REVIEW

Biological targets for isatin and its analogues: Implications for therapy

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Abstract: Isatin and its metabolites are constituents of many natural substances. They are also components of many synthetic compounds exhibiting a wide range of effects, including antiviral activity, antitumor and antiangiogenic activity, antibacterial, antitubercular, antifungal, antiapototic, anticonvulsant and anxyolytic activities. Isatin itself is an endogenous oxidized indole with a wide spectrum of behavioral and metabolic effects. It has a distinct and discontinuous distribution in the brain, peripheral tissues and body fluids and isatin binding sites are widely distributed also. Its output is increased during stress. Its most potent known in vitro actions are as an antagonist of atrial natriuretic peptide (ANP) function and NO signaling. As we understand more about its function and sites of action we may be able to develop new pharmacological agents to mimic or counteract its activity. We consider here the most promising biological targets for various isatin analogues and/or metabolites, which are employed for the development of various groups of therapeutics. It is also possible that the level of endogenous isatin may influence the in vivo pharmacological activity of compounds possessing the isatin moiety.
Keywords: isatin, isatin analogues, isatin binding proteins, isatin targets, biological and pharmacological activity

Introduction

Endogenous compounds are often used as a basis, or structural component, of pharmacological preparations. For example indirubin is the active ingredient of Danggui Longhui Wan, a mixture of plants that is used in traditional Chinese medicine to treat chronic diseases (Hoessel et al 1999) and also individual Chinese medicinal herbs *Isatis indigotica* and *Strobilanthes cusia* (Mak et al 2004). Indirubin derivatives are potent inhibitors of cyclin dependent kinases and some other pharmacologically important kinases. Indirubin may be formed during dimerization of isatin (Figure 1), another biologically active compound, which attracts much interest of pharmacologists. Isatin (indoledione 2, 3) is an endogenous indole found in the mammalian brain, peripheral tissues and body fluids (see for review Medvedev et al 1996; Glover et al 1998; Medvedev and Glover 2004; Medvedev, Igosheva et al 2005). The isatin moiety is also present in a range of compounds which can act as inhibitors of apoptosis (Lee et al 2001; Chapman et al 2002), anticonvulsants (Verma et al 2004), and other antiviral (Sriram et al 2004; Pirrung et al 2005), anti-bacterial and anti-fungal (Chohan et al 2004) agents. It is possible that drugs containing the isatin moiety, may compete with endogenous isatin, and the latter can influence their therapeutic effect. Consequently, analysis of the isatin content of the body, its metabolic routes, and its interactions may not only be of importance for understanding more about the function of this endogenous regulator, but also for understanding its possible role as a functional agonist/antagonist of drugs.

In this review we discuss the wide range of biological targets that are known to be sensitive to isatin and its analogues and the evidence that endogenous isatin may affect their responsivity.

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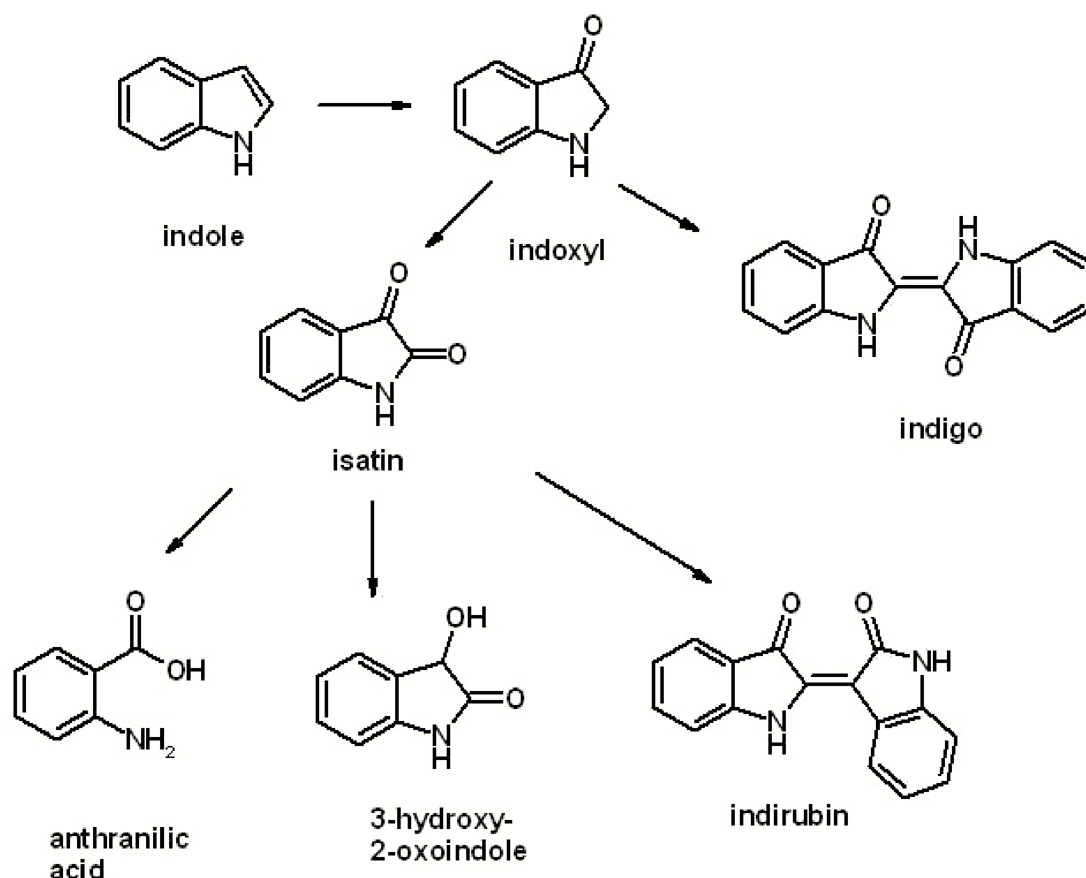

Figure 1 Scheme of isatin origin and metabolism (modified from Medvedev et al 1996).

Metabolism

Isatin was originally identified as one component of the endogenous monoamine oxidase (MAO) inhibitory activity, tribulin (Glover et al 1988). The isatin concentration in blood can exceed 1 μM (Manabe et al 1997; Mawatari et al 2001; Igosheva et al 2004). Based on tissue isatin (Watkins et al 1990) and water (Reinoso et al 1997) content calculations, basal isatin tissue concentrations are different in various organs (Table 1). Highest concentrations in the brain are in hippocampus, cerebellum and striatum (1–1.3 μM), whereas in peripheral organs the highest concentrations are in seminal vesicles and vas deferens: 47.4–79 μM. In the heart the maximal basal concentration approaches to 3 μM. In other rat organs basal isatin concentrations vary from 0.3 μM (spleen) to 1.5 μM (liver). In the rat, stress causes the 2–3 fold increase of isatin content in the brain and heart (Igosheva et al 2004), 2.5–6 fold increase of isatin output in urine (Tozawa et al 1998), and its concentration in serum reaches 2.9 ± 0.29 μM (Igosheva et al 2004). This suggests that under certain conditions isatin concentrations may be as high as 10 μM and even more (if we take into consideration exceptionally high concentrations in seminal vesicles and vas deferens).

Although the pathways of endogenous isatin formation (see Figure 1) still require better experimental support there is in vitro evidence that isatin can be formed from indole, typically produced by tryptophan catabolism in the gut, and that this involves microsomal cytochrome P450 (Gillam et al 2000). Besides excretion with urine, the catabolic pathways of isatin include further, possibly spontaneous, oxidation and dimerization yielding the indigoid pigments, indigo and indirubin (Medvedev et al 1996; Gillam et al 2000), hydrogen peroxide-dependent conversion into anthranilic acid (Medvedev and Glover 2004) and NADPH-dependent reduction to 3-hydroxy-2-oxoindole (Usami et al 2001). All these compounds have been found in urine (Usami et al 2001; Medvedev and Glover 2004). However, it remains unclear whether indigoid formation precedes urinary excretion and aerobic transformation of the urinary components. Interestingly, during in vitro oxidation of indole by cytochrome P450 enzymes, isatin was an intermediate, which was then

Table 1 Concentrations of isatin in brain and some other tissues of the rat

Tissue	Range of isatin concentrations, μM
Whole brain	0.3–0.4
Hippocampus	0.9–1.3
Cerebellum	0.8–1.2
Striatum	0.6–1.0
Heart	0.79–2.62
Liver	0.77–1.45
Kidney	0.88–1.32
Lung	0.14–1.20
Spleen	0.35–0.62
Testes	0.16–0.63
Seminal vesicles	2.37–47.4*
Vas deferens	1.58–79.0*

Calculations have been made using data on tissue isatin content (Watkins et al 1990; see also Medvedev et al 1996) and water content in various tissues of the rat (Reinoso et al 1997).
*Due to lack of data available on water content in vas deferens and seminal vesicles isatin concentrations have been calculated using data on water content in testes (0.861 ml/g) (Reinoso et al 1997).

transformed into the indigoid pigment major end products (Gilliam et al 2000).

The study of isatin content in conventional and germ-free rats has shown that at least for basal isatin, urinary but not tissue levels are largely produced by the gut flora. Urinary isatin levels in germ free rats are 50 times lower than in samples from conventional rats, but both have similar concentrations in tissues (Sandler et al 1991). This suggests that gut flora do have an important role in the production of urinary isatin, at least in the rat.

Biological targets

Distribution of isatin-binding sites

Several lines of experimental evidence suggest the existence of multiple binding sites for isatin in the brain and other tissues. [^{3}H]isatin imaging in situ has shown the pattern of isatin-binding sites in different parts of the rat brain (Crumeyrolle-Arias et al 2003). The highest labeling was found in hypothalamic nuclei, in the cortex, the hippocampus, and the cerebellum. Administration of the MAO inhibitor, pargyline, to rats caused some reduction, but did not abolish the [^{3}H]isatin-binding (Crumeyrolle-Arias et al 2003). This suggests that MAO is one of the isatin-binding sites in situ. Indeed, this has been demonstrated using high-resolution crystal structures of recombinant MAO B with isatin and its tight binding led to an improved crystal quality (Binda 2003). Other putative isatin binding sites in the rat brain include atrial natriuretic peptide (ANP) and C-type natriuretic peptide, which inhibit [^{3}H]isatin-binding to rat brain sections and isolated membrane fractions (Medvedev, Crumeyrolle-Arias et al 2005). These results suggest that some [^{3}H]isatin-binding in the brain may be to the natriuretic peptide receptors (NPR) (Medvedev, Crumeyrolle-Arias et al 2005). Using an optical biosensor technique isatin-binding sites have been demonstrated in both particulate and soluble fractions of the brain and peripheral rat tissues (Ivanov et al 2002; Medvedev and Glover 2004). In the brain isatin-binding predominated in the membrane fraction, whereas in the kidneys the highest binding was observed in the soluble fractions. The distribution of isatin-binding sites in the particulate fraction reduced in the following order: brainstem > brain hemispheres = cerebellum > heart > kidneys > liver. In the soluble fraction there was a different rank of isatin-binding.

There are biological targets that specifically bind isatin and also those sensitive to inhibition by physiologically relevant concentrations of isatin. The former group includes MAO, the cytosolic enzymes glycerol-3-phosphate dehydrogenase (Buneeva et al 2003), glyceraldehyde-3-phosphate dehydrogenase (Panova et al 2002, Medvedev et al 2006), pyruvate kinase (Buneeva et al 2006), and ubiquitin (Buneeva et al prepared for publication). Other isatin binding proteins detected in both soluble and particulate fractions of various tissues using optical biosensor technique (Ivanov et al 2002) require detailed investigation and identification (Buneeva et al in preparation).

Isatin-binding proteins

Pyruvate kinase (PK; EC 2.7.1.40) is an important glycolytic enzyme, which catalyses the reaction of a phosphoryl group transfer from phosphoenolpyruvate to ADP in the reaction of substrate phosphorylation: Phosphoenolpyruvate + ADP → (Enol)pyruvate + ATP. Rabbit muscle pyruvate kinase (this isoform is also present in the brain (Tsutsumi et al 1988; Yamada and Noguchi 1999)) did bind isatin and the K_d value of 10 μM was at the upper physiological range of isatin concentrations (Buneeva et al 2006). This interaction appeared to be specific as the other enzyme of this class, creatine kinase (EC 2.7.3.2) did not exhibit concentration dependent binding of [^{3}H]isatin (Buneeva et al 2006). Pyruvate kinase interaction with isatin was sensitive to inhibition by ATP (IC_{50} of 25 μM) and by a neuroprotective preparation, deprenyl (IC_{50} of 5 μM). Although the biological role of this interaction is not fully understood, it may be that this phenomenon underlies some non-glycolytic functions of this enzyme (Buneeva et al 2006). Indeed certain evidence exists that pyruvate kinase is the factor

of regulation of cytoskeletal proteins (Vértessy et al 1999; Kovács et al 2003), it is also involved in resistance of glial cells to hypoxia (Shimizu et al 2004).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) is a classical glycolytic enzyme, exhibiting diverse nonglycolytic functions in dependence of its subcellular localization. GAPDH specifically interacts with different RNAs in vivo and in vitro (Nagy and Rigby 1995; De et al 1996; Lin et al 2000; Yi et al 2000; Evguenieva-Hackenberg et al 2002) and may cleave some of them (Evguenieva-Hackenberg et al 2002). Overexpression of GAPDH and its subsequent nuclear translocation are implicated in the initiation of one or more apoptotic cascades and also in the aetiology of some neurological diseases (Tatton et al 2003; Berry 2004). The translocation of GAPDH into the nucleus can be an important event leading to apoptosis (Tatton et al 2003; Berry 2004), especially in neuronal cells.

There is increasing evidence that GAPDH may serve as a target for small-molecule anti-apoptotic compounds slowing/preventing progression of neurodegenerative disorders (Ishitani et al 2003; Berry 2004). GAPDH is thus a promising target for various neuroprotective drugs and good evidence now exists that the binding of deprenyl (initially introduced as a mechanism-based MAO B inhibitor) and other propargylamines to GAPDH is primarily responsible for the neuroprotective and antiapoptotic effects of these substances (Carlile et al 1998; Kragten et al 1998; Maruyama et al 2001; Tatton et al 2003; Berry 2004).

Besides these pharmacological substances GAPDH can also bind isatin (Medvedev et al 2006) with K_d values (of 12 and 3.1 μM, determined by two independent methods respectively), which are within the upper physiological limit of isatin concentrations. Such interaction was relatively specific because another oligomeric cytosolic NAD-dependent enzyme, lactate dehydrogenase, did not bind to the immobilized isatin analogue (Medvedev et al 2006). Binding of GAPDH to isatin only weakly influenced the glycolytic activity of this enzyme but inhibited GAPDH-mediated cleavage of *E. coli* tRNA.

Glycerol-3-phosphate dehydorgenase (GPDH)

Highly purified rabbit muscle GPDH effectively binds to the isatin analogue immobilised on the cuvette of IAsys optical biosensor (Buneeva et al 2003). Replacement of the cuvette medium for washing buffer did not cause total dissociation of GPDH-isatin complexes. This suggests involvement of several types of enzyme-isatin interaction including tight binding. Although 10 μM and 100 μM concentrations of isatin caused different effects on GPDH activity: the former significantly increased apparent K_m for NAD, whereas the latter decreased apparent V_{max} and increased K_m, – possible biological significance of this regulation remains unclear.

Ubiquitin

Ubiquitin is a highly conserved 8 kDa protein, which is covalently attached to lysine residues of target proteins by the ubiquitin conjugation system. This metabolic pathway is often used for the selective proteasome degradation of many short-lived proteins in eukaryotic cells. The ubiquitin-mediated degradation of regulatory proteins plays important roles in the control of numerous processes, including cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis (Hershko and Ciechanover 1998). The ubiquitin system is involved into the immune response, development, programmed cell death, and abnormalities in ubiquitin-mediated processes may be a cause for development of various pathological conditions, including malignant transformation (Hershko and Ciechanover 1998; Hoeller et al 2006), systemic lupus erythematosus (Jury et al 2003) and other autoimmune diseases (Jiang et al 2004), Parkinson's disease (Dawson 2006, Buneeva and Medvedev 2006), etc.

We have recently demonstrated that ubiquitin binds to an isatin analogue immobilized at the Biacore cell (Buneeva et al in preparation). Although the K_d value of 0.27 mM is rather high, this phenomenon may have implications for ubiquitin interaction with isatin analogues (see below).

Biological targets sensitive to inhibition by isatin

Table 2 summarizes current knowledge of the effects of physiological concentrations of isatin on molecular targets in vitro. The most sensitive in vitro targets for isatin are the ANP signaling system, which includes ANP receptor binding, ANP receptor coupled particulate guanylate cyclase (natriuretic peptide receptor type A, NPR-A) (Glover et al 1995; Medvedev et al 1998; Medvedev, Igosheva et al 2005), soluble NO stimulated guanylate cyclase (Medvedev et al 2002), and MAO B (Medvedev and Glover 2004). They are sensitive to fluctuations of physiological concentrations of isatin. Although other targets listed in this Table 1 exhibit rather low sensitivity to the highest physiological concentration of isatin it is possible that using isatin moiety as a lead structure

Table 2 Molecular targets sensitive to physiological concentrations of isatin (modified from Glover et al 1995; Medvedev, Igosheva et al 2005)

Inhibition $\geq$50% caused by 1 μM isatin	Inhibition $\geq$50% caused by 10 μM isatin	Inhibition 20%–40% caused by 10 μM isatin	Inhibition <20% caused by 10 μM isatin
Receptor binding: [^{121}I]ANP- receptor binding	*Enzyme activity:* Natriuretic peptide receptor (NPR-A) coupled guanylate cyclase MAO B	*Neurotransmitter receptor binding:* NMDA receptor AMPA receptor Dopamine D4-receptor Muscarinic (M-2) receptor Glycine (strychnine sensitive) receptor	*Neurotransmitter receptor binding:* Adenosine-1, adenosine-2; α -1 , α-2, β-ad-renergic; dopamine-1, dopamine-2; GABA A, GABA B; histamine-I, histamine-2, histamine-3; 5-HT-1, S-HT-2, 5-HT-3; muscarinic-1, muscarinic-3; nicotinic; kainic acid; glycine (strychnine insensitive); opiate (σ, μ, κ).
Enzyme activity: NO-stimulated guanylate cyclase		*Growth factor receptor binding:* Epidermal growth factor receptor	*Brain and gut peptides and growth factor receptor binding:* Angiotensin II, type 2; vasopressin-I; bombesin; CCK_A; CCK_B endothelin B; substance P; neuropeptide Y; neurotensin; somatostatin; vasoactive intestinal peptide; nerve growth factor, NPR-C
		Ion channel binding: Potassium channel	*Ion channel binding:* Chloride; sodium; potassium; calcium
		Other targets: Inositol triphosphate Protein kinase C	*Uptake site binding:* Adenosine; choline; dopamine; GABA; noradrenaline; 5-HT.
		Enzyme activity: MAO A	*Other targets:* Forskolin; leukotriene B4, leukotriene D4; thromboxane A2, ERK

it would be possible to obtain pharmacologically attractive compounds acting as effective inhibitors of these regulatory systems. There are many examples, when the presence of various substituents in the isatin moiety significantly increased biological activity to particular targets (see below).

Atrial natriuretic peptide signaling system

The natriuretic peptides are a family of related compounds, including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). These peptides and their receptors represent an important regulatory system (Anand-Srivastava and Trachte 1993; Potter et al 2006), involved not only in the regulation of fluid homeostasis and blood pressure, but also into regulation of emotional behavior (eg, anxiety and arousal) and in altered stress hormone release and autonomic nervous system activation (Wiedemann et al 2000). Natriuretic peptides interact with three types of membrane receptor. Two of them, natriuretic peptide receptor type A (NPR-A) and natriuretic peptide receptor type B (NPR-B), are transmembrane glycoproteins exhibiting ligand-dependent intrinsic guanylyl cyclase activity (Anand-Srivastava and Trachte 1993; Silberbach and Roberts 2001; Misono 2002; Trachte 2005; Potter et al 2006). ANP and BNP bind preferentially to NPR-A, whereas CNP exhibits much higher affinity for NPR-B. The third receptor, natriuretic peptide receptor type C (NPR-C), lacks the cyclase domain and binds all three peptides. NPRC receptors are coupled to adenylyl cyclase inhibition through inhibitory guanine nucleotide-regulating protein (Anand-Srivastava and Trachte 1993; Silberbach and Roberts 2001; Misono 2002; Trachte 2005; Potter et al 2006).

Isatin inhibited [^{125}I]ANP binding to guinea pig cerebellar membranes (Glover et al 1995) and the IC_{50} of 0.4 μM is within the lower limit of the physiological range of isatin concentrations (0.3–1.3 μM). ANP and CNP inhibited [^{3}H]isatin-binding to rat brain sections and isolated membrane fractions and the IC_{50} value for the ANP effect (0.2 μM) (Medvedev, Crumeyrolle-Arias et al 2005) was very close to the corresponding effect of isatin on ANP binding. Isatin also inhibited rat brain particulate guanylyl cyclase activity stimulated by ANP and BNP (Medvedev et al 1998). This suggests the involvement of NPR-A. Recently, it has been reported that isatin in situ blocks the effect of ANP on hyperpolarization-activated current in human atrial myocytes involving cyclic GMP signaling (Lonardo et al 2004). The latter provides further experimental evidence for isatin interaction with NPR-A. Isatin administration blocked cyclic GMP excretion under conditions of fluid overload, which is known to induce endogenous ANP release (Medvedev et al 2001). This provides evidence that isatin can antagonise NPR-A signaling in vivo. Indeed perfusion with 10 μM isatin administration aborted the protective effect of atrial natriuretic peptide administered just prior to reperfusion evaluated by limited infarction in rabbit hearts (Yang et al 2006). Isatin administration has been

shown to block cyclic GMP excretion under conditions of fluid overload, which is known to induce endogenous ANP release (Medvedev et al 2001). This provides further evidence that isatin can antagonise NPR-A signaling in vivo.

At high concentrations (100 μM) isatin may also inhibit natriuretic peptide signaling associated with NPR-C (Medvedev, Crumeyrolle-Arias et al 2005). This is consistent with isatin inhibition of ANP, BNP and CNP-induced behavioral effects (Bhattacharya et al 1996a, 1996b; Telegdy et al 2000; Pataki et al 2000), because the three major natriuretic peptides only act in common at one type of natriuretic peptide receptor type C, NPR-C (Anand-Srivastava and Trachte 1993; Silberbach and Roberts 2001; Misono et al 2002; Potter et al 2006).

Potter et al (2004) have reported that pretreatment with isatin (100 μg bilaterally) abolished the effect of bremazocine, a κ-opioid receptor agonist, causing enhanced total outflow facility by enhancing levels of natriuretic peptide (ANP, BNP, CNP) in aqueous humor. This is further evidence suggesting that isatin antagonizes natriuretic peptide receptors in situ and in vivo.

Nitric oxide stimulated soluble guanylate cyclase

Besides membrane bound gualylate cyclase coupled to natriuretic peptide receptors the soluble guanylate cyclase is the other enzyme catalyzing cGMP formation from GTP. The enzyme is activated by NO (formed from L-arginine by nitric oxide synthase) and various NO donors including those employed clinically (Luscher 1992; Keefer 2003; Napoli and Ignarro 2003). The enzyme plays an important role in vasodilation (Ignarro et al 1999; Ignarro 2002). It has been shown that basal activity of the human platelet guanylate cyclase is not sensitive to isatin whereas the NO-stimulated activity is inhibited by low physiological concentrations of isatin (in the 10^{-8} to 10^{-6} M range) (Medvedev et al 2002). Subsequent increase of isatin concentration was accompanied by clear decrease of its effect.

The unidirectional regulation of particulate (NPR-A) and soluble guanylate cyclases by different range of isatin concentration is suggested to be important for stress-induced switch in metabolic processes sensitive to cGMP-dependent control (Medvedev et al 2002).

Extracellular signal regulated protein kinase (ERK)

This enzyme existing as ERK1 and ERK2 isoforms is activated via phosphorylation and the activated ERK phosphorylates cytoplasmic, membrane and nuclear substrates required for sequential transition of cell cycle phases (particularly G1/S and G2/M transitions) (Chambard et al 2007). Treatment of cells with 60–100 μM isatin reduced ERK activity and significantly reduced phosphrylation of the EKR2 isoform (Cane et al 2000).

Cell cultures

Isatin can induce cell death, including apoptosis in some tumor cells. However, molecular targets responsible for this effect remain unknown. Igosheva et al (2005) have shown that isatin (50–100 μM) increased apoptosis in dopaminergic neuroblastoma SH-SY5Y cells. At higher isatin concentrations the cells died via necrosis. Cane et al (2000) observed antiproliferative effect of isatin in both tumor (human promyelocytic leukemia HL60 cells, rat adrenal pheochromocytoma PC12 cells, mouse N1E-115 neuroblastoma cells) and normal cells (mouse fibroblast BALB/c3T3 cells, bovine brain capillary BBC cells). The effective concentration, required for a 50% effect was at the upper limits of physiological concentrations or higher (10–50 μM). In both these studies the cells were cultured with low serum concentrations as a source of growth factors and it remains to be determined whether normal and/or tumor cells would be similarly vulnerable.

Isatin (10–100 μM) inhibited nitric oxide production by RAW 264.7 macrophage cells activated by a mixture of lipopolysaccharide (LPS) and interferon γ in a dose dependent manner. It also inhibited production of prostaglandin E2 (PGE2) and tumor necrosis factor (TNF-α) (Matheus et al 2007). The inhibition of NO and PGE2 production in LPS-INF-γ is obviously associated with the isatin effect on expression of inducible nitric oxide synthase and cyclooxygenase-2 (COX-2) respectively, as it has been demonstrated by a Western blot analysis (Matheus et al 2007).

Isatin metabolites and analogues and their effects on biological targets

Isatin and its metabolites are constituents of natural substances and also components of synthetic compounds exhibiting: antiviral activity (Mak et al 2004), antitumor and antiangiogenic activity (Pandeya et al 2005; Cerchiaro and da Costa Ferreira 2006), antibacterial, antitubercular, antifungal (Chohan et al 2004; Pandeya et al 2005), and other activities. Some of them are effective anticonvulsants and anxyolytics (Pandeya et al 2005). The specific biological targets responsible for these diverse effects are not all known. However there are several specific targets,

which have attracted considerable attention in relation to the regulatory or pharmacological effects of isatin and its metabolites and analogues.

Proteases

Isatin analogues possessing various substituents at R-R''' and R''''' (see Figure 2) were active against SARS coronavirus 3CL protease (belonging to the family of serine proteases), an important target for design of drugs for treatment of a new viral atypical pneumonia, known as severe acute respiratory syndrome (SARS). The IC_{50} values were within the concentration range from 0.95 to 17.50 μM (Chen et al 2005). The effect of the most active compound tested was highly specific: inhibition of other serine proteases (trypsin, chymotrypsin) and papain (cysteine protease) was observed at much higher concentrations (10 μM–1 mM) (Chen et al 2005).

N-substituted isatin derivatives (but not isatin itself) inhibited parasitic cysteine proteases identified in trypanosomes (cruzain and rhodesain) and malaria parasites (falcipain-2) (Chiyanzu et al 2003).

Webber et al (1996) synthesized isatin-based inhibitors of human rhinovirus 3C protease, a viral enzyme that is absolutely required for the proteolytic cleavage of viral precursor polyproteins to functional proteins (Wanga and Chen 2007).

Simple N-substituted isatin analogue, N-carbobenzyloxy isatin, acted as a slow binding inhibitor of chymotrypsin but not of porcine elastase (Iyer and Hanna 1995). N-substituted isatins containing complex substituents effectively inhibited human serine proteases: chymotrypsin, leukocyte elastase and plasmin (Shuttleworth et al 2000).

Caspases

Caspases are a family of cysteine-dependent aspartate-directed proteases. There are at least 14 caspases known to date, which play a significant role in apoptosis and inflammation (Earnshaw et al 1999; Yuan and Yankner 2000; Troy and Salvesen 2002). Caspases (8 and 9) function as "upstream"-initiators of the proteolytic cascade or "downstream"-effectors (caspases 3, 6, 7) (Chowdhury et al 2006). Recent studies suggest that caspases are also involved in many other physiological processes such as immunity and cell differentiation, cell cycle, regulatory mechanisms in nervous tissue, etc. (Timmer and Salversen 2007; Kuranada and Miura 2007). Numerous data show that caspases' cascades take part in the dysfunction and death of neurons in neurodegenerative disorders, including Alzheimer's, Parkinson's and Hantington's diseases (Earnshaw et al 1999; Troy and Salvesen 2002; Jellinger 2006; Mattson 2006; Mehta et al 2007). There is an increasing evidence that caspase substrates serve as molecular targets in the

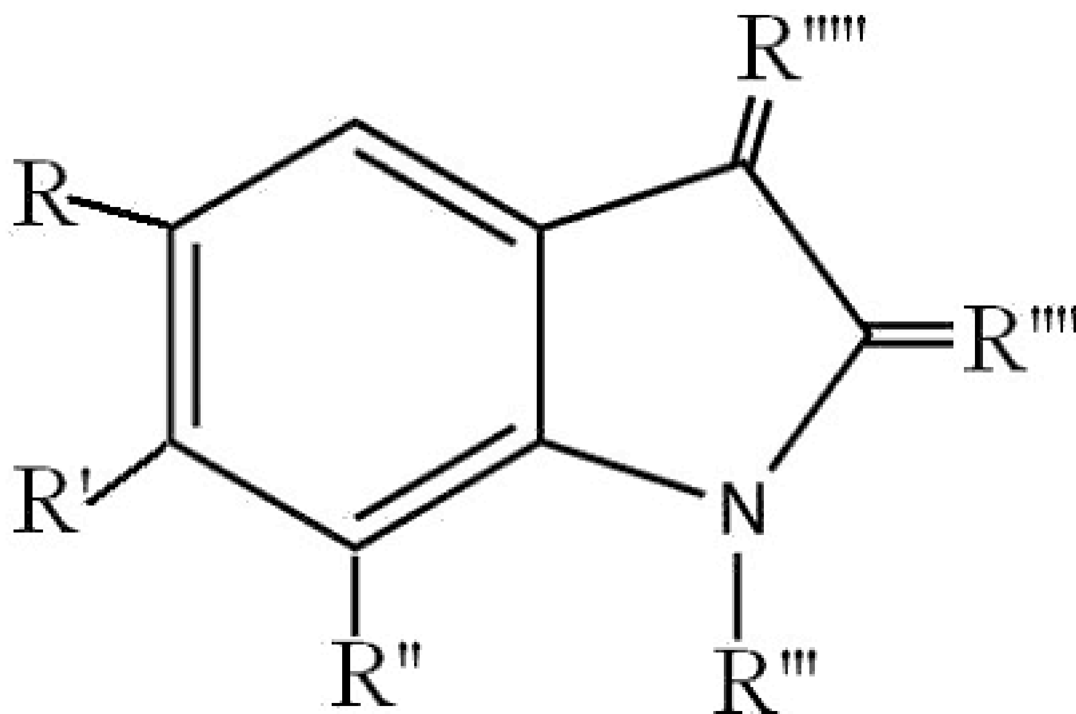

Figure 2 Key positions in the isatin ring used for various substitutions.

damaging mechanisms in pathogenesis of cardio-vascular dysfunctions, autoimmune and autoinflammatory diseases, various forms of cancer, rheumatic, ophthalmologic dysfunctions and many other diseases (Guttenplan et al 2001; Hajira et al 2004; Stoneman and Bennett 2004; Hinter et al 2005; Jin and El-Deiry 2005; De Thonel et al 2005; Rose and Martin 2005; Reeve et al 2005; Vermeulen et al 2005; Siegel 2006; Timmer and Salversen 2007; Testa and Riccioni 2007).

The simple isatin analogue, 5-nitroisatin effectively inhibited caspase-7 (K_i 0.29 μM), caspase-3 (K_i 0.50 μM), and caspase-6 (K_i 1.6 μM) (Lee et al 2000). SB-281277, a specific isatin sulfonamide inhibitor of caspase-3/-7 inhibited caspase-3 and -7 with a K_i of 15 and 47 nM, respectively. This compound exhibited 100-fold greater selectivity for highly homologous caspase-3 and -7 vs all other family members except caspase-9 (K_i of 460 nM) (Lee et al 2000). Isatin sulfonamides, selective caspases 3/7 inhibitors blocked apoptosis in murine bone marrow neutrophils and human chondrocytes (Lee et al 2000). SB-281277 prevented the ability of bisphosphonate caspase activators (zoledronic acid, clodronate or alendronate) to cause the characteristic changes in nuclear morphology of rabbit osteoclasts associated with apoptosis (Benford et al 2001).

Pyrrolidine and azetidine isatin also acted as caspase inhibitors and most of them exhibited high inhibitory potency against caspase-3 (IC_{50} of 5–10 nM) and caspase-7 (IC_{50} 15–25 nM).

Kinases

Protein kinases constitute a very large family of potential targets in a variety of diseases. Cyclin-dependent kinases (CDK) are positive regulators of cell cycle progression (Morgan 1997). They are key regulators of embryonic development and cancer and several CDK inhibitory drugs are at various stages of clinical trials as antitumor agents (Senderowicz 2003). There is evidence that cyclin-dependent kinases (CDK) are major regulators of T cell immunity and tolerance (see for review Rowell and Wells 2006; Wells 2007). These enzymes represent novel potential targets for therapy in autoimmune disease and organ transplantation.

There is evidence that indirubin, originating from the dimerization of isatin (see Figure 1) and its derivatives are potent inhibitors of CDKs1 (Hoessel et al 1999, Leclerc et al 2001). Indirubins exhibit a strong affinity for CDKs (IC_{50} values in the range of 50–100 nM) (Hoessel et al 1999, Leclerc et al 2001). Other kinases (c-Src tyrosine kinase, caseine kinase, insulin receptor tyrosine kinase, cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinases C α and β1, c-Raf, MAPK, ERK) exhibited much lower sensitivity (IC_{50} values from 1.5 μM and above) (Hoessel et al 1999).

Substituted indirubins, produced by *Escherichia coli*-expressed human P450 2A6 mutants cultivated in the presence of 5-methylindole or 5-methoxyindole inhibited human CDK-1 and -5 with IC_{50} values of 0.4–1.2 μM and glycogen synthase kinase-3α/β (Guengerich et al 2004).

Indirubins effectively inhibited glycogen synthase kinase-3β (GSK-3β) with IC_{50} values in the 5–50 nM range (Leclerc et al 2001). This kinase is involved in multiple physiological processes, including cell cycle regulation by controlling the levels of cyclin D1 (Diehl et al 1998) and β-catenin (Yost et al 1996), insulin effect on glycogen synthesis (Cohen 1999; Summers et al 1999), axonal outgrowth (Lucas et al 1998), HIV-1 Tat mediated neurotoxicity (Maggirwar et al 1999), etc. GSK-3β and CDK5 are responsible for most of the abnormal hyperphosphorylation of the microtubule-binding protein tau observed in the paired helical filaments, which are diagnostic for Alzheimer's disease (AD) (Imahori and Uchida 1997; Mandelkow and Mandelkow 1998). Thus indirubins may constitute a lead compound in the study and treatment of neurodegenerative disorders.

The endogenous activity of ERK was significantly decreased by treatment with isatin 5-hydroxyoxindole. The activity was inhibited in a concentration-dependent manner by both endogenous compounds. Concentrations of 60 mM were necessary to obtain significant inhibition with 5-hydroxyoxindole and isatin (20% and 25%, respectively). 5-hydroxyoxindole was more active than isatin in inhibition of ERK2 phosphorylation. These effects appear to be rather selective as both compounds did not influence ERK1 phosphorylation (Cane 2000).

Others

Reverse transcriptase, catalyzing the reaction of DNA synthesis on RNA template is considered as a promising target for anti-HIV drugs (De Clerq 2005; Pauwels 2006). Aminopyrimidinimino isatin derivatives inhibited replication of HIV type 1 in T4 lymphocytes (CEM cell line) and especially in MT-4 cells, however, their effectiveness was significantly lower than that of the reference standard Nevirapine (Sriram et al 2005). Although in vitro assay revealed that the compounds possessing anti-HIV-1 activity did inhibit reverse transcriptase; their IC50 values (concentration required for 50% inhibition) of 20–40 μM were several times higher than their EC50 (effective concentration

required for 50% inhibition) for the cytopathic effect of HIV-1 in MT-4 cell culture (Sriram et al 2005). Twelve isatin derivatives of this series were also active against hepatitis C virus (HCV) RNA replication showing 80% inhibition at 50 μg/ml, however, biological targets responsible for this effect have not been investigated (Sriram et al 2005). *N*-methylisatin 3-thiosemicarbazone (methisazone, Marboran) was effective in the prophylaxis of smallpox. In several trials during epidemics, methisazone reduced the attack rate by 75 to 95% (De Clerq 2001). Isatin 3-thiosemicarbazone inhibited vaccina virus multiplication in chicken embryo kidney cells or HeLa cells with IC_{50} value <1 μg/ml, but in the case of other viruses (monkeypox virus and variola virus) the IC_{50} values were higher (30–60 μg/ml). Mechanism of action of isatin 3-thiosemicarbazone remains to be clarified, certain evidence exists that it influences with viral maturation (De Clerq 2001).

1 μM N-methyl isatin β-thiosemicarbazone (Me-IBT) inhibited Rous sarcoma virus RNA-dependent DNA polymerase by 50%, whereas in combination with 1 μM $CuSO_4$, which caused minor (10%–15%) inhibition, it caused nearly total inhibition of the enzyme activity (Levinson et al 1973).

The isatin analogues, 5-Fluoroisatin, 5-Chloroisatin, 6-Chloroisatin, 7-Chloroisatin, and 5-methylisatin, but not 4-Bromoisatin and 5-Iodoisatin inhibited production of nitric oxide and PGE2 TNF-α by RAW 264.7 macrophage cells activated by a mixture of lipopolysaccharide (LPS) and interferon γ (Matheus et al 2007). However, their effectiveness insignificantly differed from the effect of isatin. As in the case of isatin the inhibition of NO and PGE2 production in LPS-INF-γ is obviously associated with the isatin effect on expression of inducible nitric oxide synthase and cyclooxygenase-2 respectively, as it has been demonstrated by a Western blot analysis (Matheus et al 2007). Production of TNF-α by the activated cells was decreased by the all isatins studied, and 5-iodoisatin exhibited the highest effectiveness (Matheus et al 2007). The authors suggest that isatin and its analogues may have chemopreventive activity against the malignant process by down-regulating COX-2 and/or iNOS (Matheus et al 2007).

Carboxylesterases are ubiquitous enzymes involved into metabolism of various xenobiotics including drugs employed clinically (eg, demerol, lidocaine, capecitabine) (Lohr et al 1998; Hyatt et al 2007). Isatin analogues containing hydrophobic groups attached at a variety of positions within these molecules, exhibited potent and specific inhibition of carboxylesterases with Ki values in the nM range (Hyatt et al 2007). Authors believe that isatin analogues may be considered as the lead compounds for the development of inhibitors modulating drug metabolism in vivo.

Interaction of isatin with its analogues and drugs

Although the interaction between isatin and its analogues, and drugs at specific biological targets still requires detailed analysis, there is evidence that isatin protects MAO B against irreversible inhibiton by mechanism based inhibitors phenelzine (Panova et al 1997) and pargyline (Medvedev et al unpublished observation). Deprenyl in its turn inhibited isatin binding to GAPDH and this effect was rather specific because the other mechanism based MAO inhibitor, tranylcypromine, was ineffective (Medvedev et al 2006). Deprenyl was somewhat more effective in inhibition of RNase activity of GAPDH than isatin, and during their combined addition the effect of RNase inhibition was somewhat less pronounced than in the case of independent action of deprenyl (Medvedev et al 2006). Thus it is possible that being less effective than the pharmacological drug, deprenyl, isatin may attenuate some of its effects.

5-Hydroxyisatin and to a lesser extent N-methylisatin inhibited ubiquitin binding to amino-isatin immobilized onto a cuvette of optical biosensor Biacore (Buneeva et al in preparation). The latter suggests that endogenous isatin may attenuate interaction of some biological targets with drugs containing isatin moiety and therefore influence expected therapeutic effect.

Conclusions

Isatin and its analogues act on a large number of biological targets and have a wide variety of actual and potential pharmacological actions. There is some evidence that isatin may attenuate the effects of certain pharmaceutical agents on specific biological targets, such as monoamine oxidase inhibitors and/or neuroprotective drugs (Panova et al 1997; Medvedev et al 2006). Isatin can be present in blood in the micromolar range and increases in response to stress (Igosheva et al 2004). This suggests that endogenous isatin, and possibly its precursors and metabolites, may affect the sensitivity of biological targets to therapeutics employed a range of diseases and disorders.

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