–9.
19. Elguero J. In comprehensive heterocyclic chemistry. Oxford: Pergamon Press; 1984.
20. Amir M, Kumar H, Khan SA. Synthesis and pharmacological evaluation of pyrazoline derivatives as new anti-inflammatory and analgesic agents. Bioorg Med Chem Lett 2008;18:918–22.
21. Gok S, Demet MM, Ozdemir A, Turan-Zitouni G. Evaluation of antidepressant-like effect of 2-pyrazoline derivatives. Med Chem Res 2010;19:94–101.
22. Siddiqui AA, Rahman MA, Shaharyar M, Mishra R. Synthesis and anticonvulsant activity of some substituted 3,5-diphenyl-2-pyrazoline-1-carboxamide derivatives. Chem Sci J 2010;8:1–10.
23. Husain K, Abid M, Azam A. Novel Pd(II) complexes of 1-N-substituted 3-phenyl-2-pyrazoline derivatives and evaluation of antiamoebic activity. Eur J Med Chem 2008;43:393–403.
24. Shih S-R, Chu TY, Reddy GR, et al. Pyrazole compound BPR1P0034 with potent and selective anti-influenza virus activity. J Biomed Sci 2010;17:13.
25. Abunada NM, Hassaneen HM, Abu Samaha ASM, Miqdad OA. Synthesis and antimicrobial evaluation of some new pyrazole, pyrazoline and chromeno[3,4-c]pyrazole derivatives. J Braz Chem Soc 2009;20:975–87.
26. Padwa A, Pearson WH, eds. Synthetic applications of 1,3-dipolar cycloaddition chemistry toward heterocycles and natural products. Hoboken, NJ: John Wiley & Sons; 2003.

27. Huisgen R. Kinetics and mechanism of 1,3-dipolar cycloadditions. Angew Chem Int Ed 1963;2:633–45.
28. Loupy A, ed. Microwaves in organic synthesis. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2002.
29. Ju Y, Varma RS. Aqueous N-heterocyclization of primary amines and hydrazines with dihalides: microwave-assisted syntheses of N-azacycloalkanes, isoindole, pyrazole, pyrazolidine, and phthalazine derivatives. J Org Chem 2006;71:135–41.
30. Kost AN, Grandberg II. Progress in pyrazole chemistry. Adv Heterocycl Chem 1966;6:347–429.
31. Corradi A, Leonelli C, Rizzuti A, et al. New ''Green'' approaches to the synthesis of pyrazole derivatives. Molecules 2007;12:1482–95.
32. Fulton JR, Aggarwal VK, De Vicente J. The use of tosylhydrazone salts as a safe alternative for handling diazo compounds and their applications in organic synthesis. Eur J Org Chem 2005;1479–92.
33. Aggarwal VK, De Vicente J, Bonnert RV. A novel one-pot method for the preparation of pyrazoles by 1,3-dipolar cycloadditions of diazo compounds generated in situ. J Org Chem 2003;68:5381–3.
34. Symeonidis T, Chamilos M, Hadjipavlou-Litina DJ, et al. Synthesis of hydroxycoumarins and hydroxybenzo[f]-or [h]coumarins as lipid peroxidation inhibitors. Bioorg Med Chem Lett 2009;19:1139–42.
35. Michaelidou A, Hadjipavlou-Litina DJ, Matsini I, Tsitsogianni E. Heterocyclic aryl(phenyl)acetic acid and aryl acetohydroxamic acids as antiinflammatory antioxidant agents and inhibitors of lipoxygenase and serine proteases. Med Chem 2007;3:439–45.
36. Dimas K, Papadaki M, Tsimplouli C, et al. Labd-14-ene-8,13-diol (sclareol) induces cell cycle arrest and apoptosis in human breast cancer cells and enhances the activity of anticancer drugs. Biomed Pharmacother 2006;60:127–33.
37. Dimas K, Demetzos C, Mitaku S, et al. Cytotoxic activity of kaempferol glycosides against human leukaemic cell lines in vitro. Pharm Res 2000;41:85–8.
38. Dimas K, Hatziantoniou S, Tseleni S, et al. Sclareol induces apoptosis in human HCT116 colon cancer cells in vitro and suppression of HCT116 tumor growth in immunodeficient mice. Apoptosis 2007;12:685–94.
39. Biobyte Corp. C-QSAR database. Claremont CA, CSlifornia. Available form: www.biobyte.com.
40. De la Cruz P, Diaz-Ortiz A, Garcia JJ, et al. Synthesis of new C-60-donor dyads by reaction of pyrazolylhydrazones with [60]fullerene under microwave irradiation. Tetrahedron Lett 1999; 40:1587–90.
41. Huisgen R, Seidel M, Wallbillich G, Knupfer H. Diphenyl-nitrilimin und seine 1.3-dipolaren additionen an alkene und alkine. Tetrahedron 1962;17:3–29.
42. Nigam S, Schewe T. Phospholipase A(2)S and lipid peroxidation. Biochim Biophys Acta 2000;1488:167–81.
43. Kulisic T, Radonic A, Katalinic V, Milos M. Use of different methods for testing antioxidative activity of oregano essential oil. Food Chem 2004;85:633–40.
44. Molyneux P. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J Sci Technol 2004;26:211–19.
45. Koleva II, Van Beek TA, Linssen JPH, et al. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem Anal 2001;13:8–17.
46. Liegois C, Lermusieau G, Colin S. Measuring antioxidant efficiency of wort, malt, and hops against the 2,2′-azobis(2-amidinopropane) dihydrochloride-induced oxidation of an aqueous dispersion of linoleic acid. J Agric Food Chem 2000;48:1129–34.
47. Pontiki E, Hadjipavlou-Litina D. Review in QSARs on LOX inhibitors. Mini Rev Med Chem 2003;3:487–99.
48. Pontiki E, Hadjipavlou-Litina D. Quantitative Structure Activity Relationships (QSARs) on lipoxygenase inhibitors. Curr Med Chem – Anti-Inflammatory Anti-Allergy Agents 2004;3:139–56.
49. Pontiki E, Hadjipavlou-Litina D. Lipoxygenase inhibitors: A comparative QSAR study review and evaluation of new QSARs. Med Res Rev 2008;28:39–117.
50. Viles-Gonzalez JF, Badimon JJ. Atherothrombosis: the role of tissue factor. Int J Biochem Cell Biol 2004;36:25–30.
51. Maraganore MD. Thrombin, thrombin inhibitors, and the arterial thrombotic process. Thromb Haemostasis 1993;70:208–11.
52. Nyby MD, Sasaki M, Ideguchi Y, et al. Platelet lipoxygenase inhibitors attenuate thrombin- and thromboxane mimetic-induced intracellular calcium mobilization and platelet aggregation. J Pharm Exp Therap 1996;278:503–9.