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RESEARCH ARTICLE

Biologically active carbazole derivatives: focus on oxazinocarbazoles and related compounds

Zouhair Bouaziz[1], Samar Issa[2], Jacques Gentili[1], Andreas Gratz[3], Andre Bollacke[3], Matthias Kassack[4], Joachim Jose[3,4], Lars Herfindal[5,6], Gro Gausdal[5], Stein Ove Døskeland[5], Catherine Mullié[7], Pascal Sonnet[7], Camille Desgrouas[8], Nicolas Taudon[8], Glaucio Valdameri[9,10], Attilio Di Pietro[9], Milad Baitiche[11], and Marc Le Borgne[1]

[1]*EA 4446 Biomolécules Cancer et Chimiorésistances, Faculté de Pharmacie - ISPB, Université Lyon 1, Lyon, France,* [2]*Ecole de Biologie Industrielle, EBInnov, Cergy-Pontoise, France,* [3]*IPMC, PharmaCampus, WWU Münster, Germany,* [4]*IPMC, Heinrich-Heine-Universität Düsseldorf, Germany,* [5]*Department of Biomedicine, University of Bergen, Norway,* [6]*Translational Signaling Group, Haukeland University Hospital, Bergen, Norway,* [7]*Laboratoire de Glycochimie, des Antimicrobiens et des Agroressources (LG2A), FRE-CNRS 3517, Université de Picardie Jules Verne, Amiens, France,* [8]*UMR-MD3, Faculté de pharmacie, Aix-Marseille-Université, Marseille, France,* [9]*Drug Resistance Mechanism and Modulation Group, Bases Moléculaires et Structurales des Systèmes Infectieux (BMSSI), UMR 5086, CNRS, Université Lyon 1, IBCP, Lyon, France,* [10]*Department of Biochemistry and Molecular Biology, UFPR, Curitiba, Brazil, and* [11]*Faculté de Technologie, Université de Sétif-1, Algérie*

Abstract

Four series of carbazole derivatives, including N-substituted-hydroxycarbazoles, oxazinocarbazoles, isoxazolocarbazolequinones, and pyridocarbazolequinones, were studied using diverse biological test methods such as a CE-based assay for CK2 activity measurement, a cytotoxicity assay with IPC-81 cell line, determination of MIC of carbazole derivatives as antibacterial agents, a *Plasmodium falciparum* susceptibility assay, and an *ABCG2*-mediated mitoxantrone assay. Two oxazinocarbazoles **Ib** and **Ig** showed CK2 inhibition with $IC_{50} = 8.7$ and 14.0 μM, respectively. Further chemical syntheses were realized and the 7-isopropyl oxazinocarbazole derivative **2** displayed a stronger activity against CK2 ($IC_{50} = 1.40$ μM). Oxazinocarbazoles **Ib**, **Ig**, and **2** were then tested against IPC-81 leukemia cells and showed the ability to induce leukemia cell death with IC_{50} values between 57 and 62 μM. Further investigations were also reported on antibacterial and antiplasmodial activities. No significant inhibitory activity on ABCG2 efflux pump was detected.

Keywords

Biological activities, carbazoles, chemical synthesis, oxazinocarbazoles, screening

History

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Introduction

Extensive research in the carbazole chemistry has been reported since the description of the parent *9H-carbazole* in 1872, by Graebe and Glaser[1]. The discovery of the antibacterial activity of murrayanine extracted from the stem bark of *Murraya Koenigii* (Rutaceae) led to the discovery of new pharmacological activities[2,3]. Wu et al.[4] described the antiplatelet and vasorelaxing activities of carbazole derivatives extracted from the *Clausena excavata* and recently, Peng et al.[5] described new derivatives excavatine B and excavatine C with cytotoxic activity and antimicrobial activities. Many examples of compounds such as 5H-benzo[*b*]carbazoles[6], furo[3,4-*b*]carbazoles[7], pyrrolo[2,3-*a*]carbazoles[8,9] as well as indolocarbazoles[10] have shown antitumor activities. Nowadays, targeted therapies have become one of the main research activities and kinases have reached the second most important drug target after G-coupled protein receptors[11]. Prudent et al.[12] have shown the activity of pyridocarbazole as casein kinase 2 (CK2) inhibitors; CK2 is a constitutively active serine/threonine protein kinase that is involved in many diseases including different types of cancer[13]. Furthermore, the challenges in cancer and infectious diseases are to find active compounds that encounter multidrug resistance frequently associated with overexpression of the ABC transporter family. Indolocarbazole derivatives represent novel classes of ABCG2 inhibitors[14]. Miscellaneous activities for carbazole derivatives have been reported such as anti-oxidative, anti-inflammatory[15], and antibacterial activities[16,17].

To our knowledge, little has been described on the biological activity of oxazinocarbazole derivatives[18,19]. We have described the synthesis and cytotoxic activities of oxazinocarbazoles[20]. In this report, we focus on a large panel of pharmaceutical applications ranging from antibacterial, antimalarial activities as well as CK2 and ABCG2 inhibition enriched with the synthesis and activity of new carbazole derivatives.

Materials and methods

Chemistry

Reagents were commercial products of the highest available purity grade. Mitoxantrone was purchased from Sigma Aldrich (Lyon, France). Melting points were determined on an Electrothermal 9200 capillary apparatus (Wolf Laboratories

Address for correspondence: Marc Le Borgne, PhD, EA 4446 Biomolécules Cancer et Chimiorésistances, Faculté de Pharmacie - ISPB, Université Lyon 1, Lyon, France. E-mail: marc.le-borgne@univ-lyon1.fr

Limited, Pocklington, UK). The IR spectra were recorded on a Perkin Elmer Spectrum Two IR Spectrometer (Perkin Elmer, Waltham, MA). ^{1}H NMR and ^{13}C NMR spectra (broadband decoupling and DEPT-135) were recorded on a Brucker Avance 400 (400 MHz for ^{1}H and 133 MHz for ^{13}C) or a Brucker Avance 500 spectrometer (Perkin Elmer, Waltham, MA) (500 MHz for ^{1}H and 126 MHz for ^{13}C) using $CDCl_3$ or DMSO-d6 as solvents. Chemical shifts (δ) are referred to that of the solvent. Low resolution mass spectra were recorded on an Agilent 1290 Infinity system (Agilent technologies, Palo Alto, CA) equipped with an Agilent 1260 DAD detector and an Agilent 6120 Quadrupole mass detector with an ESI source in a positive mode. HRMS spectra were performed on a Q-TOF Micro Water with an ESI source in the positive mode. Flash chromatography was performed on 230–400-mesh silica.

*Synthesis of 9-isopropyl-9H-carbazol-4-ol (**1**)*

In a dried tricol containing 263 mg of NaH 60% (18.75 mmol) under an argon atmosphere, 500 mg of 4-hydroxycarbazole (2.73 mmol) dissolved in 0.4 mL of DMF and 5 mL of anhydrous THF were added dropwise. Within 10 min, 0.3 mL of 2-iodopropane (3 mmol) was added. The mixture was kept at room temperature and under an argon atmosphere for 2 h. The residue was hydrolyzed and neutralized with 15% HCl. After extraction with CH_2Cl_2, the organic phase was dried over Na_2SO_4 and concentrated under vacuum. The residue was purified by column chromatography on silica gel (CH_2Cl_2/Petroleum ether: 1/1). Yield 65%; viscous oil; IR: 3450, 1631, 1599, 1580 cm^{-1}; ^{1}H NMR (δ, $CDCl_3$): 8.36 (m, 1H, Harom.); 7.53 (d, 1H, $J = 8.3$ Hz, Harom.); 7.44 (m, 1H, Harom.); 7.30–7.24 (m, 2H, Harom.); 7.13 (d, 1H, $J = 8.3$ Hz, Harom.); 6.56 (dd, 1H, $J_1 = 7.8$ Hz, $J_2 = 0.6$ Hz, Harom.); 5.41 (s, 1H, OH); 4.99 (m, 1H, CHMe$_2$); 1.72 (d, 6H, $J = 7.1$ Hz, CHMe$_2$); ^{13}C NMR (δ, $CDCl_3$): 152.4 (1Cq); 141.8 (1Cq); 139.2 (1Cq); 126.2 (1CH); 124.9 (1CH); 123.3 (1CH); 122.7 (1Cq); 119.2 (1CH); 111.8 (1Cq); 109.9 (1CH); 104.7 (1CH); 103.2 (1CH); 47.2 (1CH); 21.1 (2CH$_3$); HRMS calculated for $C_{15}H_{15}NNaO$, $MNa^+ = 248.1046$, found, $MNa^+ = 248.1046$.

*Synthesis of 7-isopropyl-3-phenyl-2,3,4,7-tetrahydro[1,3]oxazino[5,6-c]carbazole (**2**)*

A solution containing 1.5 mmol of aniline and 3 mmol of formaldehyde dissolved in 10 mL methanol was stirred for 30 min at 0 °C. A solution containing 1 mmol of hydroxycarbazole **1** dissolved in 10 mL of methanol was added dropwise. The mixture was then stirred at room temperature for 24 h. At the end of the reaction, the solvent was evaporated under vacuum and the residue was purified by column chromatography on silica gel (CH_2Cl_2/cyclohexane: 1/6). Yield 63%; mp = 132.4 °C; IR: 1627, 1601, 1578 cm^{-1}; ^{1}H NMR (δ, $CDCl_3$): 8.54 (d, 1H, $J = 7.7$ Hz, Harom.); 7.67 (d, 1H, $J = 8.3$ Hz, Harom.); 7.59 (m, 1H, Harom.); 7.47–7.37 (m, 7H, Harom.); 7.12 (m, 1H, Harom.); 5.81 (s, 2H, H-2); 5.13 (m, 1H, CHMe$_2$); 5.00 (s, 2H, H-4); 1.87 (d, 6H, $J = 7.0$ Hz, CHMe$_2$); ^{13}C NMR (δ, $CDCl_3$): 150.9 (1Cq); 149.1 (1Cq); 140.1 (1Cq); 139.2 (1Cq); 129.5 (2CH); 124.8 (1CH); 124.0 (1CH); 123.7 (1CH); 122.5 (1Cq); 121.7 (1CH); 119.0 (3CH); 112.2 (1Cq); 110.0 (1Cq); 109.7 (1CH); 103.1 (1CH); 47.0 (1CH); 80.2 (1CH$_2$); 51.0 (1CH$_2$); 21.1 (2CH$_3$); HRMS calculated for $C_{23}H_{23}N_2O$, $MH^+ = 343.1805$, found, $MH^+ = 343.1811$.

*General procedure for the aminomethylation of 9-isopropyl-9H-carbazol-4-ol (**1**)*

A solution containing 2 mmol of primary amine and 2 mmol of formaldehyde dissolved in 10 mL of methanol was stirred for 30 min at 0 °C. A solution containing 1 mmol of hydroxycarbazole **1** dissolved in 10 mL of methanol was added dropwise. The mixture was then stirred at room temperature for 3 h to give a precipitate of the aminometylated compound which was purified by column chromatography on silica gel (CH_2Cl_2/petroleum ether: 2/1).

*3-[(Benzylmethylamino)methyl]-9-isopropyl-9H-carbazol-4-ol (**3**)*

Yield 67%; mp = 150 °C; IR: 2836, 1636, 1601 cm^{-1}; ^{1}H NMR (δ, $CDCl_3$): 8.39 (d, 1H, $J = 7.7$ Hz, Harom.); 7.39 (d, 1H, $J = 8.3$ Hz, Harom.); 7.33–7.13 (m, 8H, 7Harom. and OH); 6.96 (d, 1H, $J = 8.3$ Hz, Harom.); 6.85 (d, 1H, $J = 8.3$ Hz, Harom.); 4.85 (m, 1H, CHMe$_2$); 3.84 (s, 2H, CH$_2$); 3.58 (s, 2H, CH$_2$); 2.21 (s, 3H, NMe); 1.60 (d, 6H, $J = 7.1$ Hz, CHMe$_2$); ^{13}C NMR (δ, $CDCl_3$): 154.8 (1Cq); 141.2 (1Cq); 139.4 (1Cq); 137.4 (1Cq); 129.8 (2CH); 128.9 (2CH); 127.9 (1CH); 126.1 (1CH); 124.6 (1CH); 123.7 (1CH); 123.3 (1Cq); 119.0 (1CH); 112.4 (1Cq); 110.9 (1Cq); 109.5 (1CH); 100.9 (1CH); 61.7 (1CH$_2$); 61.4 (1CH$_2$); 47.0 (1CH); 41.5 (1CH$_3$); 21.1 (2CH$_3$); HRMS calculated for $C_{24}H_{27}N_2O$, $MH^+ = 359.2118$, found, $MH^+ = 359.2120$.

*3-{[(2-Chlorobenzyl)methylamino]methyl}-9-isopropyl-9H-carbazol-4-ol (**4**)*

Yield 63%; mp = 61 °C; IR: 2925, 1636, 1603 cm^{-1}; ^{1}H NMR (δ, $CDCl_3$): 8.44 (d, 1H, $J = 7.7$ Hz, Harom.); 7.48–7.19 (m, 8H, 7Harom. and OH); 7.06 (d, 1H, $J = 8.3$ Hz, Harom.); 6.94 (d, 1H, $J = 8.3$ Hz, Harom.); 4.93 (m, 1H, CHMe$_2$); 3.98 (s, 2H, CH$_2$); 3.83 (s, 2H, CH$_2$); 2.32 (s, 3H, NMe), 1.69 (d, 7.0 Hz, 6H, CHMe$_2$); ^{13}C NMR (δ, $CDCl_3$): 154.6 (2Cq); 141.2 (1Cq); 139.3 (1Cq); 135.2 (2Cq); 131.9 (1CH); 130.2 (1Cq); 129.3 (1CH); 127.3 (1CH); 126.2 (1CH); 124.6 (1CH); 123.7 (1CH); 123.2 (1Cq); 119.0 (1CH); 110.9 (1Cq); 109.5 (1CH); 100.9 (1CH); 61.5 (1CH$_2$); 59.1 (1CH$_2$); 47.0 (1CH$_3$); 41.4 (1CH); 21.1 (2CH$_3$); HRMS calculated for $C_{24}H_{26}ClN_2O$, $MH^+ = 393.1728$, found, $MH^+ = 393.1728$.

*3-{[(3-Chlorobenzyl)methylamino]methyl}-9-isopropyl-9H-carbazol-4-ol (**5**)*

Yield 47%; mp = 182 °C; IR: 2967, 2833, 1633, 1598 cm^{-1}; ^{1}H NMR (δ, $CDCl_3$): 8.46 (d, 1H, $J = 7.7$ Hz, Harom.); 7.48 (d, 1H, $J = 8.3$ Hz, Harom.); 7.42–7.21 (m, 7H, 6Harom. and OH); 7.05 (d, 1H, $J = 8.3$ Hz, Harom.); 6.95 (d, 1H, $J = 8.3$ Hz, Harom.); 4.99 (m, 1H, CHMe$_2$); 3.94 (s, 2H, CH$_2$); 3.64 (s, 2H, CH$_2$); 2.31 (s, 3H, NMe), 1.69 (d, 6H, $J = 7.0$ Hz, CHMe$_2$); ^{13}C NMR (δ, $CDCl_3$): 154.6 (1Cq); 141.3 (1Cq); 139.5 (1Cq); 139.4 (1Cq); 134.6 (1Cq); 130.3 (1CH); 129.9 (1CH); 128.1 (1CH); 127.9 (1CH); 126.1 (1CH); 124.7 (1CH); 123.7 (1CH); 123.2 (1Cq); 119.1 (1CH); 112.4 (1Cq); 110.7 (1Cq); 109.6 (1CH); 101.0 (1CH); 61.5 (1CH$_2$); 61.0 (1CH$_2$); 47.0 (1CH$_3$); 41.6 (1CH); 21.1 (2CH$_3$); HRMS calculated for $C_{24}H_{26}ClN_2O$, $MH^+ = 393.1728$, found, $MH^+ = 393.1726$.

*3-{[(4-Chlorobenzyl)methylamino]methyl}-9-isopropyl-9H-carbazol-4-ol (**6**)*

Yield 67%; mp = 139 °C; IR: 2985, 2954, 2845, 1633, 1603 cm^{-1}; ^{1}H NMR (δ, $CDCl_3$): 8.46 (d, 1H, $J = 7.9$ Hz, Harom.); 7.48 (d, 1H, $J = 8.1$ Hz, Harom.); 7.43–7.21 (m, 7H, 6Harom. and OH); 7.04 (d, 1H, $J = 8.1$ Hz, Harom.); 6.95 (d, 1H, $J = 8.3$ Hz, Harom.); 4.94 (m, 1H, CHMe$_2$); 3.93 (s, 2H, CH$_2$); 3.63 (s, 2H, CH$_2$); 2.30 (s, 3H, NMe), 1.69 (d, 6H, $J = 7.2$ Hz, CHMe$_2$); ^{13}C NMR (δ, $CDCl_3$): 154.6 (1Cq); 141.3 (1Cq); 139.4 (1Cq); 135.9 (1Cq); 133.7 (1Cq); 131.1 (2CH); 129.0 (2CH); 126.1 (1CH); 124.7 (1CH); 123.6 (1CH); 123.2 (1Cq); 119.1 (1CH); 112.4 (1Cq); 110.7 (1Cq); 109.6 (1CH); 101.0 (1CH); 61.4 (1CH$_2$); 60.8

($1CH_2$); 47.0 ($1CH_3$); 41.5 (1CH); 21.1 ($2CH_3$); HRMS calculated for $C_{24}H_{26}ClN_2O$, $MH^+ = 393.1728$, found, $MH^+ = 393.1727$.

*3-{3-[(Benzylmethylamino)methyl]-9-isopropyl-9H-carbazol-4-yloxy}propan-1-ol (**7**)*

A solution containing 100 mg (0.28 mmol) of **3**, 38 mg (0.28 mmol) of 3-bromopropan-1-ol, 77 mg (0.56 mmol) of K_2CO_3 and 10.3 mg (0.028 mmol) of tetrabutylammonium iodide (TBAI) dissolved in 5 mL of CH_3CN was stirred at 80 °C for 4 h. The mixture was then filtered on celite and washed with CH_3CN. The filtrate was evaporated and the residue was purified by column chromatography on silica gel (EtOAc/petroleum ether: 2/1) to give **7** as a liquid product. Yield 65%; IR: 3381, 2930, 2875, 1622, 1596 cm^{-1}; ^1H NMR (δ, $CDCl_3$): 8.19 (d, 1H, $J = 7.9$ Hz, Harom.); 7.46–7.13 (m, 10H, Harom.); 4.89 (m, 1H, CHMe$_2$); 4.25 (t, 2H, $J = 5.8$ Hz, CH_2); 3.96 (t, 2H, $J = 5.8$ Hz, CH_2); 3.72 (s, 2H, CH_2); 3.51 (s, 2H, CH_2); 3.05 (bs, 1H, OH); 2.17 (s, 3H, NMe); 2.13 (m, 2H, CH_2); 1.62 (d, 6H, $J = 7.0$ Hz, CHMe$_2$); ^{13}C NMR (δ, $CDCl_3$): 154.0 (1Cq); 141.2 (1Cq); 139.6 (1Cq); 139.0 (1Cq); 129.6 (2CH); 129.5 (1CH); 128.5 (2CH); 127.3 (1CH); 125.3 (1CH); 123.1 (1CH); 121.9 (1Cq); 120.7 (1Cq); 119.3 (1CH); 116.8 (1Cq); 110.1 (1CH); 106.3 (1CH); 71.3 ($1CH_2$); 62.0 ($1CH_2$); 60.6 ($1CH_2$); 57.4 ($1CH_2$); 47.1 ($1CH_3$); 42.8 (1CH); 33.2 ($1CH_2$); 21.1 ($2CH_3$); HRMS calculated for $C_{27}H_{33}N_2O_2$, $MH^+ = 417.2537$, found, $MH^+ = 417.2531$.

Preparation of recombinant human CK2 holoenzyme

The preparation of the human recombinant CK2 holoenzyme was performed according to the protocol previously described by Olgen et al.[21]. The α-subunit (CSNK2A1) and β-subunit (CSNK2B) of the human protein kinase CK2 were expressed in a bacterial pT7-7 system in *Escherichia coli* BL21 (DE3). Newly transformed starter cultures were grown overnight at 37 °C in LB-medium until they reached the stationary phase. With the separate starter cultures, new medium was inoculated for both subunits and cultivated until an OD_{500} of 0.6 was reached. Protein expression was induced by addition of IPTG (1 mM final concentration) and carried out at 30 °C for 5–6 h for CSNK2A1 and at 37 °C for 3 h for CSNK2B. Bacterial cells were harvested by centrifugation (6000 × g for 10 min at 4 °C) and disrupted by sonification (three times 30 s on ice). Preparations were then centrifuged to remove the cell debris and the bacterial extracts for both subunits and combined before purification by a three-column procedure. The fractions containing active CK2 holoenzyme were determined by activity measurement using the synthetic peptide substrate RRRDDDSDDD. Finally, fractions exhibiting CK2 activity were combined and analyzed by SDS-PAGE and Western Blot.

Capillary electrophoresis-based assay for the testing of inhibitors of the human CK2

The recently established capillary electrophoresis CK2 activity assay was used for testing the inhibitors[22]. Therefore, 2 µL of the dissolved inhibitors (stock solution in DMSO) were mixed with 78 µL of CK2 supplemented kinase buffer which was composed of 1 µg CK2 holoenzyme, 50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. The reaction was initiated by the addition of 120 µL assay buffer, which was composed of 25 mM Tris/HCl (pH 8.5), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 100 µM ATP, and 0.19 mM of the substrate peptide RRRDDDSDDD. The reaction was carried out for 15 min at 37 °C and stopped by the addition of 4 µL EDTA (0.5 M). Subsequently the reaction mixture was analyzed by a PA800 capillary electrophoresis from Beckman Coulter (Krefeld, Germany). Acetic acid (2 M, adjusted with conc. HCl to a pH of 2.0) was used as the electrolyte for electrophoretic separation. The separated substrate and product peptide were detected at 214 nm using a DAD-detector. Pure solvent was used as a negative control (0% inhibition) and assays devoid of CK2 were used as a positive control (100% inhibition). For primary testing an inhibitor concentration of 10 µM was used. Compounds that revealed at least 50% inhibition at 10 µM were used for IC_{50} determinations. The IC_{50} inhibition was determined using nine inhibitor concentrations ranging from 0.001 µM to 100 µM. IC_{50} values were calculated from the resulting dose–response curves.

Cytotoxicity assay

IPC-81 acute myelogenic leukemia rat cells[23] were maintained at logarithmic growth in the medium (DMEM) supplemented with 10% horse serum. For cytotoxicity testing, the IPC-81 cells were seeded in 96 well tissue culture plates at 150 000 cells/mL and exposed to various concentrations of CK2 inhibitors or vehicles for 24 h. The cells were next fixed in 2% buffered formaldehyde (pH 7.4) with the DNA-specific dye Hoechst 33342 (Polysciences Inc., Eppelheim, Germany) and scored for death by fluorescence microscopic evaluation of apoptotic chromatin condensation. At least 100 cells in three different microscopic fields were counted. IPC-81 cells were also analyzed for intactness of metabolic activity (formazan oxidation) by the WST-1 assay (Roche Diagnostics, Basel, Switzerland) following the supplier's instructions.

Determination of antibacterial activity

The following reference strains were used for testing the antibacterial activity: *E. coli* DSM 1103 and *Pseudomonas aeruginosa* DSM 1117 for Gram negative bacteria (Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany) and *Staphylococcus aureus* CIP 103.429 and *Enterococcus faecalis* CIP 103.214 (Collection de l'Institut Pasteur, Paris, France) for Gram-positive bacteria. Bacteria were grown overnight at 35 °C in Tryptic Soy Broth and streaked on Tryptic Soy Agar (AES, Bruz, France). From these isolation plates, inocula were prepared according to CLSI recommendations[24]. The broth microdilution technique was carried out as advised using selected carbazole derivatives dissolved in DMSO at concentrations ranging from 0.0625 to 128 µg/mL. Ciprofloxacin and DMSO were used in each series of experiments as a positive and a negative control, respectively. The MIC was determined as the lowest drug concentration at which wells remained visually clear.

Plasmodium falciparum susceptibility assay

The *in vitro* activities of oxazinocarbazole derivatives were tested over a concentration range of 0.78–10 000 nM against the susceptibility to chloroquine (CQ) strain *P. falciparum* 3D7. The traditional labeled hypoxanthine method was used to assess antimalarial activity, as described by Desjardins and coworkers[25]. CQ was routinely included as positive controls as well as negative controls using a solvent. The resulting IC_{50}s were calculated using Pk-Fit software[26] (Simulations Plus, Inc., Lancaster, CA). Inhibition growth (I) and corresponding drug concentration (C) were fitted according a sigmoid model, described as

$$I = (I_{max} \cdot C^{\gamma}) / \left(C^{\gamma} + IC_{50}^{\gamma}\right)$$

where I_{max} is the maximum inhibition growth and gamma the sigmoid factor of the curb.

Inhibitory activity on human ABCG2-transfected cells

All compounds were dissolved in DMSO and then diluted in the DMEM high glucose medium. The stock solution was stored at

−20 °C and warmed to 25 °C just before use. The human fibroblast HEK293 cell line transfected with *ABCG2* was obtained as previously described[27]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM high glucose), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and drug supplemented with 0.75 mg/mL G418. To perform the *ABCG2*-mediated mitoxantrone assay, HEK293-*ABCG2* cells were seeded at a density of 1×10^5 cells/well into 24-well culture plates. After 48 h incubation, the cells were exposed to 5 µM mitoxantrone for 30 min at 37 °C, in the presence or absence of compounds at various concentrations. The cells were then washed with phosphate buffer saline (PBS) and after being trypsinized and subsequently resuspended in ice-cold PBS (0.2 mL), they were kept on ice until analysis by flow cytometry. The data of intracellular drug fluorescence were monitored with a FACS Calibur cytometer (Becton Dickinson, Franklin Lakes, NJ) using a FL4-H channel. At least 10 000 events were collected, for which the maximal fluorescence (100%) was the difference between geometric mean fluorescence of cells incubated with 5 µM of GF120918 as a control (100% inhibition) and without a inhibitor.

Results and discussion

Chemistry

Among the 43 carbazole derivatives described in this study, the synthesis of only **2–7** has never been reported until now (Figure 1).

For the other series, experimental works are previously described. The *N*-substituted hydroxycarbazoles **8** and **9** were prepared in one step by a chemoselective *N*-alkylation of 2- or 3-hydroxycarbazole with 2.5 eq of NaH in DMF/THF mixture under argon atmosphere at room temperature and subsequent treatment with the alkylating agent[20,28]. The tricyclic carbazolequinones **10–12** were obtained starting from the corresponding hydroxycarbazoles by oxidation with Frémy's salt (Figure 2)[29,30].

Syntheses of pyridocarbazolediones **13** and **14** have been achieved using regiospecific hetero-Diels–Alder reaction between an azadiene and an appropriate bromocarbazolequinone[29]. Syntheses of isoxazolocarbazolediones **15** and **16** have been accomplished through a regioselective 1,3-dipolar cycloaddition of nitrile oxide with an appropriate bromocarbazolequinone (Figure 3)[31].

The oxazinopyrimidocarboline derivatives **17** and **18** were synthesized through a Mannich reaction employing 5-hydroxy-β-carboline, 4 eq of formaldehyde and 2 eq of a primary amine in MeOH at room temperature (Figure 4)[32].

Dimers **19** and **20** were obtained as by-products through a dimerization process in the course of the Mannich condensation among N-substituted 4-hydroxycarbazole, formaldehyde, and 2-(aminomethyl)pyridine as the primary amine (Figure 5)[20].

The oxazino[5,6-*c*]carbazoles **Ia–p** were prepared by a Mannich type condensation of N-substituted 4-hydroxycarbazole with 1.5 eq of various primary amines and 3 eq of formaldehyde. The Mannich reaction of N-substituted 2-hydroxycarbazole with the primary amines and formaldehyde led to a mixture of the two regioisomers, the oxazino[6,5-*b*]carbazoles **IIa–c** and the oxazino[5,6-*a*]carbazoles **IIIa–e** which were isolated by chromatography. Identification of the regioisomeric oxazinocarbazoles was unambiguously established from their ^{1}H NMR 300 MHz spectra (Figure 6)[20]. Thus, for oxazinocarbazoles **II**, the ^{1}H NMR spectra shows two singlets at 7.63 and 6.78 ppm for the aromatic protons H-5 and H-10 while in the regioisomers **III**, it exhibits two doublets at 7.83 and 6.78 ppm for the aromatic protons H-5 and H-6 with 8.5 Hz as a coupling constant value.

The N-isopropylated compound **1** was chemoselectively prepared by treatment of 4-hydroxycarbazole with sodium hydride in the presence of isopropyl iodide in the solvent system THF-DMF. Then, compound **2** was prepared by a Mannich reaction of **1** with 1.5 eq of aniline and 3 eq of formaldehyde in MeOH at room temperature. In contrast, syntheses of **3–6** have been carried out using a Mannich reaction of **1** with 2.5 eq of the corresponding secondary amines and 2.5 eq of formaldehyde in methanol at room temperature. The *O*-alkylation of **3** with 1 eq of 3-bromopropan-1-ol in the presence of 0.1 eq of tetrabutylammonium iodide (TBAI) and 2 eq of anhydrous K_2CO_3 in CH_3CN at 80 °C gave the compound **7** (Scheme 1).

Biological results

Carbazoles derivatives as potent inhibitors of human protein kinase CK2

Thirty-seven compounds were evaluated by the capillary electrophoresis (CE)-based assay[22] for human protein kinase CK2 activity measurement and inhibitor screening (Table 1). With this recently developed assay, the produced and purified recombinant human CK2 holoenzyme was used. The synthetic peptide RRRDDDSDDD was used as the substrate, which is reported to

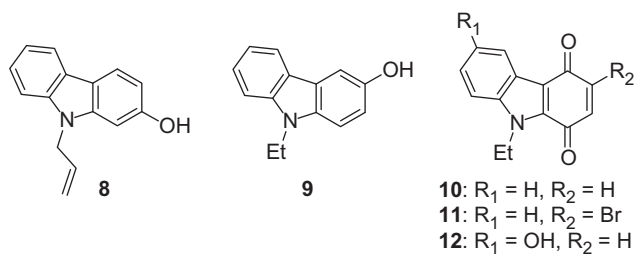


Figure 2. Structures of tricyclic hydroxycarbazole and carbazolequinone derivatives **8–12**.

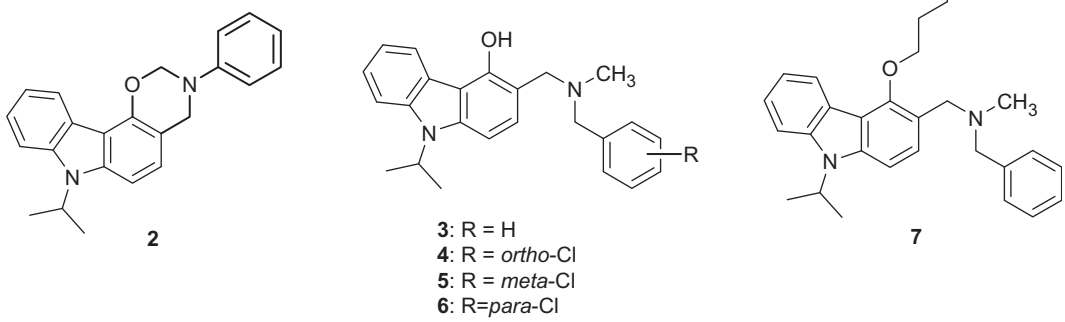


Figure 1. Structures of oxazinocarbazole **2** and aminomethylated carbazoles **3–7**.

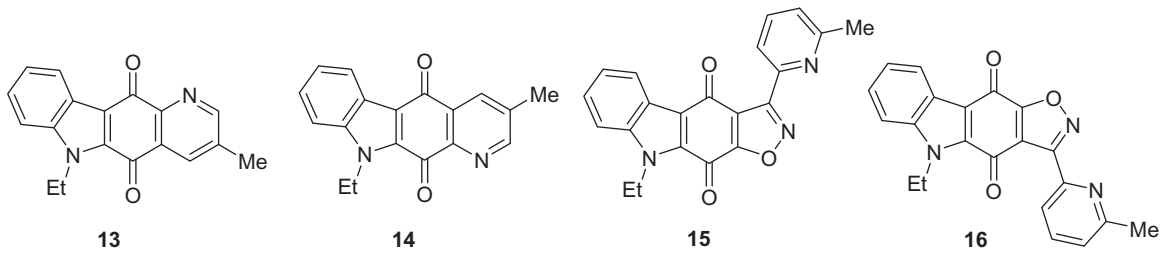


Figure 3. Structures of pyrido and isoxazolocarbazolediones **13–16**.

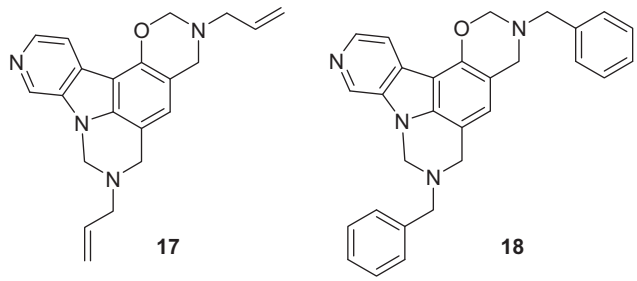


Figure 4. Structures of oxazinopyrimidocarboline derivatives **17** and **18**.

Figure 5. Structures of *N,N*-bis(carbazolylmethyl)amines **19** and **20**.

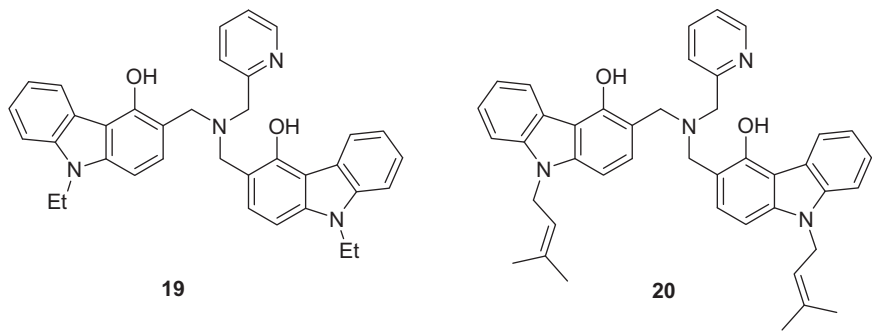
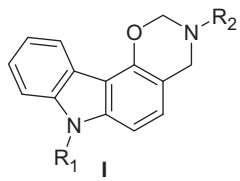
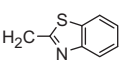


Figure 6. Structures of oxazinocarbazoles **Ia–p**, **IIa–c**, and **IIIa–e**.



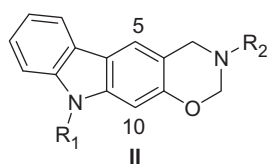
Ia: R_1 = Me, R_2 = $CH_2CH{=}CH_2$
Ib: R_1 = Me, R_2 = C_6H_5
Ic: R_1 = Me, R_2 = $CH_2C_6H_5$
Id: R_1 = Me, R_2 = $CH_2C_6H_4$(*p*-CH_3)

Ie: R_1 = Me, R_2 = 

If: R_1 = Et, R_2 = $CH_2CH{=}CH_2$
Ig: R_1 = Et, R_2 = C_6H_5
Ih: R_1 = Et, R_2 = $CH_2C_6H_5$
Ii: R_1 = Et, R_2 = $CH(CH_3)C_6H_5$
Ij: R_1 = $CH_2CH{=}CH_2$, R_2 = $CH_2CH{=}CH_2$
Ik: R_1 = $CH_2CH{=}CH_2$, R_2 = $CH_2C_6H_5$

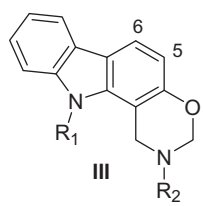
Il: R_1 = $CH_2CH{=}CH_2$, R_2 = 

Im: R_1 = $CH_2CH{=}C(CH_3)_2$, R_2 = C_6H_5
In: R_1 = $CH_2CH{=}C(CH_3)_2$, R_2 = $CH_2C_6H_5$
Io: R_1 = $CH_2CH{=}C(CH_3)_2$, R_2 = $CH_2C_6H_4$(*p*-CH_3)
Ip: R_1 = $CH_2CH{=}C(CH_3)_2$, R_2 = $CH(CH_3)C_6H_5$



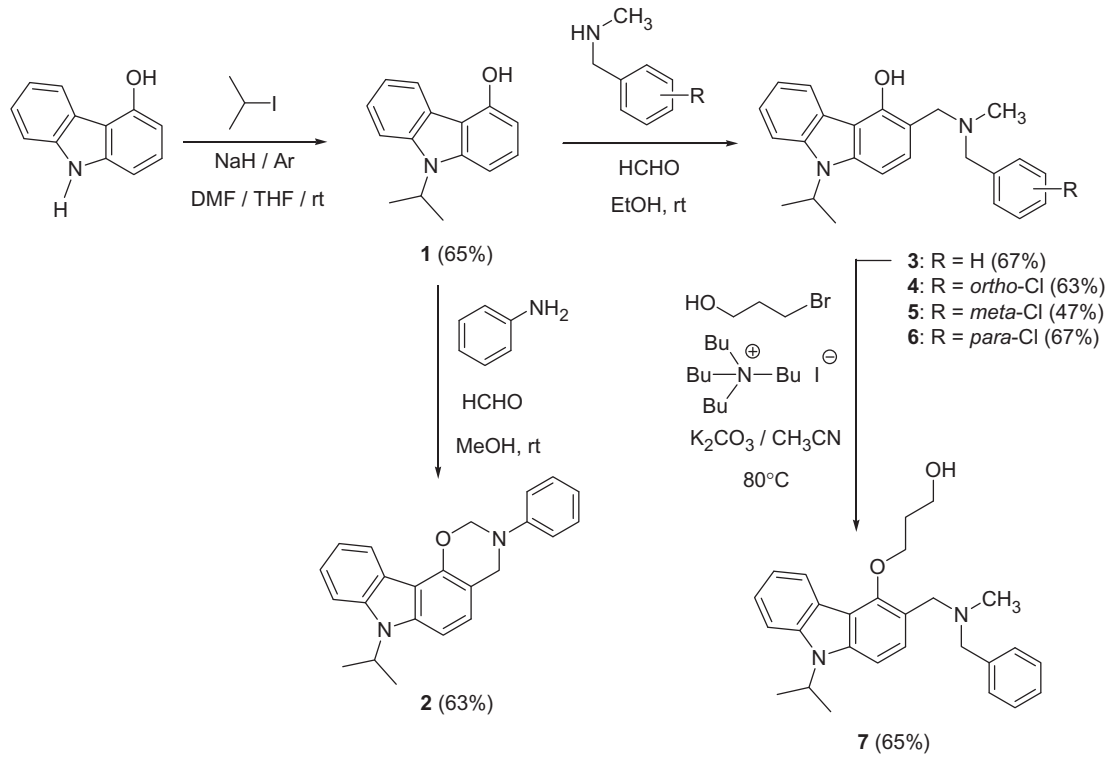
IIa: R_1 = Me, R_2 = CH_2-$CH{=}CH_2$
IIb: R_1 = Me, R_2 = C_6H_5

IIc: R_1 = Me, R_2 = 



IIIa: R_1 = Me, R_2 = CH_2-$CH{=}CH_2$
IIIb: R_1 = Me, R_2 = $CH_2C_6H_5$
IIIc: R_1 = Me, R_2 = $CH_2C_6H_4$(*p*-CH_3)
IIId: R_1 = Me, R_2 = $CH(CH_3)C_6H_5$

IIIe: R_1 = Me, R_2 = 



Scheme 1. Synthesis of compounds **1–7**.

Table 1. Inhibition of human protein kinase CK2 by carbazole derivatives **10–16**, **Ia–p**, **IIa–c**, and **IIIa–e**.

Compound	hCK2 (%) Inhibition at 10 μM	hCK2 IC_{50} (μM)
10	26	–
11	20	–
12	19	–
13	24[a,b]	–
14	Not soluble	–
15	18[a,b]	–
16	29[a,b]	–
Ia	20	–
Ib	52	8.7
Ic	29	–
Id	18	–
Ie	26	–
If	21	–
Ig	58	14.0
Ih	14	–
Ii	17	–
Ij	09	–
Ik	15	–
Il	25	–
Im	26	–
In	07	–
Io	15	–
Ip	34	–
IIa	33	–
IIb	18	–
IIc	15	–
IIIa	17	–
IIIb	26	–
IIIc	25	–
IIId	31	–
IIIe	17	–
emodin	99	0.58

[a]All target compounds were tested at 10 μM, except for compounds **13**, **15**, and **16** (tested with saturated solutions appeared to be below 10 μM).
[b]Compounds were tested in triplicates, except for compounds **13**, **15**, and **16** (only once experiment).

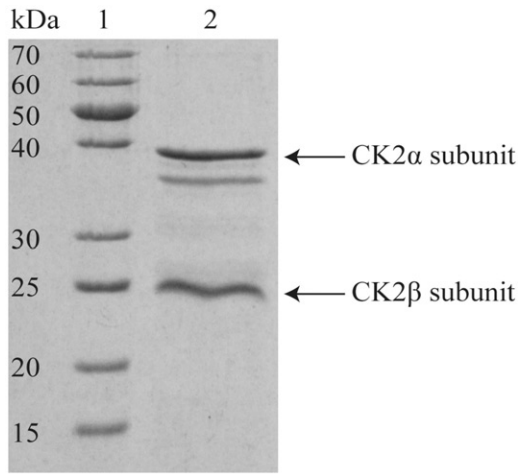


Figure 7. SDS-PAGE of purified human protein kinase CK2 holoenzyme. About 15 μL of purified protein solution (0.25 μg/μL) were separated on a 12.5% acrylamide gel and stained with Coomassie Brilliant Blue G250. At the left, the apparent molecular mass of the marker proteins (lane 1) is given. Lane 2 shows the purified human CK2 enzyme holoenzyme (3.75 μg). The band below the CK2α belongs to the well-known degradation product of the α-subunit CK2α (amino acids 1–335), which is supposed to be enzymatically active and using occurs during purification[33].

be most efficiently phosphorylated by CK2. The purity of the CK2 holoenzyme was higher than 99% (Figure 7)[33]. We can directly monitor the degree of CK2 substrate phosphorylation without pretreatment of the kinase reaction before capillary electrophoresis. This enabled us to reliably determine CK2 activity/inhibition.

For initial testing, inhibition was determined relative to the control at inhibition concentration of 10 μM of compounds dissolved in DMSO. Negative control cells were cultured with pure DMSO that never exceeded 1% as a final concentration and

Table 2. Inhibition of human protein kinase CK2 by oxazinocarbazole **2** and aminomethylated carbazoles **3–7**.

	hCK2	
Compound	(%) Inhibition at 10 μM	IC_{50} (μM)
2	71	1.401
3	26	–
4	31	–
5	29	–
6	32	–
7	16	–

set to 0% inhibition. Reactions without CK2 were used as the positive control and set to 100% inhibition. Compounds with more than 50% inhibition at a concentration of 10 μM were subjected to an IC_{50} determination. For this purpose, inhibition was measured at final concentrations ranging from 0.001 to 100 μM in appropriate intervals. IC_{50} were calculated from the resulting dose–response curves. Each value was determined at least in triplicate in independent experiments[22].

Tricyclic carbazolequinones **10–12** showed weak CK2 inhibition (% inhibition values <26%). Series of pyridocarbazolequinones **13**,**14** and isoxazolocarbazolequinones **15**,**16** were not very soluble at 10 μM in pure DMSO. Nevertheless, the saturated solutions of derivatives **13**, **15**, and **16** were tested and showed weak CK2 inhibitory activity (% inhibition values <29%), having in mind that these compounds could have stronger inhibitory activities without the problem of solubility. In the series of oxazinocarbazoles **I–III**, all compounds were soluble and the assay was performed in triplicate. Two oxazino[5,6-*c*]carbazoles **Ib** and **Ig** appeared to be potential CK2 inhibitors (% inhibition values >50%). They are structurally characterized by a short alkyl chain in R_1 (methyl for **Ib** and ethyl for **Ig**) and a common phenyl group in R_2. The IC_{50} values were calculated: 8.7 and 14.0 μM, respectively.

Further pharmacomodulation works were realized to optimize these first results. In a recent SAR study[34] on another tetracyclic scaffold (indeno[1,2-*b*]indole), the best inhibitory activity against CK2 was observed by introducing an isopropyl group on indolic nitrogen. Then the oxazinocarbazole **2** exerted the best CK2 inhibition with an IC_{50} value of 1.40 μM (Table 2) and could be used as lead compound for further structural exploration. In contrast, the opening of the oxazine ring did not improve the activity. In fact, aminomethylated carbazoles **3–7** showed a weak level of inhibition (<32%).

Oxazinocarbazoles as antileukemic agents

We tested the oxazinocarbazoles potency against IPC-81 leukemia cells. The IPC-81 cell line is derived from the transplantable BNML model of acute myelogenic leukemia (AML), known to be a reliable predictor of the clinical efficiency of antileukemic agents, like the first-line AML anthracycline drug daunorubicin (DNR)[35]. The oxazinocarbazoles had IC_{50} values between 57 and 62 μM (Figure 8). All the tested compounds had a similarly steep dose dependency, and were capable of inducing 100% IPC cell death at concentrations above 80 μM (Figure 8). The cell death morphology had apoptotic features, with nuclear condensation and surface membrane blebs (not shown), which are typical for such cells treated with various apoptosis inducers and antileukemic drugs[36]. We also tested the cell's mitochondrial formazan conversion (WST-assay, see Methods section). This assay agreed with the morphological assessment of cell death for long incubation times. For short incubation periods, the microscopic assay was superior, presumably because the already formed

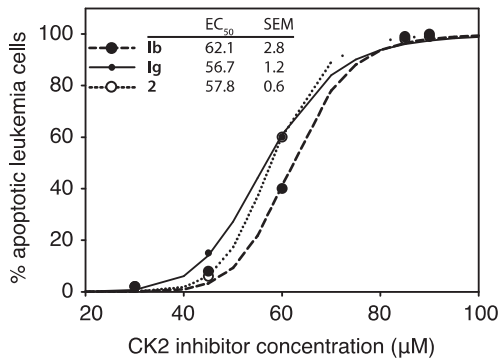


Figure 8. The ability of oxazinocarbazoles to induce IPC-81 leukemia cell death. IPC-81 cells were incubated with various concentrations of the compounds **Ib**, **Ig**, and **2**, and apoptosis assessed by microscopic evaluation of leukemia cell morphology after 24 h.

Table 3. Antimicrobial activities (MIC μg/mL) of carbazole derivatives **8–10**, **12**, **17–20**, **Ic**, **Ik**,**l**, **Ip**, and **IIc**.

	MIC (μg/mL)			
Compound	*Staphylococcus aureus* CIP 103429	*Enterococcus faecalis* CIP 103214	*Pseudomonas aeruginosa* DSM 1117	*Escherichia coli* DSM 1103
8	32	32	>128	>128
9	64	32	>128	>128
10	16	16	>128	>128
12	>128	>128	>128	>128
17	>128	>128	>128	>128
18	>128	>128	>128	>128
19	>128	>128	>128	>128
20	>128	>128	>128	>128
Ic	>128	>128	>128	>128
Ik	>128	>128	>128	>128
Il	>128	>128	>128	>128
Ip	>128	>128	>128	>128
IIc	>128	>128	>128	>128

mitochondrial formazan converting enzymes only lose their activity when the cell undergoes secondary necrosis with loss of ATP and general proteolysis.

We conclude that the oxazinocarbazole derivatives were indeed able to induce leukemia cell death at concentrations well below 80 μM.

Oxazinocarbazoles as antibacterial agents

Most of the tested compounds did not display any significant antibacterial activity at the tested concentrations (Table 3). Only hydroxycarbazoles **8**,**9** and non-substituted carbazolequinone **10** were found to be somewhat effective, the latter being the most efficient, but only on Gram-positive bacteria (i.e. *S. aureus* and *E. faecalis*). These molecules had in common a low molecular weight, as compared to the ineffective ones. Surprisingly, compound **12**, which only differs from compound **10** by the addition of a hydroxyl substituent, lost all activity at concentrations up to 128 μg/mL despite a rather low molecular weight. The overall lack of efficiency on Gram-negative bacteria can be hypothesized to be linked with an impermeability of the Gram-negative cell wall to the tested compounds.

Oxazinocarbazoles as antimalarial agents

Sixteen compounds were evaluated *in vitro* for their antimalarial activity against *P. falciparum* 3D7 strain according with the IC_{50} values as shown in Table 4. As the strain used in this assay was

Table 4. Antiplasmodial activities (IC_{50} nM) of carbazole derivatives **2**, **8–10**, **12**, **17–20**, **Ib**,**c**, **Ig**, **Ik**,**l**, **Ip**, and **IIc**.

Compound	*Plasmodium falciparum* 3D7 IC_{50} (nM)
2	3380 ± 108
8	0675 ± 190
9	>4500
10	>4500
12	>4500
17	>4500
18	0551 ± 181
19	>4500
20	>4500
Ib	4244 ± 653
Ic	>4500
Ig	4131 ± 482
Ik	>4500
Il	>4500
Ip	0659 ± 180
IIc	0430 ± 390
Chloroquine	018.9 ± 0.53

well described, chloroquinosensible and chloroquine were used for the validation of the assay. The susceptibility to available antimalarial drugs for this strain following the used isotopic method is close to the range 2–200 nM with end points corresponding to dihydroartemisinin and quinine, respectively. The susceptibility to doxycycline which is only used for malaria prophylaxis is closed to 10 μM. Four compounds showed interesting results with IC_{50} under 1 μM: hydroxycarbazole derivative **8** ($IC_{50} = 675$ nM), oxazinopyrimidocarboline derivative **18** ($IC_{50} = 551$ nM) and two oxazinocarbazole derivatives **Ip** ($IC_{50} = 659$ nM) and **IIc** ($IC_{50} = 430$ nM).

Oxazinocarbazoles and ABCG2-mediated drug transport

To investigate the activity of carbazole derivatives in multidrug resistance, oxazinocarbazoles **2**, **Ia–c**, **Ig**, **Ij**,**k**, **In**, **IIa**,**c**, **IIIa**,**e**, and dimers **19**,**20** were screened for their inhibition of mitoxantrone efflux in *ABCG2*-transfected cells. Ko143 was used as other control and showed an inhibition of 115%. Only very low inhibition (20% for compound **IIIe**) was observed at 10 μM for the tested compounds.

Conclusions

Five different biological assays were used to explore new pharmacological potencies of carbazole derivatives: inhibition of CK2, IPC-81 cell line cytotoxicity, antibacterial activity, antimalarial activity, and inhibitory activity on HEK293-*ABCG2* cells. The best results were obtained on CK2 assay with the oxazinocarbazoles **Ib** ($IC_{50} = 8.7$ μM), **Ig** ($IC_{50} = 14.0$ μM), and **2** ($IC_{50} = 1.4$ μM). Cytotoxicity activity of these CK2 inhibitors was determined on IPC-81 leukemia cells (IC_{50} lower than 80 μM). From the point of view of the structure, oxazinocarbazoles **Ib**, **Ig**, and **2** have a short indolic N-substituted side chain (e.g. methyl, ethyl, and isopropyl) and a phenyl group in the oxazine ring. New pharmacomodulations on oxazinocarbazole template are currently studied and will later be published. In contrast, investigation concerning the antiplasmodial activities showed encouraging results with carbazole derivatives **8** ($IC_{50} = 675$ nM), **18** ($IC_{50} = 551$ nM), **Ip** ($IC_{50} = 659$ nM), and **IIc** ($IC_{50} = 430$ nM). Further structural explorations should be envisaged to determine a new pharmacophore model for antimalarial agents. No carbazole derivatives showed significative activities on bacteria and ABCG2 efflux pump for further investigations. Finally carbazole and its congeners (e.g. oxazinocarbazoles) continue to be central templates for drug design of new small molecules.

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Declaration of interest

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