From Tyrian Purple to Kinase Modulators: Naturally Halogenated Indirubins and Synthetic Analogues

Konstantina Vougogiannopoulou, Alexios-Leandros Skaltsounis

Department of Pharmacognosy and Natural Product Chemistry, Faculty of Pharmacy, National and Kapodistrian University of Athens, Athens, Greece

Abstract

Indirubins represent a small category of compounds with significant pharmacological activity focusing on the inhibition of protein kinases. A series of derivatives has been developed during the last 15 years aiming the investigation and amelioration of the indirubin scaffold in terms of activity, selectivity, and drug-likeness. The current article focuses on the naturally brominated indirubins present in the famous historic dye of Tyrian purple, attempting to gather all available literature regarding biosynthesis, isolation, and synthesis of related analogues. Halogenated indirubins are by far one of the most important subcategories of indirubins, with its main representatives 6-bromoadindirubin (6BI) and 6-bromoadindirubin-3′-oxime (6BIO) possessing an increased selectivity against GSK-3. This review attempts to summarize concisely structure/activity relationships among closely related halogenated analogues in terms of protein kinase inhibition and selectivity, while it also focuses on the various biological applications arising from the interactions of halogenated indirubins with molecular targets. Those include effects of halogenated indirubins on stem cells, cardiac, renal, and pancreatic cells, on leukaemia and solid tumors, and on neurodegeneration.

Introduction – Tyrian purple

Indirubin (1), indigo (2), and isoindigo (3) are the core representatives of a rather small category of bisindole alkaloids referred to as indigoids (Fig. 1). Their fascinating history begins before even their exact chemical structure was elucidated. These compounds are the colored constituents of the natural dyes indigo and the famous molluscan Tyrian purple, used throughout the centuries for textile dying and so providing a significant commercial benefit to communities which produced it.

Indigo dye was used as a blue natural dye from the Bronze Age (~7000). It has been established that the treatment of indigo bearing plants (Brassicaceae, Polygonaceae, Fabaceae) for the production of the blue dye was a common practice in the past, almost worldwide. In Europe, indigo was predominantly produced from the woad of Isatis tinctoria (Brassicaceae) [1]. According to Pliny the Elder, the inhabitants of Britain used to paint their faces with a blue dye (indigo) in order to appear more intimidating to the enemy. The cultivation and process of woad and the trading of indigo were extremely vital economical elements of the renaissance commerce. The main production and trade centers were Albi/Toulouse in France, Somerset in Great Britain, Thüringen in Germany, and Florence in Italy.

In India, Pakistan, South America, and Africa, Indigofera tinctoria (Fabaceae) was cultivated and processed for the production of indigo dye [2] while the Mayas have combined indigo and natural clays to prepare the pigment Maya blue [3]. In China, Korea, and Japan, Polygonum tinctorium (Polygonaceae) was used for the preparation of indigo dye, although the species was considered poor in terms of indigo content [4].

While indigo was considered to be a «dye of the poor», Tyrian purple was used widely to declare emperors used it to color ceremonial robes while a variation of Tyrian purple [5], the blue Tekhelet is mentioned in the Jewish bible as the dye used in the clothes of the High Priest [6]. The oldest application of molluscan purple dye dates
has been treated in the traditional Chinese medicine with the recipe Danggui Longhui Wan, a mixture of 11 herbal medicines. The active ingredient was found to be a dark blue powder, Qing Dai, prepared from the leaves of indigo producing plants. Eventually, the antileukemic activity was attributed to indirubin, which was detected in the mixture of Danggui Longhui Wan as a minor constituent [11].

Since then, indirubin and its halogenated analogues have exerted a vast range of biological effects in stem cells [12], cardiac, renal, and pancreatic cells. In addition, brominated indirubins have been utilized as tools for the exploration of neurodegeneration, cancer, and as potential therapeutic agents for parasitic diseases. In most of the cases, all of the above effects can be associated with the interaction of indirubins with important molecular targets such as members of the family of protein kinases (GSK-3 [13], CDKs [14], and Aurora kinases [15]) and the aryl hydrocarbon receptor [16,17], placing them among the most promising nature-derived drug candidates [18].

Chemistry of Halogenated Indirubin Analogues

Natural sources

The name “indirubin” was first introduced in 1855 by Edward Schunck [19] to describe a red coloring ingredient present in indigo producing plants. Extensive studies performed thereafter, have proven that indirubin is present in diverse natural sources such as the indigo producing plants of Isatis spp. [20], Indigofera spp., and Polygonum spp., recombination bacteria, [21] mammalian – including human – urine [22], and Tyrian purple producing marine mollusks [23] (© Fig. 2).

The pigments present in the plant-derived indigo dye are formed with the dimerization of indole glucosidic precursors, under the treatment of the plant for the production of the dye [24]. The main precursors involve indican (4), isatan A (5), and isatan B (6) [25], while their presence prior to the production of the dye is largely dependent on the post-harvest treatment of the plant [24]. Indirubin has been successfully isolated from the leaves of Isatis with the use of “green” techniques as supercritical fluid extraction (SFE) [26].

On the other hand, indirubins of molluscan origin are present in the purple pigment of Tyrian purple which is produced by organisms of the Muricidae family. The simultaneous presence of non-brominated and brominated indigoids in Tyrian purple was reported for the first time in 1909 with the isolation of 6,6′-dibro-moindigo (7) from Hexaplex trunculus [27]. The predominant indigoid ingredient of the dye depends greatly on the species used for the production as well as the conditions under which it was produced. Among the most commonly used mollusks for the production of the dye, Hexaplex trunculus was found to possess the greatest variety of brominated indigoids: indigo, 6-bromomoi-digo (8), 6,6′-dibromoindigo (7), as well as indirubin (2), 6-bromoindirubin (9, 68), 6,6′-dibromoindirubin (10), and 6′-bromoindirubin (11) are all present in the DMF extract thereof [28]. Interestingly, indigoids are not present in the mollusk itself rather than being synthesized in the procedure of dye production, which involves alkaline treatment of the mollusk and exposure to sunlight. This process was later on partially elucidated with the isolation from Dicathais orbita of the colorless ultimate precursor tyrindoxyl sulfate (12) [29,30] as well as several intermediates from this species and other Muricidae such as tyrindoxyl (14), tyrindoleninone (15), and tyriverdin (16) [31–33]. In H.

Fig. 1 Structures of indirubin (1), indigo (2), and isoidigo (3). The Tyrian purple producing mollusk Hexaplex trunculus (Muricidae) and details of the fresco entitled “The Saffron Gatherers” located in the archaeological site of Akrotiri, Thera, Greece.
trunculus, which for the moment exhibits the greatest variety of indigoids, indoxyl sulfate (13) has also been proposed as another ultimate precursor, [34] a fact that is reflected upon its variety of non-, mono- and dibrominated indigoids. Nowadays, we can attribute the formation of the marine indigoids to a series of oxidative, photochemical, enzymatic transformations and dimerizations, although a concise concept of their genesis is yet to be clarified. Recent advances suggest that their origin is likely to be sex-specific and related to reproduction [35], as purple pigmentation has been detected in the egg masses of several gastropods [36, 37].

Bioguided isolation of brominated indirubins and precursors

The general interest in indirubin scaffolds due to their use in traditional medicine and their identification as kinase inhibitors led to the investigation of brominated indirubins as bioactive agents. The isolation of natural mono- and dibrominated indirubins, along with indirubin, has been performed from the whole body mass of *H. trunculus* after exposure to light and oxygen, lyophilization, and extraction with dichloromethane. Removal by precipitation of the insoluble indigo derivatives affords an indirubin-enriched dichloromethane extract (0.25 mg of indirubin content in 1 kg of dried mollusks), of which with the aid of MPLC fractionation four fractions corresponding to indirubin and the aforementioned derivatives can be obtained [38].

After screening of the fractions representing indirubin and the natural 6-brominated analogues on a set of 3 kinases (CDK1/cyclinB, CDK5/p35, and GSK-3β), 6-bromoindirubin (9, 6BI) was identified as a potent and selective GSK-3 inhibitor [39]. It was the first time 6BI was isolated from a natural source as a minor indirubin constituent of Tyrian purple, although it has been detected numerous times in Muricidae extracts and artifacts dyed with Tyrian purple via chromatographic analytical techniques [40, 41].

On the other hand, the interest in indirubin precursors focuses not on kinase inhibition but strong antimicrobial activity. Under this scope, organic solvent extracts of the egg masses of *D. orbita* were examined for their bacteriostatic activity against human and marine pathogens (*E. coli, S. aureus, P. aeruginosa*). Bioguided isolation of the precursors led to the isolation and identification of tyriverdin (16) as a strong antimicrobial agent at a concentration of 1–0.5 µg/ml [42]. Moreover, tyrindoleninone (15) and its oxidation product 6-bromoisatin (17) are identified as anticancer agents [43] while extracts containing indole Tyrian purple precursors have a potential chemopreventive role in colorectal cancer [44].
Total synthesis of indirubins and related analogues

Total synthesis of indirubin was performed for the first time in 1881 by A. Baeyer [45], a few years after its isolation from indigo dye. The original method was based on the reaction of indoxyl with isatin under alkaline conditions, while during the 20th century the procedure was modified by the use of the more stable acetoxindole [46]. Even though many analogues of indirubin have been reported, the basic synthetic preparation has been to a large extent conserved. Synthesis of 6BI [47] is based on the combination under mild alkaline conditions of acetoxindole with 6-bromoisatin (17), the latter being easily prepared from 6-bromoaniline (18) through the 2-step Sandmeyer synthesis [48]. First, the aniline is converted to the corresponding isonitrosoacetanilide (19) under treatment with chloral hydrate and hydroxylamine, while in the second step the acetanilide undergoes cyclization in concentrated sulfuric acid resulting in the formation of the isomeric 6-bromoisatin and 4-bromoisatin, which are separated under fractional precipitation in an acidic environment. Shifting of the 6BI bromine atom to positions 5 and 7, results in the formation of 5-bromoidirubin (20, 5BI) and 7-bromoidirubin (21, 7BI), synthesized similarly to 6BI from 5-bromoisatin and 7-bromoisatin, respectively. Five and 7 bromosubstituted indirubins are not naturally derived in terms of the bromine position, as no report of them as natural products is present in current literature.

A large series of indirubin analogues bearing halogens or simple substituents on the benzene rings has been achieved with the aforementioned procedure (Fig. 3), starting from the corresponding isatin and acetoxindoles [49–51]. Those analogues involve methoxylated indirubins [52], 5,7-bisubstituted aniline analogues [53], 5-nitro analogues [54, 55], 5-carboxylates bearing unsaturated and aromatic side chains [56], and 5,5-bisubstituted analogues with halogenated and hydroxylated substituents [57, 58].

One of the most promising modifications performed on the indirubin core, so far concerning the modulation of activity and solubility properties, is the conversion of the 3’ carbonyl group into a quaternary carbon (Fig. 3). Thus, in the case of indirubin and the brominated 5BI, 6BI, and 7BI, treatment with hydroxylamine hydrochloride in pyridine results in the formation of the corresponding oximes, namely indirubin-3’-oxime (22, 10), 5-bromo-indirubin-3’-oxime (23, 5BIO), 6-bromoindirubin-3’-oxime (24, 6BIO), and 7-bromoindirubin-3’-oxime (25, 7BIO), molecules with a vast range of biological activity and in the case of 6BIO, enhanced potency and selectivity towards GSK-3β [51].

Several analogues of halogenated indirubins have been developed aiming the improvement of biological properties on the one hand and enhanced drugability on the other, given the fact that simple indirubin analogues are characterized by low solubility. LogD values of 6BIO (2.59) [59], 5BI, and indirubin (3.7 and 2.5, respectively) [61] reflect the low hydrophilicity of simple indirubins despite the presence of an oxime group. A series of 6BIO analogues possessing amino-aliphatic chains on the 3’ oxime group exerted selectivity against GSK-3β and also a more favorable solubility in water with logD values varying from 1.90 (36) to ~0.87 for the simple piperazine analogue (33) (Fig. 4, products 27–37) [59, 60]. The introduction of those hydrophilic chains is achieved through the intermediate formation of the 3’-oxime ether bearing a terminal bromine atom (26), which is afterwards substituted with commercially available secondary amines. Similarly, 7BIO analogues of the same type have been synthesized bearing varying long hydrophilic chain substituents on position 3’ [61].

Under the same perspective of enhancing the solubility of bioactive indirubins, sugar moieties have been introduced to the basic core. Retaining the synthetic methodology of dimerization, sugar moieties have been incorporated in positions 1 and 1’, originating from glycosylated isatins and indoxyls, respectively [62, 63]. Finally, one of the most radical interventions performed so far to indirubin has been the introduction of a heterocyclic nitrogen atom to the benzene ring originating from isatin. This attempt to simulate the presence of a bromine atom in position 7 resulted in the synthesis of 7-azaindirdurbin, an isostere of the natural indirubin with antiproliferative properties [64, 65].

For the class of 5-brominated indirubins, more soluble 5-substituted analogues have been developed simulating the brominated core, with the main representatives indirubin-5-sulfonate (E622) and 5-carboxyindirubin (39) being the lead compounds in a series of 5-substituted analogues [60]. On this basis, compounds bearing polar hydroxylated chains on position 3’, basic sulfonamide (Fig. 4, products 40–44), and carboxamide (Fig. 4, products 45–55) groups on position 5 have been developed with remarkable water solubility (logD ~ 2.1 for E622) and significant cytotoxicity [66]. Finally, a series of 5-substituted non-planar indirubins has been developed via the transformation of the 3’ carbonyl group into a quaternary carbon (Fig. 4, products 56–57), a change very effective in terms of solubility [60].

Biological Properties of Halogenated Indirubins and Analogues

Protein kinase inhibition

Protein kinases (PKs) consist in a vast group of enzymes catalyzing the reversible phosphorylation of protein substrates [67]. Due to this vital function, they have been found to participate in most of the signal transduction processes in the eukaryotic cell [68], while their deregulation has been established in a number of diseases such as cancer [69], neurodegeneration, and protozoan infections [70]. Indirubins are considered ATP-competitive PK inhibitors, while screening of 85 kinases of the ProQinase “selectivity panel” revealed a selectivity trend for 5O, 5BIO, 6BIO, and 7BIO [71].

A very important group of the human kinome (as the sum of the kinases expressed from humans is referred [72]) is represented by the CDKs (cyclin dependent kinases). They are serine/threonine kinases which are to a large extent conserved, and require the binding with a cofactor for their activation (e.g., cyclins). They play a vital role in the cell cycle by controlling its progression through a succession of activation and deactivation events [73, 74]. Most of the CDKs have been associated to various forms of cancer, thus making the discovery of new and specific inhibitors an intriguing target during the past years [75, 76]. Indirubins in general are considered to be inhibitors of CDK1, CDK2, and CDK5 [77], the former being of uttermost importance to the general cell cycle progression while the latter is expressed mostly in neurons [78].

GSK-3 (glycogen synthase kinase), although originally discovered for its implication in diabetes through phosphorylation of glycogen synthase [79], has been brought to attention due to its abundance in brain cells and neurons and its ability to abnormally phosphorylate tau protein in the Alzheimer’s disease (AD) pathway [80]. Tau’s aggregation is responsible for the formation of the
Fig. 3  Simple substituted indirubin analogues bearing at least one halogen atom in their core and inhibitory activity against CDK1/cyclin B, CDK2/cyclin E, GSK-3α/β, CDK5/p25, Auroras A, B, C, and FLT3. The colour scale represents the range of activity indicated in the bottom. Data gathered from literature cited: [13–15, 17, 38, 51, 54, 57, 58, 60, 61, 71, 162].

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Fig. 3  Simple substituted indirubin analogues bearing at least one halogen atom in their core and inhibitory activity against CDK1/cyclin B, CDK2/cyclin E, GSK-3α/β, CDK5/p25, Auroras A, B, C, and FLT3. The colour scale represents the range of activity indicated in the bottom. Data gathered from literature cited: [13–15, 17, 38, 51, 54, 57, 58, 60, 61, 71, 162].
neurofibrillary tangles (NFTs) and the β-amyloid deposition observed in AD [81], while the role of GSK-3 in inflammation pathology of AD is under investigation [82].

The beta-isoform of GSK-3 (GSK-3β) is found to be associated through various signaling pathways with mood disorders [83] and schizophrenia [84], osteoporosis [85] and cancer (Wnt signaling) [86], atherosclerosis, cardiac hypertrophy, hypertension [87], and signal transduction [88]. The natural 6BI and its semi-synthetic analogue 6BIO are both potent and selective GSK-3β inhibitors, a fact that gave rise to the commercialization of 6BIO under the name “BIO” and “GSK-3 inhibitor IX” [89] and the development of analogues with a vast range of biological applications.

Apart from the aforementioned kinases, indirubins also target the Aurora kinases [15], FLT3 (Fms-like tyrosine kinase 3) [58, 90], JAKs (Janus kinases) [91], and according to molecular modeling studies PDK1 (pyruvate dehydrogenase kinase 1), with specificity and potency depending on their chemical structure [92].

Fig. 4 Synthesis of 6-bromoindirubin and related substituted analogues.

- **a** Chloral Hydrate, Na2SO4, NH2OH·HCl, H2O, H+, b conc. H2SO4, c 3-acetoxyindole, Na2CO3, MeOH, d NH2OH·HCl, py, reflux, e 1,2-dibromoethane, Et3 N, DMF, RT [51]. For the preparation of the 6-Br amine analogues: f DMF, RT, secondary amines namely, dimethylamine (27), diethylamine (28), diethanolamine (29), 3-(methylamino)propane-1,2-diol (30), morpholine (31), 6-BIMYEO, pyrrolidine (32), piperazine (33), 1-methylpiperazine (34), 1-(2-hydroxyethyl)piperazine (35), 1-[2-(2-hydroxyethoxy)ethyl]piperazine (37) [59]. For the preparation of the 5-sulfonylamide analogues: g 2 steps, SOCl2, 80 °C and DMAP (cat) with amines namely, dimethylamine (40), diethanolamine (41), 4-hydroxypiperidine (42), N,N,N′-trimethylethylenediamine (44) [60]. For the preparation of the 5-carboxamide analogues: h two steps, PFF-trifluoroacetate, DMAP, py, DMF, and DMAP, dioxane with the appropriate amine namely, piperazine (45), 1-methylpiperazine (46), ethanolamine (47), diethanolamine (48), N,N,N′-trimethylthiethylenediamine (49), N,N-dimethyl-ethane-1,2-diamine (50), N,N-dimethyl-2-(4-methyl-1-piperazinyl)ethanamine (51), N,N-dimethyl-p-phenylenediamine (52), 3-aminopyridine (53), 4-(4-methyl-1-piperazinyl)aniline (54), 1-amino-1-deoxy-D-glucitol (55) [66]. For the preparation of the 3′-quaternary analogues: i Grignard reactions in THF or py, -20 °C, with alkyl-magnesium bromides, namely methylmagnesium bromide (56), allylmagnesium bromide (57) [60].

Vougogiannopoulou K and Skaltsounis A-L. From Tyrian Purple... Planta Med 2012; 78: 1515–1528

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nally, it is worth mentioning that *Leishmania sp.* possesses protein kinases sharing certain homology to the mammalian ones (CRK3, LdGSK-3, and protozoan MAPks), whose functional role in the life cycle of parasites can be even more important than in mammalian cells [70].

Interaction of indirubins with molecular targets such as the PKs causes the modulation of various physiological pathways. Inhibition of GSK-3β affects the progression of parental pathways Wnt and Hedgehog (Hh) [93]. Wnt is a signal transduction pathway controlling differentiation in the stage of embryonic development, stem cell fate in adults, neuronal development, and neuroprotection [94]. GSK-3β has been found to phosphorylate several components of Wnt, with β-catenin being one of the most important. In canonical Wnt signaling and in the absence of Wnt proteins, β-catenin is phosphorylated by GSK-3β and thus degraded by the proteasome. Inhibition of GSK-3 leads to β-catenin intracellular accumulation/stabilization and through a series of intracellular events triggers the transcription of target genes associated with apoptosis and cell proliferation [95]. Furthermore, inhibition of GSK-3β by indirubins, through its implication in the phosphatidylinositol 3-kinase Akt signaling pathways (PI3K/Akt), is capable of modulating the expression of factors associated with hypoxia and ischemia [96] and apoptosis in serum-deprived conditions [97].

GSK-3β is also relevant to the effect of indirubins on Notch-1 signaling, a pathway participating in cell cycle progression, invasion, migration, and apoptosis. Dereglulation of Notch is observed in many types of human cancers and tumorgenesis. IO has been found to suppress Notch-1 signaling through downregulation of GSK-3β [98], while 5′-nitroindirubin-3′-oxime induces cell cycle arrest possibly through blockage of Notch-1 signaling [99]. Finally, a less studied but very promising field for the implication of indirubins in biological processes involves the regulation of STAT3 signaling. STAT3 is a family of different transcription factors playing an important role in tumor survival/proliferation and inflammatory responses [100]. In STAT3, JAKs phosphorylate STAT3 and activate signaling for the transcription of specific target genes. Except JAKs, many other PKs implicate the activation of STAT3, like members of the Src family, PKC, EGFR, etc. [101]. Indirubin and derivatives such as IO and 5IO have been found to block STAT3 signaling through the inhibition of implicated PKs [102–104]. Recently, it has been shown that STAT3 activation is highly dependent on GSK-3β, as specific inhibitors of the latter block the STAT3 DNA binding ability [105].

Structural diversity and selectivity
Since the identification of indirubin as a protein kinase inhibitor, several analogues have been designed and synthesized targeting the kinase. After several years of research, the vast range of analogues existing allows for structure/activity relationships to be established. Halogenated indirubins share a special place among those analogues as they offer a versatile tool for the exploration of specific kinase inhibition [106], and also a matrix upon more selective and active analogues was later on developed (Fig. 3). By reviewing the literature existing so far on indirubins and kinase inhibition, the shifting from the mediocriely active and non-specific indirubin to variably substituted indirubins with enhanced kinase inhibition involves the identification of natural 6BI as a GSK-3β specific inhibitor [39]. Earlier reports on synthetic 5-halogenated indirubins indicated an antimotor activity [107], although this was not correlated to kinase inhibition until indirubins were collectively acknowledged as kinase inhibitors [15].

During the last decade, lead indirubins have been established for the most important PK targets identified, namely 6BI for GSK-3β inhibition [38] and indirubin-5-sulfonate for CDK2 (E622) [65], focusing on two different axes targeting cell proliferation on the one hand and neurodegeneration on the other. It is also worth mentioning that although E622 is not halogenated, its design was based on 5-halogenated indirubins with antimotor properties, with the halogen being replaced by a group giving-enhanced druggability to the scaffold. The latter is also the case with 3′ oxide analogues and 6BI, which were developed in terms of rendering the indirubin scaffold more soluble.

Fig. 3 provides a quick overview on the kinase inhibitory properties of simple substituted indirubins, which possess at least one halogen atom in their core. For indirubin itself, the nonspecificity especially among the examined CDks is evident. Substitution on position 5 generally enhances the PK inhibition potency, although it eliminates selectivity. This is particularly true for 5-iodo analogues, which exhibit nanomolar range activity both in CDks and GSK-3β. Recently, a series of new 5,5′ bisubstituted analogues were developed showing great potency towards CDK2 [57].

As substitution is shifting towards position 6, greater selectivity towards GSK-3β is accomplished peaking for 5,6 bisubstituted analogues with the bromine atom on position 6. The affinity of 6-bromo-substituted indirubins and 6BI in particular, with GSK-3β in comparison with CDks, was elucidated with the crystallographic studies of the complex 6BI/GSK-3β [51], taking into account the previous X-ray structures of indirubin-5-sulfonate (E226) with CDK2 [15] and the complex CDK2/cyclin A [108] and indirubin-3′-oxide with CDK5/p25 [39]. Further crystallographic data [109] confirm the pharmacophore of the indirubin scaffold in most of the analogues due to the fact that PKs are to a large extent conserved.

The pharmacophore of the indirubin scaffold consists of the lactam amide nitrogen, lactam amide oxygen, and cyclic pyrrole nitrogren (Fig. 5 B). In the case of E226 and CDK2 (both inactive and activated by cyclin A), the lactam and pyrrole nitrogen atoms act as hydrogen bond donors to the oxygen atoms of Glu81 and Leu83, respectively, while the amine group of Leu83 forms an additional bond with the scaffold’s lactam oxygen. In the case of IO and CDK5/p25, the corresponding amino acid residues are Glu81 and Cys83, while for 6BI and GSK-3β, they correspond to Asp133 and Val135. In all of the tested kinases, the indirubin scaffold is inserted into the ATP binding pocket located between the two lobes of the enzyme. For analogues methylated on the lactam nitrogen (N-methylindirubins), PK inhibitory activity is lost, due to its incapability to act as a hydrogen bond donor, and therefore such analogues are used as negative controls for indirubin kinase inhibition (Fig. 5 B).

As proposed from crystallographic studies and molecular modeling studies, the selectivity of 6BI to GSK-3β versus CDks is related to minor differences in the binding pocket of the enzymes. GSK-3β with the relatively small Leu132 provides a more spacious environment for the bromine atom to be inserted in the back of the cavity, whereas in CDks 1, 2, and 5, this area is restricted due to the bulkiier Phe80 (Fig. 5 A). By taking into account the topology of the binding pockets, the results of Fig. 3 can be rationalized for all indirubin analogues discussed. The highly unspecific 5-halogenated indirubins are able to associate with all of the competitive kinases to some extent, as the 5-sub-

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stituent is directed outside of the binding pocket. 2D and 3D QSAR studies on halogenated indirubins show that affinity with GSK-3β is enhanced with the substitution in positions 5/6 with electron-withdrawing atoms such as halogens, while similar substitution on positions 4/7 is not favorable [110]. As seen in Fig. 3, 5- and 6-bromo or iodo, as well as 5,6-bisubstituted analogues possess the greatest activity towards GSK-3β. Astonishing is the case of 7-substituted analogues, which stand out among halogenated indirubins as cases of no significant PK inhibition by 6BIO [71]. This is also supported by the low potential of 7-brominated analogues to insert into the binding pockets of the kinases as the substituent is directed in the less spacious interior of the cavity (Fig. 5A).

**Effect on stem cells and progenitors**

The establishment of 6BIO as a potent and selective GSK-3 inhibitor was followed by a very promising discovery concerning its effect on stem cells. 6BIO was found to maintain the undifferentiated phenotype of both human and mouse embryonic stem cells (HESCs and MESCs, respectively), sustaining their pluripotency possibly through Wnt activation [111], and also to decrease MESCs proliferation rates, not due to apoptosis but rather accumulation of the cells in the G1 phase [112]. Recently, it has been proposed that this delay of the cell cycle progression is due to the downregulation of cyclin D1 and the upregulation of p57 by 6BIO [113]. Results from different research groups, report that 6BIO appears to also stimulate the LIF (leukemia inducing factor) signal, which acts synergistically with Wnt activation in terms of maintaining the undifferentiated state of MESCIs [114]. In the absence of LIF/Wnt signaling, it has been proposed that ESCs renewal could be a result of elevated myc levels and subsequent stem cell stability [115].

GSK-3 function is also a key factor in hematopoiesis and the expansion of hematopoietic stem cells into mature blood cells. 6BIO has been found to promote and inhibit the ex vivo expansion of umbilical cord blood hematopoietic stem cells (UCB HSCs) in low and high concentrations, respectively [116, 117]. GSK-3 inhibition by 6BIO also causes a decrease in proliferation of adult olfactory epithelial human neural precursors accompanied by an increase of differentiation markers, thus suggesting the promotion of early neuronal differentiation [118].

Similarly, human mesenchymal stem cells (hMSCs) from bone marrow are regarded as putative osteoblast progenitors differentiating into osteoblasts in vitro. 6BIO induces the cell cycle inhibition of hMSCs while enhancing the early stage of osteogenesis, as mineralization is observed after treatment [119]. In particular with osteoblasts, the latter is supported by in vivo experiments on bone mass loss after extensive glucocorticoid treatment, during which treatment with 6BIO resulted in the attenuation of bone mineralization loss [120].

Despite the vague mechanism of action concerning stem cells and progenitors, 6BIO was found to inhibit the differentiation of T cells while arresting the development of CD8+ T cells into effector cells [121] and also inhibit the proliferation of HMADSCs (human adipose derived stem cells) and their adipogenic differentiation [122]. Furthermore, 6BIO significantly enhances the ability of ESCs to reprogram somatic cells after fusion thus allowing the dedifferentiation of the hybrids [123]. Finally, 6BIO prevents the process of epithelial to mesenchymal spontaneous transition (EMT) of HESCs when cultured also under feeder–free conditions, although it was not able to expand HESCs in a long–term culture system [124]. Paradoxically, 6BIO was found to be associated with reduced cell proliferation of human islet–derived precursor cells (HIPCs), which are characterized as mesenchymal stem cells, able to differentiate into islet–like structures [125].

Another interesting application of 6BIO discovered recently is the ability to facilitate the derivation of ESCs from blastocysts when used alone or in combination with LIF [126, 127]. When an inner cell mass mass of blastocysts (ICM) able to provide ESCs is incubated with 2 μM 6BIO, all of the formed colonies provide ESCs giving a 4-fold increase in the efficiency of the derivation. Recent studies report a fivefold increase of ESCs derivation when multiple factors are utilized along with 6BIO [128]. In addition, 6BIO in combination with fibroblast growth factor (FGF) can contribute in the formation of porcine embryonic germ cells (EGCs) colonies, increasing the mitosis index and maintaining the undifferentiated state [129]. Finally, 6BIO was found to increase the expression of genes and pluripotency markers in ESCs suggesting that upregulation of stemness genes keeps the cells in a self–renewing pluripotent state [130].
Effect on leukemia and solid tumors
Anticancer properties of halogenated indirubins and related analogues seem to focus on three basic concepts: inhibition of CDKs and cell cycle arrest, restrictions on signaling pathways and especially STAT3, and the induction of non-apoptotic cell death by 7BIO and certain 7-halogenated analogues [60, 131]. Potential kinase inhibition, although it is not yet established, probably lies behind the antitumor properties of 5-carboxamide analogues (45–55) against LXFLS29L lung cancer cells, with IC50 in the low μM range [66]. In addition, bromo- and methoxy-indirubin analogues have been examined for their capability of inducing apoptosis in neuroblastoma cells, although the mechanism of apoptosis is not yet clarified [52]. Furthermore, 5-substituted indirubin derivatives (E622, 40–44), besides the potent inhibition of CDKs, have been shown to block STAT3 signaling, inhibit Srf, and finally induce apoptosis in human breast cancer cells [132]. Most importantly, 6BIO induces apoptosis in human melanoma cells accompanied with inhibition of STAT3 signaling while suppressing in vivo tumor growth in xenograft human melanoma models [133]. In addition, the synergy between all these factors is possibly the cause of the inhibition of proliferation observed under treatment with 6BIO of malignant lymphoid cells [134].

GSK-3 inhibitors are still under investigation as antileukemic factors [116] since limited literature has been published on this topic. 6BIO exerts an in vivo curative effect against leukemia animal models as well as specific cytotoxicity in vitro against rapidly dividing leukemia blasts [135]. In addition, GSK-3β inhibition by 6BIO was found to inhibit MLL leukemia cell proliferation and transformation [136]. An assumption of GSK-3β inhibition leading to apoptosis is made, although indirubins are also potential inhibitors of FLT3, which is often mutated in patients with acute myeloid leukemia (AML) [58, 88]. Finally, indirubin type inhibitors of GSK-3β have been found to improve survival in glomabearing mice [137] while 6BIO is suppressing telomerase activity probably via GSK-3β inhibition, without showing an overt toxicity [138].

Effect on cardiac cells
Results by several studies reveal that 6BIO also affects cardiac cells, both differentiated and undifferentiated, as a potent GSK-3β inhibitor [139]. Specifically, 6BIO enhances the survival of human cardiac stem cells (HSCs) while stimulating their growth kinetics [140], in addition to the fact that 6BIO treatment of postmitotic highly differentiated cardiac cells promoted their proliferation [141, 142]. 6BIO via inhibition of GSK-3β is found to expand the pivotal role of Isl1+ cardiovascular progenitors to cardiogenesis in a dose-dependent manner without significant suppression of apoptosis [143]. All those findings are of great importance concerning the repair and diversification of the heart [144]. Another aspect of the effect of halogenated indirubins on cardiac cells is portrayed in studies concerning the neuronal or myocardial damage induced by ischemia/hypoxia. 6BIO was found to prevent ischemic neuronal death in oxygen/glucose deprivation conditions [145], while in a similar in vitro model of neuronal progenitors it was found to rescue neurons either as a preconditioning technique or as a post-injury system [146]. Moreover, treatment of hypertrophied rabbit hearts with 5IIO was found to increase tolerance to ischemia through GSK-3β inhibition, suggesting a practical treatment in the protection of hypertrophied hearts during open heart surgeries [147].

Furthermore, under the scope of investigating how histone deacetylase-2 (Hdac2) deficiency attenuates cardiac hypertrophy in mice, it was found that intraperitoneal admission of 6BIO in mice is capable of inhibiting in vivo GSK-3β, leading to increased heart–body weight ratios [148].

Effect on renal and pancreatic cells
6BIO through mediation in the Wnt and Akt signaling has a significant effect on kidney and pancreatic tissues. When diabetic Wistar rats were administered 6BIO subcutaneously, it was found that GSK-3 signaling was modulated and apoptosis of the cells adjacent to globeruli was reduced in the diabetic kidney followed by reduced urinary protein secretion [149]. In addition, exposing mouse kidney mesenchymes in 6BIO triggers nephrone segregation and epithelial differentiation [150]. Finally, inhibition of GSK-3β by 6BIO after the treatment of mice with endoxaemce renal failure resulted in the reduction of nephrotoxicity and mortality by sepsis [151]. 6BIO also is found to promote the replication and survival of pancreatic beta cells [152] and the proliferation of facultative hepatic stem/progenitor cells [153], proposing that inhibition of GSK-3 and small molecule inhibitors could have applications in regenerative therapies.

Effect on neurodegeneration
6BIO’s ability to affect CNS cells derives from its most important property of being a selective and potent GSK-3β inhibitor. Abnormal phosphorylation events related to GSK-3β activity have been established in neurodegenerative states, and those findings lead to the further investigation of GSK-3β inhibitors as neuroprotective agents [154]. 6BIO was found to reverse okadaic acid-induced multi-substrate phosphorylation [155], tau phosphorylation, and apoptosis in cultured cortical neurons, with very limited toxicity [156, 157]. Most importantly, this pattern of neuroprotection was repeated with the use of three more 6BIO 3’-substituted derivatives, even though they are not as potent GSK-3β inhibitors as 6BIO [59, 158]. GSK-3β inhibition with 5IIO has shown a neuroprotective effect and a stress response reduction in human neurons [159]. A similar effect is also observed with HIV-induced neurotoxicity to human neurons where 6BIO was found to significantly reduce the activity of proapoptotic caspas 3,7 [160], and with cortical neuron cells suffering endoplasmic reticulum stress where 6BIO treatment resulted in attenuation of CHOP expression, suggesting a role of this factor in neuronal cell death [161]. In vivo experiments in mice suffering from kainate acid–induced neurotoxicity have shown that brominated indirubin analogues (6BIO, 5BIO, and 5A6B1) reduce mortality and striatal astrogliosis [162].

Although GSK-3β inhibition is considered a putative target for neurogeneration, results from different research groups suggest that strong GSK-3β inhibition from the acetoxime analogue of 6BIO, which is even more potent against GSK-3β, leads to inhibition of hippocampal axon growth [163] and neurite axon growth [164]. This effect is observed in a dose-dependent manner, thus leaving open the possibility of a therapeutic effect of inhibitors in low doses.

Effect on protozoans and other parasites
The antiprotozoan properties of halogenated indirubins are to a large extent associated with the potential of inhibiting kinases, like the leishmanian homologues of CDK1 (CRK3), GSK-3 (LdGSK-3), and MAPKs, whose functional role in the life cycle of...
the parasite can be even more important than in mammalian cells.

After screening of a panel of indirubins, the 6-brominated analogues proved to be the most effective against the growth of amastigotes and promastigotes of Leishmania donovani, a fact attributed to kinase inhibition. Interestingly, 6BIO was found to possess greater affinity with CRK3 (leishmanial homologue of CDK-1) than LdGSK-3 (homologue of GSK-3), while the bisubstituted 5Me6BIO (78) associated greatly with the latter [165]. On the other hand, 5IO was found to inhibit the growth of promastigotes and amastigotes of L. mexicana though without any significant potency against CRK3 [166] while docking studies indicated potential of leishmanial MAPK inhibition by 5-iodo substituted indirubins, placing them as candidates for antileishmanian treatment [167]. Indirubin analogues also have shown modest in vitro activity against Toxoplasma gondii tachyzoites in the micromolar range [168].

The activity of 6BIO expands also to arachnoids of Rhipicephalus microplus, in which the homologue of GSK-3 has been elucidated and found to play an important role in embryonic processes. 6BIO was found to cause a decrease in larval hatching and oviposition of females [169].

Interaction of indirubins with the aryl hydrocarbon receptor

Aryl hydrocarbon receptor (AhR), also known as dioxin receptor, is a cotranscription factor mediating the toxicological and biological properties of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), PAHs, and HAHS (polycyclic and halogenated aromatic hydrocarbons) [170]. Binding of the ligand to the receptor is essential for the manifestation of toxicological response including hepatotoxicity, immunotoxicity, and tumor promotion [171]. It remained an orphan receptor without an endogenous ligand being identified, up to 2001, when indirubin identified in human urine was found to contribute to the activity of the AhR [16, 172, 173]. Paradoxically, while long-term exposure to xenobiotics leads to an increased risk of malignancies [174], acute TCDD toxicity has been found to inhibit solid tumor proliferation through upregulation of endogenous CDK inhibitors [175]. The role of AhR in tumorigenesis is still to a large extent unidentified and under considerable investigation [176].

Conclusion

Indirubins represent a very robust scaffold among naturally derived compounds and exhibit an outstanding versatility both as biological tools and bioactive factors. Small variations on the basic skeleton, as in the case of halogenated indirubins, have been proven to modulate significantly biological activity, leading to more active and selective PK inhibitors with fascinating applications as in the field of stem cells. All of the above, along with their charming history through the ages of natural product research and development, places them in the front line of nature-inspired drug discovery.

Conflict of Interest

There are no conflicts of interest among the authors of this manuscript.


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A detailed analysis of glycogen synthase kinase-3beta (GSK3beta) inhibitors and their role in various diseases, with a focus on Alzheimer's disease.

Glycogen synthase kinase-3beta (GSK3beta) is a serine/threonine kinase that plays a crucial role in various cellular processes, including cell cycle regulation, metabolism, and signaling. It is a target for developing therapeutics for several diseases, including Alzheimer's disease, cancer, diabetes, and cardiovascular diseases.

GSK3beta is involved in cell survival, proliferation, and differentiation. It regulates the activity of various proteins, including the Wnt/β-catenin signaling pathway, which is critical in the development of the nervous system. GSK3beta is also involved in the regulation of the epithelial-mesenchymal transition (EMT), which is important in cancer progression.

In Alzheimer's disease, GSK3beta inhibition with indirubin derivatives has shown promise in reducing amyloid plaques and tau pathology. However, the development of GSK3beta inhibitors faces several challenges, including off-target effects, toxicity, and efficacy.

In conclusion, GSK3beta inhibitors have the potential to be effective in the treatment of diseases, but further research is needed to overcome the challenges associated with their development.

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