Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Indirubin Core Structure of Glycogen Synthase Kinase-3 Inhibitors as Novel Chemotype for Intervention with 5-Lipoxygenase

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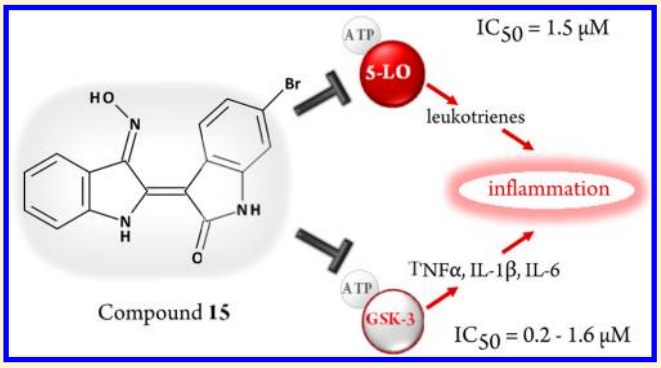
Supporting Information

ABSTRACT: The enzymes 5-lipoxygenase (5-LO) and glycogen synthase kinase (GSK)-3 represent promising drug targets in inflammation. We made use of the bisindole core of indirubin, present in GSK-3 inhibitors, to innovatively target 5-LO at the ATP-binding site for the design of dual 5-LO/GSK-3 inhibitors. Evaluation of substituted indirubin derivatives led to the identification of (3*Z*)-6-bromo-3-[(3*E*)-3-hydroxyiminoindolin-2-ylidene]indolin-2-one (**15**) as a potent, direct, and reversible 5-LO inhibitor (IC_{50} = 1.5 μM), with comparable cellular effectiveness on 5-LO and GSK-3. Together, we present indirubins as novel chemotypes for the development of 5-LO inhibitors, the interference with the ATP-binding site as a novel strategy for 5-LO targeting, and dual 5-LO/GSK-3 inhibition as an unconventional and promising concept for anti-inflammatory intervention.



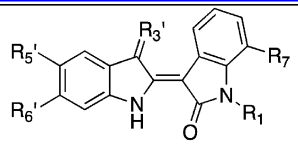
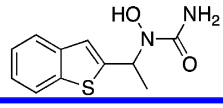
INTRODUCTION

5-Lipoxygenase (5-LO) is an iron-containing dioxygenase that initiates the biosynthesis of leukotrienes (LT) from arachidonic acid (AA), by the incorporation of molecular oxygen to form 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HpETE) and the subsequent dehydration to leukotriene $(LT)A_4$.[1] In the cells, AA is liberated by the cytosolic phospholipase $(cPL)A_2$ and delivered to 5-LO by the 5-LO-activating protein (FLAP). The unstable epoxide LTA_4 is then metabolized to LTB_4 or to cysteinyl-containing LT_S (C_4, D_4, and E_4; cysLTs), which act via specific receptors (BLT and CysLT receptors 1 and 2) to promote inflammation and regulate immunity (LTB_4) or to induce smooth muscle contraction and plasma extravasation (cys-LTs).[2] Thus, $CysLT_1$ antagonists (e.g., montelukast) are effectively used to treat asthma and allergy, while clinical trials for BLT_1 antagonists have not been successful so far, despite excellent preclinical results.[3] As compared to LT-receptor antagonism, however, targeting LT biosynthesis offers the obvious advantage of suppressing all LTs and appeals for its potentially higher efficacy as anti-inflammatory therapy.[4]

Thus, different sources (e.g., nature, synthesis) and diverse medicinal chemistry approaches (e.g., lead identification,[5] scaffold-hopping,[6] structure-based drug design,[7] dynamic modeling,[8] structural optimization[9]) have long been explored to identify potent 5-LO inhibitors.[4] These compounds interfere with 5-LO mainly in three different ways: (I) active-site iron chelation, (II) uncoupling of the redox cycle of the active site iron, and (III) substrate/fatty acid competition at the active site. Accordingly, most 5-LO inhibitors are metal ion chelators, reducing agents, or fatty acid mimetics.[4] Despite intensive research, however, only the iron ligand-type 5-LO inhibitor compound **25** (zileuton, 1-(1-benzothiophen-2-ylethyl)-1-hydroxy-urea, Table 1) has been approved so far for asthma treatment, though its use is limited due to liver toxicity. Other lead compounds or concepts have instead failed during development, as a result of severe side effects (e.g., methemoglobinemia for redox active compounds) or lack of efficacy (e.g., ineffectiveness under oxidative stress for non-redox type inhibitors),[4] thereby generating demand for novel chemotypes and mechanisms of intervention with 5-LO, as well as for novel therapeutical strategies. In fact, considerable efforts have been directed toward the identification of dual-inhibition approaches, mainly targeting 5-LO and another enzyme of the AA cascade, in order to improve therapeutic efficacy and to reduce side effects. Thus, dual-inhibition of 5-LO/cyclooxygenase (COX),[10] 5-LO/microsomal prostaglandin E_2

Received: November 12, 2013
Published: April 3, 2014

Table 1. Inhibition of 5-LO Product Formation by IO and 7-Bromo Indirubin Derivatives

cmpd						5-LO activity at 3 μM compound (% of control)[a]	
	R_1	R_7	$R_{3'}$	$R_{5'}$	$R_{6'}$	cell-free[b]	intact cells[c]
1 (IO)	H	H	N-OH	H	H	78.4 ± 5.8	92.2 ± 2.6
2 (7BIO)	H	Br	N-OH	H	H	68.9 ± 4.9***	43.0 ± 13.7***
3	CH_3	Br	N-OH	H	H	97.7 ± 8.1	122.0 ± 8.0
4	H	Br	O	H	H	85.4 ± 4.3	109.6 ± 4.9
5	H	Br	N-OMe	H	H	82.9 ± 6.4	120.1 ± 3
6	H	Br	N-OCOMe	H	H	79.2 ± 2.3	111.8 ± 4.5
7	H	Br	O	CH=N-OH	H	80.0 ± 8.0	95.1 ± 3
8	H	Br	N-OH	H	COOH	77.1 ± 7.5	122.8 ± 7.9
9	H	Br	O	H	COOH	80.0 ± 7.5	105.2 ± 6.4
10	H	Br	N-OH	$COOCH_3$	H	72.8 ± 3.3	99.1 ± 37.8
25						28.1 ± 5.2***	37.9 ± 2.0***

[a]Values are means ± SE, n = 3 or 4. **p < 0.01; ***p < 0.001 vs control; ANOVA + Bonferroni. [b]Cell-free: isolated human recombinant 5-LO. [c]Intact cells: intact human neutrophils stimulated with 2.5 μM A23187 ionophore.

synthase-1 (mPGES-1),[11] or 5-LO/soluble epoxide hydrolase (sEH)[12] has been examined with promising results.

Here, we present the bisindole core of the natural alkaloid indirubin as a novel chemotype for the development of synthetic 5-LO inhibitors with a new molecular mode of action of 5-LO interference, i.e., targeting the ATP-binding site. Since these compounds have been revealed as potent inhibitors of glycogen synthase kinase (GSK)-3,[13,14] a central kinase in immunity and inflammation, an unconventional 5-LO/GSK-3 dual-inhibition strategy is proposed.

RESULTS AND DISCUSSION

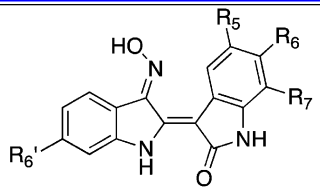
Several natural compounds suppress 5-LO product formation,[15] but most of them act by unselective antioxidant activity, possess metabolically labile moieties, and exert severe side effects. Certain natural substances (e.g., hyperforin, tryptanthrin), however, allowed the identification of novel 5-LO inhibitory mechanisms,[16,17] but their synthesis is often hardly accessible and/or presents limitation for structural optimization. In a recent study aimed to investigate the effect of the natural indirubin derivative **1** (also known as indirubin-3′-oxime, IO) on vascular inflammation as a promising vasoprotective compound, we have identified a direct, though moderate, effect of **1** on 5-LO activity, causing suppression of LT biosynthesis in inflammatory cells.[18] Interestingly, the 3,2′-bisindol core of **1** represents a novel structure as compared to known natural and/or synthetic inhibitors of 5-LO and may prevent classical limitations of natural 5-LO inhibitors, since it lacks obvious antioxidant properties, while being prone to structural modifications. Importantly, compound **1** has been a lead compound for the development of inhibitors of kinases (such as GSK-3; IC_{50}, 22 nM in a cell-free assay),[13,14,19] due to interference with the ATP-binding site. Remarkably, 5-LO also binds ATP,[1] though this interaction has remained thus far unexplored in terms of development of 5-LO inhibitors. In fact, ATP-binding to 5-LO, apparently via the C2-like domain, is exploited for purification of 5-LO using an ATP affinity column.[20] However, the exact position of the ATP-binding site of 5-LO is unknown, thus precluding molecular modeling approaches, and this site has only been analyzed with probes for photoaffinity labeling.[20,21] Consequently, we here aimed to analyze (i) whether indirubin analogues may serve as novel chemotypes for the development of 5-LO inhibitors with improved potency as compared to that of **1**, (ii) the possible interference with the ATP-binding site of 5-LO as a new molecular strategy for 5-LO inhibition, and (iii) whether dual inhibition of 5-LO and GSK-3, which are both attractive targets to control inflammation, may take place at similar compound concentrations in relevant assays.

A comprehensive evaluation of 5-LO inhibitors requires both cell-free and cellular test systems.[4] We thus performed assays with human recombinant 5-LO and with Ca^{2+}-ionophore-stimulated human neutrophils, respectively. Compound **1** inhibited 5-LO activity with an IC_{50} of 15.0 (cell-free) and 11.1 (cellular) μM. To identify 5-LO inhibitors more potent than **1**, we thus performed an initial screening of synthetic indirubin derivatives at a concentration of 3 μM. At this concentration, no significant inhibition was observed for **1** (Table 1), while the reference compound **25** worked as expected (60−70% inhibition). To fulfill the objective, a set of indirubins from our in-house library was rationally selected. Thus, our selection included compounds known to interact with the kinase ATP cavity in different fashions and with direct or inverted binding modes, as previously shown for effects on GSK-3 (**14**, **15**),[22] Aurora kinases (**2**, **6**, **11**, **12**),[23] and dual-specificity tyrosine-regulated kinases (DYRKs; **7**, **8**, **9**, **10**).[24] Overall, all known types of interaction of indirubins with the ATP cavity were covered in a small set of compounds.

The presence of bromine in 7-position (**2**, also known as 7-bromo-indirubin-3′-oxime, 7BIO), which increased the anti-

dx.doi.org/10.1021/jm401740w | J. Med. Chem. 2014, 57, 3715−3723

Table 2. Inhibition of 5-LO Product Formation by IO Derivatives

cmpd					5-LO activity at 3 μM compound (% of control)[a]	
	R_5	R_6	R_7	R_6'	cell-free[b]	intact cells[c]
11	H	H	Cl	H	65.8 ± 3.4**	67.4 ± 8.3*
12	H	H	F	H	58.1 ± 4.4***	50.4 ± 17.9**
13	H	H	CF_3	H	68.0 ± 1.7*	92.6 ± 1.9
14 (5BIO)	Br	H	H	H	62.3 ± 0.9***	51.1 ± 8.7**
15 (6BIO)	H	Br	H	H	32.4 ± 1.5***	38.0 ± 5.3***
16	H	H	H	Br	43.9 ± 4.1***	111.0 ± 22.2
25					28.1 ± 5.2***	37.9 ± 2.0***

[a]Values are means ± SE, $n = 3$ or 4. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs control; ANOVA + Bonferroni. [b]Cell-free: isolated human recombinant 5-LO. [c]Intact cells: intact human neutrophils stimulated with 2.5 μM A23187 ionophore.

tumoral effect of indirubins,[25] improved 5-LO inhibition in comparison to **1** in both cell-free and cellular test systems (Table 1), which let us to further consider 7-bromo-indirubin derivatives. *N*-Methylation in the 1-position is used as negative control in kinase inhibition assays, because of a steric clash between the *N*-methyl group and amino acid residues of the ATP pocket.[22] Importantly, the *N*-methylated compound **3** was also inactive for 5-LO, highlighting a possible similar steric hindrance on 5-LO. Replacement of the 3′-oxime by a keto group (**4**), 3′-methoxyme (**5**), or acetoxyme (**6**) as well as moving the oxime from the 3′- to 5′-position (**7**) was detrimental, essentially indicating the requirement for the free oxime moiety in the 3′-position and for the hydrogen in position N1 for an improved inhibitory activity on 5-LO. In addition, incorporation of a carboxylic acid in 6′-position (**8**, **9**) or of a methyl ester in the 5′-position (**10**) also did not improve 5-LO inhibition in both cell-free and cellular assays. Note that several known LT synthesis inhibitors of different classes are lipophilic compounds with an acidic moiety,[4] which has been correlated to high plasma protein binding and, as observed for example for boswellic acids,[26] to ineffectiveness *in vivo*. Moreover, those findings rather exclude an inverted mode of interaction between indirubins and 5-LO, as **8**, **9**, and **10** were initially designed to interfere with the ATP cavity of DYRKs via inverted binding.

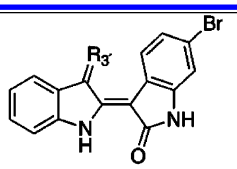
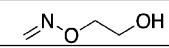
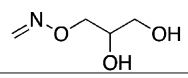
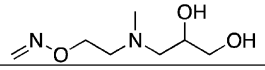
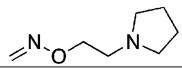
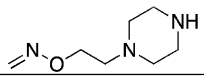
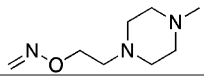
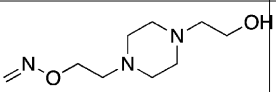
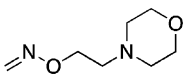
5-LO has been discussed as possible drug target in cancer, and therefore the compound **2** (7BIO) may be an interesting lead in this respect. However, for further SAR studies, we aimed to test whether the bromine in the 7-position was required or if a different substitution pattern leading to less cytotoxic compounds may be tolerated or even advantageous in terms of 5-LO inhibition. Indeed, replacement of the bromine by chlorine (**11**) or fluorine (**12**) was tolerated, while replacement with trifluoromethyl (**13**) was detrimental in intact cells (Table 2). Note that incorporation of the bromine in the 5-position (**14**, 5BIO) and especially in the 6-position (**15**, 6BIO) instead of the 7-position increased the 5-LO inhibitory potency. These data indicate the need of a halogen for high efficiency and position 6 as preferred. Interestingly, insertion of the bromine in the 6′-position (compound **16**) instead of the 6-position was detrimental for inhibition of 5-LO, but only in intact cells. Note that compound **15** has been previously reported to reduce SH-SY5Y cancer cells viability only at high concentrations (EC_{50} > 30 μM for compound **15** vs 8 μM for compound **2**).[25] Thus, **15** is more potent as 5-LO inhibitor than **2**, but less cytotoxic.

As **15** (6BIO) has been found to be a potent GSK-3 inhibitor (IC_{50} in cell-free assay, 5 nM),[22] we then explored the inhibitory potential on 5-LO of 6BIO analogues with improved solubility, possessing hydroxy- (**17**−**19**) and/or amine-moieties (e.g., pyrrolidine-, piperazine-, morpholino-, **20**−**24**) (Table 3) and known to be more potent on GSK-3 than the parent **15**.[14] However, these structural modifications resulted in a lower inhibition as compared to compound **15**, and significant effects were observed only for the 1-glycerol oximether **18** and the morpholino-*N*-ethyl oximether **24**, suggesting a possible difference in the size and shape of 5-LO binding site as compared to the ATP cavity of GSK-3. Table 4 summarizes the IC_{50} values of the most relevant derivatives for inhibition of human recombinant 5-LO and 5-LO product formation in human neutrophils. Compound **15** showed the same potency in cell-free and cellular assays (IC_{50} = 1.5 μM), indicating an improved potency of about 10-fold over that of parental compound **1**, and was equipotent to the reference compound **25** (IC_{50} = 0.6−3 μM, Table 4). The high potency of **15** was also confirmed after addition of exogenous AA to the cellular test system, which bypasses the need for $cPLA_2$. Note that a reduced potency after addition of exogenous AA was instead observed for FLAP inhibitors.[27] Hence, our findings suggest that **15** is likely not targeting $cPLA_2$ or FLAP (in the low micromolar range) but instead shows features of a direct and cell-permeable 5-LO inhibitor.

To evaluate interference with the putative ATP-binding site, we first assessed whether **15** interferes with the binding of 5-LO to ATP-agarose. As shown in Figure 1A, human recombinant 5-LO was eluted from ATP-agarose column by incubation with **15**, while the inactive *N*-methylated indirubin-derivative **3** was ineffective. As expected, no elution was observed by **25**, while ATP eluted 5-LO. Next, we evaluated whether 5-LO inhibition by **15** was affected by ATP. We observed a significant reduction of both potency (right shift of inhibitory curve, higher IC_{50}

dx.doi.org/10.1021/jm401740w | J. Med. Chem. 2014, 57, 3715−3723

Table 3. Inhibition of 5-LO Product Formation by 6-Bromo Indirubin Derivatives

cmpd	 R_3'	5-LO activity at 3 μM compound (% of control)[a] cell-free[b]	intact cells[c]
17		88.3 ± 5.4	81.5 ± 26.4
18		58.0 ± 16.1*	77.7 ± 3.4*
19		71.5 ± 3.1	87.0 ± 17.8
20		75.1 ± 3.3	127.1 ± 37.7
21		76.2 ± 2.6	64.7 ± 21.3
22		82.1 ± 2.4	91.1 ± 5.1
23		89.3 ± 5.0	119.2 ± 25.3
24		60.3 ± 3.1***	62.7 ± 12.0**

[a]Values are means ± SE, $n = 3$ or 4. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs control; ANOVA + Bonferroni. [b]Cell-free: isolated human recombinant 5-LO. [c]Intact cells: intact human PMNL stimulated with 2.5 μM A23187 ionophore.

Table 4. IC_{50} Values for Inhibition of 5-LO Product Formation by Indirubin Derivatives and Positive Control 25

	5-LO activity (IC_{50} values)[a]		
		intact cells[c]	
compd	cell-free[b]	A23187	A23187/AA
1	15.0 ± 2.1	11.1 ± 1.3	8.2 ± 1.5
2	5.8 ± 0.8	1.7 ± 0.5	3.6 ± 0.9
11	4.9 ± 0.6	4.9 ± 0.7	3.1 ± 0.3
12	3.7 ± 0.4	5.0 ± 0.8	3.3 ± 1.7
14	5.6 ± 0.4	2.6 ± 0.6	3.3 ± 0.8
15	1.5 ± 0.1	1.5 ± 0.5	1.5 ± 0.3
25	0.6 ± 0.1	1.7 ± 0.7	3 ± 1

[a]The IC_{50} values were calculated after sigmoidal concentration−response fitting (variable slope) by GraphPad Prism software. Values are means ± SE, $n = 3$. [b]Cell-free: isolated human recombinant 5-LO. [c]Intact cells: intact human neutrophils stimulated with 2.5 μM A23187 ionophore in the absence or presence of 20 μM AA.

values) and efficacy (lower magnitude of inhibition) by increasing ATP concentrations in both the S40 fraction of *E. coli* transformed with pT3−5LO (Figure 1B) and isolated 5-LO (Figure 1C), while no significant differences were observed for the iron ligand-type inhibitor **25** (Supplementary Figure S1).

Notably, the observation that the potency of **15** was not impaired in the cell-free assay under non-reducing conditions (Table 4), which is instead typical for non-redox inhibitors,[4] and its ineffectiveness as radical scavenger (Figure 2A) essentially excluded classical non-redox and redox mechanisms of 5-LO inhibition. Interestingly, **15** inhibited 5-LO in a reversible fashion (Figure 2B) and showed efficiency also in human monocytes (Figure 3), indicating that its suppressive effect on LT synthesis is not restricted to only a certain cell type. Human monocytes also express 15-LO, which is closely related to the murine leukocyte-type 12-LO (also known as 12/15-LO), and allow assessing inhibitory effects on 12- and 15-HETE formation. However, **15** was not active in this respect (Figure 3), indicating a selective effect on 5-LO. Note that 12/15-LO fails to bind ATP.[28] Importantly, **15** also inhibited 5-LO product formation when neutrophils or monocytes were stimulated with the pathophysiologically relevant stimuli lipopolysaccharide (LPS) and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (IC_{50} = 1.5 ± 0.7 and 0.5 ± 0.2 μM for 5-HETE reduction in neutrophils and monocytes, respectively) (Supplementary Figure S2A). It should be noted that no reduction in cell viability was observed after incubation with **15** (Supplementary Figure S2B), excluding possible toxic effects at this level. We also tested whether **15** simultaneously targets other enzymes of the AA cascade that have been previously considered for dual inhibitory strategies. However, in cell-free assays, **15** at 10 μM did not significantly inhibit COX-1 or COX-2, and only partial inhibition was observed for mPGES-1 and sEH, while the positive controls indomethacin (indo, for COXs), MK886 (for mPGES-1), and 12-(3-adamantane-1-yl-ureido)-dodecanoic acid (AUDA, for sEH) inhibited as anticipated (Figure 4).

Together, **15** shows a novel and reversible mechanism of interference with 5-LO, seemingly via the ATP-binding site, with relevance in different cells and assay conditions, and selectivity over other LOs. We propose that binding to the ATP site may cause structural alteration of 5-LO that results in reduced catalytic activity. For instance, changes in the relative location/orientation of the regulatory C2 domain of 5-LO toward the catalytic domain has been also proposed as mechanism for the novel type 5-LO inhibitor hyperforin.[16] Targeting the ATP-binding site represents a novel molecular strategy for 5-LO inhibition without affecting other iron-containing enzymes and proteins or fatty acid metabolizing enzymes (including 12/15-LOs that do not bind ATP). In fact, the development of 5-LO inhibitors acting by classical mechanism of 5-LO inhibition (i.e., iron chelation, redox interference, AA competition) has essentially been hampered by severe side effects due to the reducing properties or to unspecific iron-chelating features of the compounds. Also, *in vivo* ineffectiveness because of rapid oxidation of reducing agents or because of competition with fatty acids (or their hydroperoxides, present in high amounts in plasma) for non-redox "substrate analogues" have been observed.[4] Compounds acting at the putative ATP-binding site may present the potential advantages to offer novel chemotypes with favorable pharmacokinetic profile and may also be useful to gain insights into structural information on the 5-LO ATP binding site, which, as mentioned remains still to be characterized.

IO derivatives have been previously reported to inhibit GSK-3. Interestingly, 6-bromo substitution also resulted in an improved inhibitory potency on GSK-3 in vitro (cell-free), with IC_{50} values of 5 nM for compound **15** vs 22 nM for compound **1**, while *N*-methylation inactivated the compounds.[13] Since 5-LO and GSK-3 are both relevant targets for an anti-inflammatory therapy, we aimed to analyze whether **15** affects

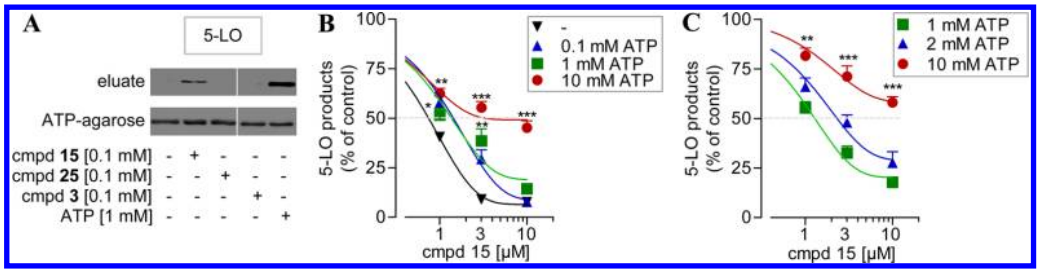


Figure 1. (A) Compound **15** elutes 5-LO bound to ATP. Results are representative for three comparable experiments. (B,C) 5-LO inhibition by **15** in (B) the S40 fraction of *E. coli* transformed with pT3−5LO or (C) isolated 5-LO is impaired by increasing ATP concentrations. Values are means + SE, $n = 3$. 5-LO products in 100% controls correspond to (ng/mL): (B) S40 fraction, 538.1 ± 90.3, 690.1 ± 87.5, 722.1 ± 108.3, and 27.9 ± 8.8, for incubations without (−) or containing 0.1, 1, and 10 mM ATP, respectively; (C) isolated 5-LO, 1147.5 ± 50.1, 1090.0 ± 40.2, and 177.9 ± 28.7, incubations containing 1, 2, and 10 mM ATP, respectively. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs corresponding concentration (B) without ATP (−) or (C) with 1 mM ATP; ANOVA + Bonferroni.

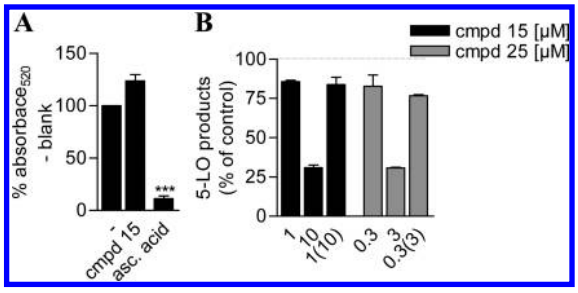


Figure 2. (A) Radical scavenging properties. Compound **15** (10 nmol) or ascorbic acid (asc. acid, 5 nmol) was incubated with 5 nmol of DPPH in 100 μL of EtOH for 30 min at RT, and the absorbance at 520 nm was measured. (B) Inhibition of 5-LO activity by compounds **15** and **25** is reversible. Purified 5-LO (about 10 μg/mL) was incubated with 1 μM or 10 μM compound **15** or with 0.3 μM or 3 μM compound **25**, respectively, for 10 min at 4 °C. Aliquots of the 10 μM compound **15** sample and of the 3 μM compound **25** sample were diluted with assay buffer 10-fold (1(10) and 0.3(3), respectively), while the concentration of the inhibitors in the other aliquots was not altered (by 10-fold dilution with assay buffer containing the corresponding concentrations of the inhibitors). Then, samples were prewarmed for 30 s at 37 °C, and 20 μM AA and 2 mM $CaCl_2$ were added. After 10 min, 5-LO product formation was analyzed as described. 5-LO products in 100% controls correspond to 799.1 ± 147.4 ng/mL. Data are expressed as percentage of control (100%), means + SE, $n = 3$.

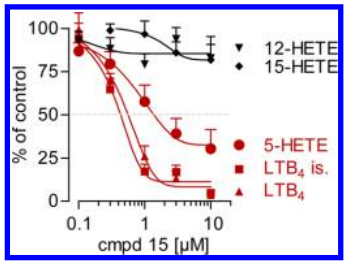


Figure 3. Effects of compound **15** on the formation of products of 5-LO (LTB_4, LTB_4 isomers, 5-HETE; red) and of 15-LO (12-HETE, 15-HETE; black) in human monocytes stimulated for 10 min at 37 °C with A23187 plus AA (2.5 and 10 μM, respectively). The preincubation with vehicle (0.1% v/v DMSO) or with compound **15** was performed for 10 min at 37 °C. Samples were analyzed by UPLC−MS/MS, and data (peak area) were normalized on the internal standard PGB_1 and are expressed as percentage of control, means + SE; $n = 3$.

cellular GSK-3 activity and downstream events at concentrations comparable to cellular 5-LO inhibition. GSK-3 is a

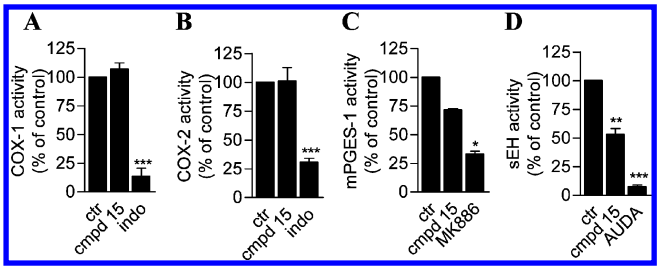


Figure 4. Effects of compound **15** on the activity of (A) COX-1, (B) COX-2, (C) mPGES-1, and (D) sEH. Compound **15** (10 μM) or reference inhibitors (10 μM indomethacin, indo; 10 μM MK886; 100 nM AUDA) were added to the respective enzymes (A, B, D) or microsomal enzyme preparation (C) 10 min prior starting the enzyme reaction. Data are expressed as percentage of the remaining activity of the uninhibited vehicle (0.1% v/v DMSO) control (100%). 100% controls correspond to (A) 108.9 ± 6.5 ng/mL 12-HHT; (B) 171.3 ± 43 ng/mL 12-HHT; (C) 1.2 ± 0.2 nmol PGE_2 in 100 μL reaction buffer; (D) Ex_{330}, Em_{465}: 28,724 ± 8,022. Values are means ± SE, $n = 3$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs control; ANOVA + Bonferroni.

major component of the β-catenin degradation complex and plays a key inhibitory role in the Wnt pathway. In the cell, GSK-3 phosphorylates β-catenin in a constitutive manner, thus targeting it for ubiquitination and degradation by the proteasome,[29] which has been described also in monocytes.[30] Accordingly, inhibition of GSK-3 induces stabilization of β-catenin that is considered as a direct outcome and a downstream activation of the canonical Wnt signaling pathway. Thus, we performed reporter gene assays in HEK293 containing the Wnt/β-catenin activated reporter pSuperTOP-FLASH (STF293 cells) as a read-out for inhibition of cellular GSK-3 activity and observed significant effects by **15** at concentrations of 0.5−1 μM (Figure 5A). Accordingly, **15** (1

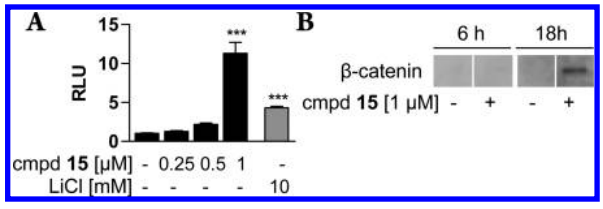


Figure 5. Compound **15** inhibits cellular GSK-3 activity as analyzed by (A) a Wnt reporter gene assay in STF293 cells and (B) by β-catenin stabilization in human monocytes. Data are means + SD, $n = 3$, duplicates.

dx.doi.org/10.1021/jm401740w | J. Med. Chem. 2014, 57, 3715−3723

μM) induced stabilization of β-catenin in human monocytes (Figure 5B). This event was accompanied by a significant reduction of pro-inflammatory cytokines (Table 5), with effects in the high nanomolar to low micromolar range. Together, **15** is a dual inhibitor of 5-LO and GSK-3 with comparable potency in intact cells.

Table 5. IC_{50} Values[a] (μM) for Inhibition of Cytokine Release by Compound 15

TNFα	IL-6	IL-8	IL-1β
0.5 ± 0.03	1.6 ± 0.7	>3	0.2 ± 0.1

[a]The IC_{50} values were calculated after sigmoidal concentration–response fitting (variable slope) by GraphPad Prism software. Values are means ± SE, n = 3. Intact human monocytes stimulated with 10 ng/mL LPS after preincubation for 30 min at 37 °C with vehicle (0.1% DMSO) or compound **15**. Cytokine amount in 100% (LPS-stimulated and vehicle-treated) controls was (ng/mL) 1.7 ± 0.3, 38.1 ± 0.3, 24.6 ± 2.9, and 20.9 ± 3.1 for TNFα, IL-6, IL-8, and IL-1β, respectively.

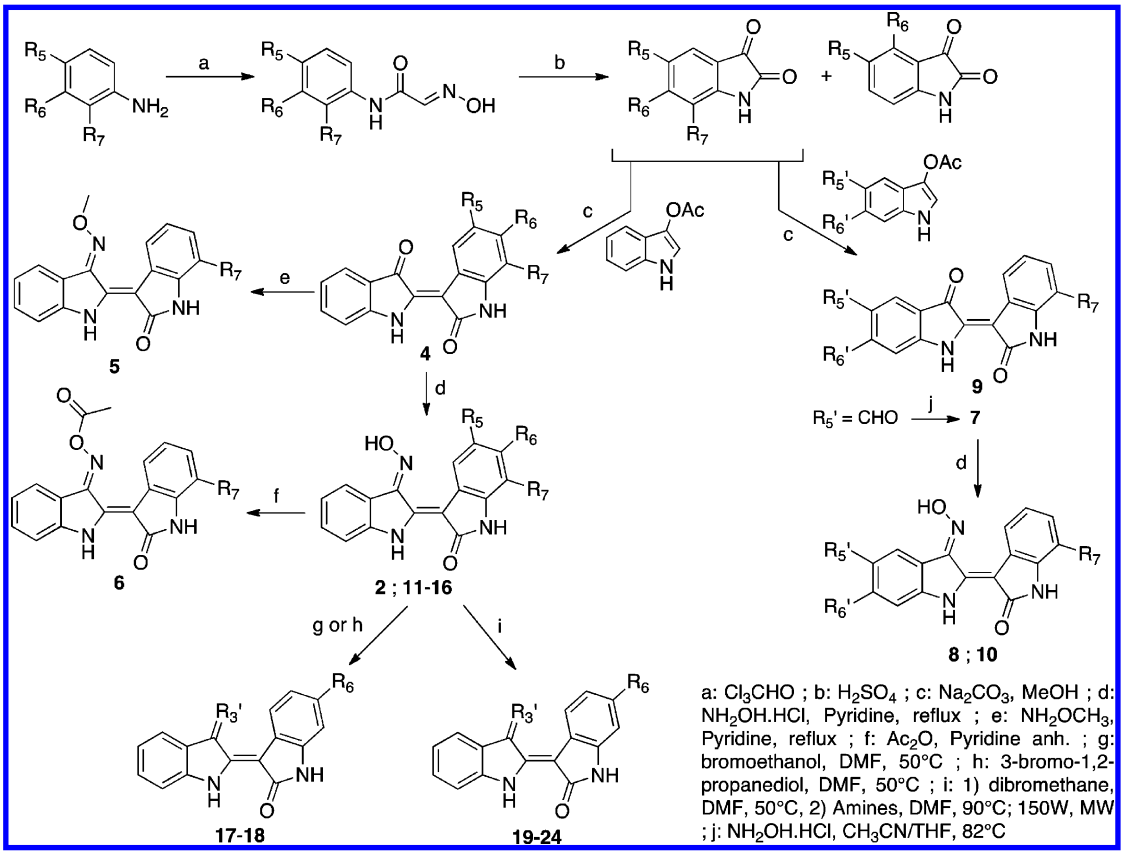
CONCLUSIONS

We identified the 3,2′-bisindol core of the natural product indirubin as a privileged and innovative chemotype for the development of 5-LO inhibitors. Starting from indirubin-3′-oxime (**1**) and considering several types of substitution, the 6-bromine substitution turned out to be advantageous and led to **15** (6BIO), a GSK-3 inhibitor, with 10-fold improvement in the potency (IC_{50} = 1.5 μM) being about equipotent to the only approved 5-LO inhibitor zileuton (**25**). Compound **15** is a direct and cell-permeable 5-LO inhibitor interfering with the ATP-binding site and showed selectivity over other AA-metabolizing enzymes and high efficiency under different stimulatory conditions. We classify this dual 5-LO/GSK-3 inhibition as a novel unconventional concept for anti-inflammatory intervention. Though inhibition of GSK-3 has been shown to attenuate asthma in mice,[31] a possible benefit of a dual 5-LO/GSK-3 inhibition in respiratory diseases is unknown. On the other hand, this novel combination of inhibitory activities may represent a promising strategy for those diseases where both GSK-3 and 5-LO are involved, such as Alzheimer's disease, which could also result in new therapeutic indications as compared to single approaches and should be further investigated in future studies. However, as GSK-3 inhibition causes up-regulation of the Wnt-pathway, caution should be taken for patients e.g. with hyperproliferative diseases where Wnt-signaling may play a crucial role.

EXPERIMENTAL SECTION

General Methods. The indirubin derivatives **2**–**6**, **11** and **12** were synthesized as previously described (see Scheme 1).[32] Their structures have been confirmed by NMR and MS and conformed to the literature. Derivatives **7**–**10** and **13** have been synthesized as previously described.[24] Their structures have been confirmed by NMR and MS and conformed to the literature. Compounds **14**, **15**, and **16** were synthesized according to procedures described in the literature.[22,25,33] For the preparation of ethers **17**–**24**, compound **15** was dissolved in anhydrous DMF under argon. Dibromoethane was then added, and the mixture was stirred for 48 h at 50 °C. Addition of water and subsequent filtration afforded the corresponding (2′*Z*-3′*E*)-6-bromoindirubin-3′-[*O*-(2-bromoethyl)-oxime]. The latter was then dissolved in anhydrous DMF under argon, and the corresponding amine was added. The mixture was then submitted to microwave

Scheme 1. General Synthesis of Indirubins Derivatives



dx.doi.org/10.1021/jm401740w | J. Med. Chem. 2014, 57, 3715–3723

irradiation, in a CEM Single Mode microwave, at 90 °C (150 W) for 40 min to afford the corresponding ethers **17**–**24**.[14] The structures were confirmed by NMR and MS and were in accordance with literature. Condensation of correctly substituted isatin and 3-acetoxyindoles afforded the indirubins in good yields (80–90%). Oxime on the 3′-carbonyl has been quantitatively introduced by reaction with hydroxylamine in refluxing pyridine.[14] Derivatives **18** and **19** have been obtained in good yield (70–80%) by reaction of the corresponding indirubin-3′-oxime with bromoethanol and 3-bromo-1,2-propanediol, respectively. Derivatives **20**–**25** have been synthesized in good yields (90–100%) in an already established two-step procedure.[14] The purity (>95%) of the compounds was determined by combustion analysis.

Materials. Compound **1** was purchased from Enzo Life Sciences (Lörrach, Germany). Compound **25** was purchased from Sequoia Research Products (Oxford, U.K.). All other chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany), unless stated otherwise. HPLC and UPLC solvents were from VWR (Darmstadt, Germany).

Expression and Purification of 5-LO. 5-LO was expressed in *E. coli* Bl21 (DE3) cells transformed with pT3–5LO,[34] as indicated in the Supporting Information. For ATP competition experiments, *E. coli* cells were lysed, and the 40,000 × *g* supernatant (S40 fraction) was preincubated with 0, 0.1, 1, or 10 mM ATP for 10 min a 4 °C. Then, vehicle (0.1% v/v DMSO) or test compounds were added for 10 min at 4 °C prior to addition of Ca^{2+} and AA (end concentrations, 1 mM free Ca^{2+} and 20 μM AA) for 10 min at 37 °C. The reaction was stopped, samples were extracted, and 5-LO products were analyzed by HPLC as indicated in the Supporting Information. For ATP-agarose binding experiments, the S40 fraction was incubated with ATP-agarose (Sigma; Deisenhofen, Germany) for 10 min at 4 °C. After centrifugation (1000 × *g*, 1 min, 4 °C), pellets were washed 3 times with PBS containing 1 mM EDTA (PBS-EDTA) and then incubated for 30 min at 4 °C with vehicle (0.1% DMSO) or test compounds, as indicated. Then, samples were centrifuged (2000 × *g*, 5 min, 4 °C), and pellets and supernatants were separated. Supernatants (eluates) were centrifuged again and then mixed with SDS-PAGE sample loading buffer. Pellets (ATP-agarose) were washed with PBS-EDTA and incubated with SDS loading buffer to release bound 5-LO. Samples were then analyzed by Western blotting as described in the Supporting Information. Alternatively, 5-LO was partially purified by ATP-agarose column for activity assays, as previously reported.[34,35] After washing of the column (bed volume, 1 mL) with PBS-EDTA, 5-LO enzyme was eluted with 10 mL of PBS-EDTA buffer containing 22 mM ATP (resulting in an eluate containing 20 mM ATP). The eluate was diluted 1:25 to 1:100 with PBS-EDTA buffer for activity test, and ATP concentration was accordingly adjusted to 1 mM, as standard conditions.[34,35] For ATP competition experiments on isolated 5-LO, ATP concentrations were adjusted to 1, 2, and 10 mM ATP, as indicated. Samples (0.5–2 μg of partially purified 5-LO, resulting in about 750 ng/mL 5-LO products in an activity test performed with 20 μM AA) were incubated 10 min at 4 °C with vehicle or test compounds, prewarmed for 30 s at 37 °C, and 2 mM $CaCl_2$ and the indicated concentrations of AA were added. The reaction was stopped, and 5-LO products were analyzed as indicated in the Supporting Information.

Determination of 5-LO Product Synthesis. Isolation and stimulation of neutrophils and monocytes and determination of 5-LO product formation were performed as described[36] and as indicated in the Supporting Information. Incubations were performed with 5 × 10^6 and 2 × 10^6 cells/mL for neutrophils and monocytes, respectively. Analysis of LO products was performed by HPLC or by UPLC–MS/MS. For the values given in Tables 1–4, 5-LO products in vehicle (DMSO) controls were cell-free, 787.2 ± 61.5 ng/mL; intact cells, 236.5 ± 101.8 and 514.8 ± 48.8 ng/mL for A23187 and A23187 + AA stimulated neutrophils, respectively.

Reporter Gene Assay. HEK-293 cells carrying a stably integrated luciferase reporter construct under the control of 7 LEF/TCF binding sites (STF293 cells,[37] provided by Dr. Jeremy Nathans, The Johns Hopkins University, Baltimore) were treated for 24 h with vehicle or the indicated compounds and lysed, and Firefly luciferase activities were measured in duplicate as reported previously.[38]

Analysis of Cytokine Production in Monocytes. Monocytes were incubated with vehicle (0.25% v/v DMSO) or compound **15** for 30 min at 37 °C and then left untreated or stimulated with LPS (10 ng/mL; 18 h). Cytokines were measured in the cell supernatants by ELISA, according to the manufacturer's instructions (R&D systems, Minneapolis, Minnesota). The expression of β-catenin was evaluated by Western blotting as described in the Supporting Information.

COX-1, COX-2, mPGES-1, and sEH Assays. Activities of isolated ovine COX-1 and human COX-2, of human mPGES-1 from A549 cells, and of purified human sEH were analyzed as described in the Supporting Information.

Statistics. Results are means ± standard error (SE) or standard deviation (SD) of *n* independent experiments. The IC_{50} values were determined by sigmoidal concentration–response equation (GraphPad Prism, San Diego, CA). Statistics was performed by repeated measures one-way analysis of variance (ANOVA) followed by Bonferroni, using a two-sided alpha level of 0.05 (*$p < 0.05$).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting methods and figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Harald Zimmer, Petra Wiecha, Monika Listing, and Anna Lazari for assistance. This work was supported by the Commission of the European Community through the INsPiRE project (EU-FP7-REGPOT-2011-1, proposal 284460).

■ ABBREVIATIONS USED

AA, arachidonic acid; AUDA, 12-(3-adamantane-1-yl-ureido)-dodecanoic acid; BIO, bromoindirubin-3′-oxime; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; DYRK, dual-specificity tyrosine-regulated kinases; FLAP, 5-lipoxygenase-activating protein; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GSK-3, glycogen synthase kinase-3; 5-HpETE, 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; IO, indirubin-3′-oxime; 5-LO, 5-lipoxygenase; LT, leukotriene; mPGES-1, microsomal prostaglandin E_2 synthase-1; sEH, soluble epoxide hydrolase

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