N-Substituted Indole-3-imine Derivatives and their Amine Congeners: Antioxidant and Src Kinase Inhibitory Effects

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Current evidences demonstrated that the activity of protein kinases can be controlled through oxidative stress induced by reactive oxygen species (ROS) and normalized by antioxidants. Recent studies with ROS, generated by mitochondria, suggested the potential signalling role of these species, where ROS, especially hydrogen peroxide, were proposed as membrane-related signalling components. The protein regulation by cellular redox states has shown that protein tyrosine kinase members, such as Src kinase and some of the members of the Src family kinases (SFKs), are proteins regulated by the cellular oxidation and reduction status. In this context, the oxidant or antioxidant potential of the synthetic Src kinase inhibitors previously synthesized and studied by our research group, such as *N*-substituted indole-3-imine and -amine derivatives, were investigated employing various acellular *in vitro* methods including microsomal NADPH-dependent inhibition of lipid peroxidation (LP), interaction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and scavenging of superoxide anion radicals. Here, we report that some of the synthetic inhibitors designed for Src kinase target have both antioxidant and kinase inhibition properties.

Key words: Reactive Oxygen Species, Tyrosine Kinase Inhibitors, *N*-Substituted Indole-3-imine and -amine Derivatives, Antioxidant Properties

Introduction

Reactive oxygen species (ROS) are the incomplete metabolites of oxygen-bearing molecules in a cell, such as superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$), hydrogen peroxide H_2O_2, and singlet oxygen ($^1O_2$). Being more reactive than the oxygen molecule itself, their excessive generation in a cell results in oxidative stress, yielding a loss of a cell function, followed by apoptosis or necrosis (Gogvadze *et al.*, 2009), implicated in cancer initiation, aging, arthritis, myocardial infarction, atherosclerosis, diabetes, neurological disorders, and many other chronic diseases (McCord, 2000). Although the detailed mechanism is still unknown, in general it is defined that ROS may act as tumour promoters by initiating the mechanisms to activate kinases which phosphorylate the residue(s) on host proteins initiating malignant transformation (Gopalakrishna and Jaken, 2000; Giannoni *et al.*, 2005). However, hydrogen peroxide and the superoxide anion, identified for decades as the toxic by-products of respiration, have been recently considered as the integral part of the signal transduction pathway through membrane receptors, hypoxia regulators, cytosolic and mitochondrial oxidases (Gulati *et al.*, 2001; Chiarugi *et al.*, 2003). This hypothesis was supported by studies revealing the growth-regulatory and tumour-promoting activity of oxygen species, as well as the antiproliferative and antitumoural function of antioxidant agents acting on ROS-producing mechanisms. One of the well identified mechanisms mediated by ROS is the regulation of the function of oxidation-susceptible protein by virtue of reversible protein oxidation through cysteine residues, upon accumulation of active oxygen compounds (Chiarugi and Giannoni, 2005; Bell and Chandel, 2007; Klimova and Chandel, 2008). Some of these oxidation-vulnerable proteins are transcription factors, protein tyrosine kinases (PTKs), some receptor tyrosine kinases (RTKs), and protein tyrosine phosphatases (PTPs). Among the protein kinases, Src family kinases (SFKs) were also found to be

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oxidation-vulnerable, and their activity alters with the alterations in the oxidative status of the cellular environment. Src kinase (c-Src) as a member of this nonreceptor tyrosine kinase family, under normal conditions, can be switched from an inactive to an active state through control of phoshorylation at two major conserved sites, Tyr419 (Y419, human) and Tyr530 (Y530, human). Phosphorylation of the former residue (Y419) activates c-Src, while phosphorylation of the latter one inactivates this kinase (Warmuth *et al*., 2003). Although the mechanism is not clarified yet, previous studies indicated that members of the Src kinase family also participate in ROS-mediated signal transductions in a way that they either are activated or inactivated by H_2O_2 or any other ROS present (Abe *et al*., 1997; Gopalakrishna and Jaken, 2000; Yoshizumi *et al*., 2000; Khadaroo *et al*., 2004; Tang *et al*., 2005; Giannoni *et al*., 2005; Hao *et al*., 2006a; Ingley, 2008; Chiarugi, 2008; Kemble and Sun, 2009). The oxidative stress-mediated activation of c-Src was previously reported to be essential for the mitogen-activated protein kinase 1 (BMK1) activation through the H_2O_2-involved mechanism (Abe *et al*., 1997). Similarly, oxidative stress-induced protein kinase D (PKD) activation was shown to be reduced partially in the presence of Src tyrosine kinase inhibitors (Waldron *et al*., 2004).

A study showing that oxidative stress induced *in vivo* causes SFK activation (Khadaroo *et al*., 2004) was later contradicted by reports revealing that Src kinase activation occurs *in vitro* but not *in vivo* (Tang *et al*., 2005). It was suggested that activation or inactivation of SFKs is due to the level of reactive oxygen compounds, or simply H_2O_2 present, and inactivation of Src and other tyrosine kinases was assumed as the protective mechanism to prevent cells from inflammatory activation (Kemble and Sun, 2009). Recently this mechanism was explained by the activation of kinases by virtue of oxidation on a conserved cysteine residue, and that is why only three of the SFKs were vulnerable to be activated or deactivated depending on the reduced or oxidized status of the cellular environment (Tang *et al*., 2005; Chiarugi, 2008; Kemble and Sun, 2009). Several studies indicated that receptor protein tyrosine phosphatase-α (RPTPα) is a positive regulator of SFKs which is critical for ROS signal transduction (Hao *et al*., 2006b), and overexpression of the RPTPα was reported to result in persistent activation of $pp60^{c\text{-}Src}$ kinase, with concomitant cell transformation and tumourigenesis (Zheng *et al*., 1992; Hao *et al*., 2006b).

Recent studies focused on the role of ROS as redox regulators of intracellular signaling, since these species are highly reactive and can directly react with all sulfhydryl-containing molecules, with protons being important in signal transduction (Nordberg and Arner, 2001). ROS and metal ions primarily inhibit phosphoserine-threonine-, phosphotyrosine- and phospholipid-phosphatases, most probably by interacting with sulfhydryl groups of their cysteine residues, which are oxidized to from either intramolecular or intermolecular disulfide bonds (Poli *et al*., 2004). These structural changes alter the protein conformation which leads to upregulation of several signaling cascades, most important growth factor kinase-, Src/Abl kinase-, MAPK-, and PI3-kinase-dependent signaling pathways (Valko *et al*., 2006). All these evidences demonstrate that protein kinases can be activated by oxidative stress and turned our attention to study the role of antioxidants in these mechanisms. In our previous study (Aboul-Enein *et al.*, 2005), it was shown that the Src kinase inhibitors 3-substituted indolin-2-one and indolin-2-thione derivatives prevent antioxidant action similar to SOD in the system generating $O_2^{\cdot-}$. These compounds also showed protective action against deoxyribose degradation by $HO^{\cdot}$ and reacted with $ROO^{\cdot}$ radicals. These findings suggested that the analyzed compounds protect against redox stimulation of cellular protein kinases. This result also presented a new insight in the current state of knowledge regarding redox regulation of signaling molecules such as kinases (Aboul-Enein *et al.*, 2005). Another study showed that some diindolylmethane derivatives possess potent radical scavenging activities as well as inhibitory effects in a primary anticancer assay *in vitro* (Benabadji *et al.*, 2004). Prompted by the above-mentioned evidences, which demonstrate that some indole derivatives possess both antioxidant and anticarcinogenic properties, it is worth to evaluate the reactivity of novel *N*- and 5-substituted indole-3-imines and their amine congeners (Fig. 1) in the inhibition of lipid peroxidation (LP), superoxide dismutase (SOD), and $pp60^{c\text{-}Src}$ tyrosine kinase, as well as the reduction of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.

Experimental

Lipid peroxidation assay

The effect of synthesized compounds on rat liver homogenate which was induced by $FeCl_2$-ascorbic acid and LP was determined. Male Albino Wistar rats (200–225 g) were fed with standard laboratory rat chow and water *ad libitum.* The animals were starved for 24 h prior to execution by decapitation under anesthesia. The liver homogenates were immediately prepared as described in the literature (Mihara *et al.*, 1980). LP of the homogenate was measured spectrophotometrically by estimation of thiobarbituric acid reactive substances (TBARS). Amounts of TBARS were expressed in terms of nanomoles of malondialdehyde (MDA) per gram of tissue. The optimized assay mixture contained 0.5 ml of liver homogenate, 0.1 ml of Tris-HCl buffer (pH 7.2), 0.05 ml of 0.1 mM ascorbic acid, 0.05 ml of 4 mM $FeCl_2$, and 0.05 ml of various concentrations of the synthesized compounds or α-tocopherol (vitamin E). The mixture was incubated for 1 h at 37 ºC. After incubation, 3.0 ml of H_3PO_4 and 1.0 ml of 0.6% thiobarbituric acid were added, shaken vigorously and boiled for 30 min. After cooling, *n*-butanol was added, mixed well, and the *n*-butanol phase was separated by centrifugation at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm against a blank, which contained all reagents except liver homogenate.

Superoxide radical scavenging activity

The superoxide radical scavenging capacity of indole imine and amine derivatives was determined spectrophotometrically on the basis of inhibition of cytochrome *c* reduction. Superoxide anions were generated in the xanthine/xanthine oxidase system. The reaction mixture contained, in a final volume of 1.0 ml, 0.05 M phosphate buffer, pH 7.8, 0.32 units/ml xanthine oxidase, 50 μM xanthine, 60 mM cytochrome *c*, and different concentrations of synthesized imine and amine derivatives in 100 μl. Xanthine oxidase was added to start the reaction, and the absorbance was measured spectrophotometrically at 550 nm for cytochrome *c* reduction. Each experiment was performed in triplicate, and the results were expressed as the percentage of the control.

DPPH free radical scavenging activity

This assay has often been used to estimate the antiradical activity of antioxidants. DPPH was dissolved in methanol to give a 100-μM solution.

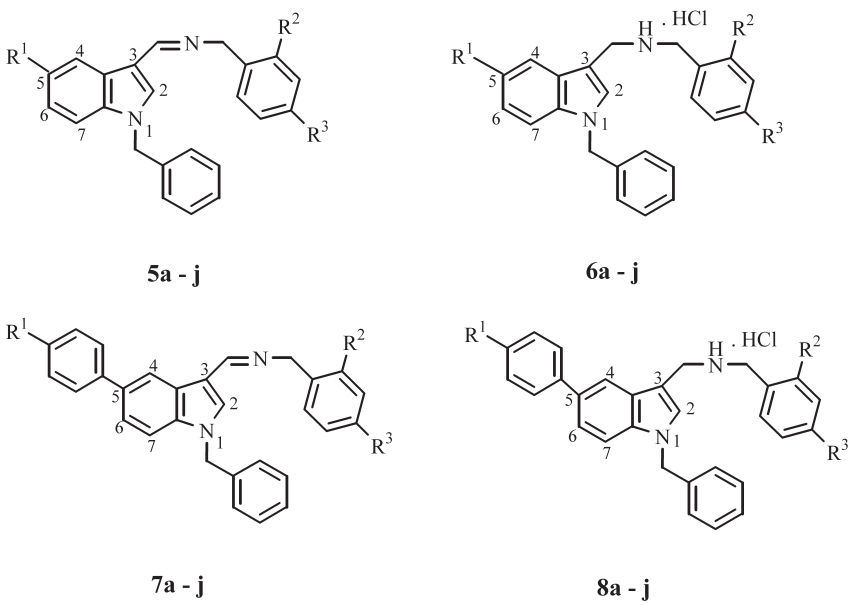

Fig. 1. Indole-imine and -amine derivatives as antioxidant and tyrosine kinase inhibitors.

1.0 ml of the methanolic solution of DPPH was added to 0.1 ml of the test compounds in DMSO. Absorbance at 517 nm was determined after 30 min at room temperature, and the scavenging activity was calculated as the percentage of radical reduction. Each experiment was performed in triplicate. DMSO was used as a control. The radical scavenging activity was calculated using the following equation:

$$\text{radical scavenging activity} = [(OD_{control} - OD_{sample})/OD_{control}] \cdot 100,$$

where $OD_{control}$ is the optical density of DMSO/DPPH, and OD_{sample} is the optical density of compounds/DPPH.

Results and Discussion

In this study, the *in vitro* antioxidant effects of novel 1,3,5-trisubstituted indole derivatives, namely *N*-benzyl 5-substituted indole-3-imines and their corresponding amine congeners (Fig. 1), on rat liver microsomal NADPH-dependent lipid peroxidation (LP) levels (Mihara *et al*., 1980) and their free radical scavenging properties were investigated (Crapo *et al*., 1978). The free radical scavenging activities of the test compounds were examined based on their ability to bleach the stable radical DPPH (Wettasinghe and Shahidi, 2000), and the results are summarized in Tables I and II. The biological activity of the compounds was evaluated by the *in vitro* tyrosine kinase assay that measures the changes in the enzymatic activity of $pp60^{c\text{-}Src}$ tyrosine kinase by virtue of following the alterations in the phosphorylation level of immobilized substrate with respect to DMSO (vehicle) control (Ölgen *et al*., 2008).

In general, imine compounds were found more effective against LP than their amine congeners. Comparing the antioxidant effects of *N*-benzyl indoles **5a**–**e** and *N*-benzyl-5-bromo indoles **5f**–**j** demonstrated that 5-bromo substitution caused lower inhibition of both SOD and LP (Table I). The amine congeners of these compounds **6a**–**j** exhibited better inhibition against SOD and LP. Among the *N*-benzyl indole and *N*-benzyl-5-bromo indole-3-imine and -amine derivatives, compounds **5d** and **5e**, having halogen substitution in the aromatic ring position 3, were found to be the most active inhibitors of LP, with 99% and 98% LP inhibition, respectively. This might be a special effect of the substitution feature at the 3-position whereas the electron-withdrawing groups might result in better LP inhibition. Moreover, the bromo substitution at 5-position of the indole ring had a negative impact on LP inhibition. The activity results of the *N*-benzyl-5-phenyl indole-3-substituted imines **7a**–**e** and *N*-benzyl-5-(*p*-fluorophenyl)indole-3-substituted imine compounds **7f**–**j** showed that *p*-fluoro substitution of the phenyl ring at 5-position of the indole ring resulted in an activity loss of LP which may be due to the phenyl substitution at 5-position of the indole ring which provides certain lipophilicity (Table II). It was also found that compounds **7a**–**j** did not have any significant activities on SOD, whereas the amine congeners **8a**–**j** exhibited only slight inhibition of SOD and LP.

In the kinase assays (Kılıç *et al*., 2009a, b), it was found that the *N*-benzyl indole amine compounds **6a**–**j** had higher inhibition than the *N*-benzyl-5-bromo indole imine compounds **5f**–**j**. With respect to substitution on position 5, it was observed that the *N*-benzyl-5-bromo indoles **5f**–**j** and **6f**–**j** had higher activities than the *N*-benzyl indoles **5a**–**e** and **6a**–**e** for both imine and amine derivatives (Kılıç *et al*., 2009a). It was considered that the volume of the bromo substituent may be important to bring compounds in suitable geometric orientation or favourable energetic states which alter the activity of an enzyme. In general, the introduction of halogen atoms in the benzyl ring at the third position enhanced the activity of the compounds, and imine derivatives showed less activity than their amine congeners in both substituted and unsubstituted derivatives. In this series of compounds halogen substitution increased both antioxidant and tyrosine kinase inhibitory activities. Bromo substitution at 5-position of the indole ring did not show parallel results for antioxidant and tyrosine kinase inhibition. In our previous study (Kılıç *et al*., 2009b), it was shown that while screening of *N*-benzyl-5-phenyl (**7a**–**e**) and *N*-benzyl-5-(*p*-fluoro)phenyl (**7f**–**j**) indole derivatives, all compounds had some activity against the kinase target, except **7a**–**d**. It was found that compound **8c**, 1-(1-benzyl-5-phenyl-1*H*-indole-3-yl)-*N*-(4-fluorobenzyl)methanamine hydrochloride, was the most potent inhibitor of $pp60^{c\text{-}Src}$ tyrosine kinase with an IC_{50} value of (4.69 ± 1.23) μM, followed by compounds **8f**, **8g**, and **8h** with IC_{50} values of (74.79 ± 1.43), (75.06 ± 1.24), and (84.23 ± 1.19) μM, respectively. Analyzing the 5-substitution at the indole ring, it was found that

Table I. Antioxidant activity of *N*-benzyl indole and *N*-benzyl-5-bromo indole-imine and -amine derivatives.

Compound	R^1	R^2	R^3	Concentration in incubation medium [M]	Inhibition of SOD[a] (%)	Inhibition of LP[a] (%)
5a	H	H	H	10^{-3}	35 ± 4.2	50 ± 2.1
				10^{-4}	36 ± 3.5	25 ± 1.4
5b	H	H	Cl	10^{-3}	NE	25 ± 3.5
				10^{-4}	NE	5 ± 0.7
5c	H	H	F	10^{-3}	35 ± 4.2	50 ± 3.5
				10^{-4}	30 ± 3.5	20 ± 2.8
5d	H	Cl	Cl	10^{-3}	NE	99 ± 0.7
				10^{-4}	NE	60 ± 1.4
5e	H	F	F	10^{-3}	NE	98 ± 2.8
				10^{-4}	NE	40 ± 2.1
5f	Br	H	H	10^{-3}	NE	NE
				10^{-4}	NE	NE
5g	Br	H	Cl	10^{-3}	NE	32 ± 1.4
				10^{-4}	NE	NE
5h	Br	H	F	10^{-3}	NE	NE
				10^{-4}	NE	NE
5i	Br	Cl	Cl	10^{-3}	NE	NE
				10^{-4}	NE	NE
5j	Br	F	F	10^{-3}	NE	NE
				10^{-4}	NE	NE
6a	H	H	H	10^{-3}	21 ± 1.0	35 ± 4.2
				10^{-4}	NE	30 ± 3.5
6b	H	H	Cl	10^{-3}	44 ± 1.6	NE
				10^{-4}	NE	NE
6c	H	H	F	10^{-3}	39 ± 4.0	NE
				10^{-4}	NE	NE
6d	H	Cl	Cl	10^{-3}	53 ± 0.8	22 ± 1.4
				10^{-4}	NE	NE
6e	H	F	F	10^{-3}	38 ± 1.5	9.0 ± 2.1
				10^{-4}	NE	NE
6f	Br	H	H	10^{-3}	49 ± 1.3	42 ± 1.4
				10^{-4}	NE	NE
6g	Br	H	Cl	10^{-3}	26 ± 1.2	45 ± 2.1
				10^{-4}	NE	NE
6 h	Br	H	F	10^{-3}	34 ± 1.7	9 ± 0.7
				10^{-4}	NE	NE
6i	Br	Cl	Cl	10^{-3}	18 ± 0.8	15 ± 1.4
				10^{-4}	NE	NE
6j	Br	F	F	10^{-3}	33 ± 1.0	17 ± 1.4
				10^{-4}	NE	NE
Vit. E				10^{-3}	99 ± 1.4	92 ± 2.8
				10^{-4}	98 ± 0.7	90 ± 1.4

NE, not effective.
[a] Each value represents the mean ± S.D. of three experimental results.

5-(4-fluoro)-phenyl substitution, regardless of any substituent at other positions of the indole ring, improved the kinase inhibitory activity of the compounds compared with their corresponding 5-phenyl substituted compounds (Kılıç *et al.*, 2009b). This tendency was also true for both imine and amine derivatives of these compounds, and the remarkable differences were seen from the screening at low doses (50 μM). Among the 5-(*p*-fluoro)phenyl indole amine derivatives, the compound activity was higher for the unsubstituted or mono-halogen-substituted derivatives **8f**, **8g** and **8h** than their corresponding derivatives **8i** and **8j** with dihalogen substitutions. Here, interestingly, the active mono-halogen-bearing derivatives were those having *p*-fluoro or *p*-chloro substituents at position 3 of the benzyl ring of the indole scaffold. It was concluded that these

Table II. Antioxidant activity of *N*-benzyl-5-phenyl and *N*-benzyl-5-(*p*-fluorophenyl)indole-imine and -amine derivatives.

Compound	R^1	R^2	R^3	Concentration in incubation medium [M]	Inhibition of SOD[a] (%)	Inhibition of LP[a] (%)
7a	H	H	H	10^{-3}	NE	98 ± 0.7
				10^{-4}	NE	99 ± 1.4
7b	H	H	Cl	10^{-3}	NE	99 ± 4.4
				10^{-4}	NE	98 ± 3.5
7c	H	H	F	10^{-3}	NE	99 ± 4.2
				10^{-4}	NE	99 ± 2.8
7d	H	Cl	Cl	10^{-3}	NE	98 ± 1.4
				10^{-4}	NE	99 ± 0.7
7e	H	F	F	10^{-3}	NE	NE
				10^{-4}	NE	NE
7f	F	H	H	10^{-3}	NE	NE
				10^{-4}	NE	NE
7g	F	H	Cl	10^{-3}	NE	NE
				10^{-4}	NE	NE
7h	F	H	F	10^{-3}	NE	NE
				10^{-4}	NE	NE
7i	F	Cl	Cl	10^{-3}	NE	32 ± 2.1
				10^{-4}	NE	NE
7j	F	F	F	10^{-3}	NE	11 ± 0.5
				10^{-4}	NE	NE
8a	H	H	H	10^{-3}	26 ± 0.7	37 ± 1.4
				10^{-4}	NE	NE
8b	H	H	Cl	10^{-3}	27 ± 1.2	41 ± 0.7
				10^{-4}	NE	NE
8c	H	H	F	10^{-3}	53 ± 1.8	49 ± 2.1
				10^{-4}	NE	NE
8d	H	Cl	Cl	10^{-3}	26 ± 0.7	35 ± 1.4
				10^{-4}	NE	NE
8e	H	F	F	10^{-3}	27 ± 0.3	49 ± 1.4
				10^{-4}	NE	NE
8f	F	H	H	10^{-3}	38 ± 1.4	39 ± 1.4
				10^{-4}	NE	NE
8g	F	H	Cl	10^{-3}	20 ± 1.3	43 ± 0.7
				10^{-4}	NE	NE
8h	F	H	F	10^{-3}	26 ± 1.0	52 ± 1.4
				10^{-4}	NE	NE
8i	F	Cl	Cl	10^{-3}	20 ± 0.3	29 ± 1.4
				10^{-4}	NE	NE
8j	F	F	F	10^{-3}	23 ± 0.2	41 ± 2.1
				10^{-4}	NE	NE
Vit. E				10^{-3}	99 ± 1.4	92 ± 2.8
				10^{-4}	98 ± 0.7	90 ± 1.4

NE, not effective.
[a] Each value represents the mean ± S.D. of three experimental results.

different influences of substituents on the activity might be due to their favourable contribution to lipophilic and electronic factors of the compounds. While no significant correlation was observed, it was identified that all active compounds have electron-withdrawing groups at position 3 of the benzyl ring of the indole ring.

Comparing the tyrosine kinase inhibitory activity and antioxidant capacity of *N*-benzyl-5-phenyl (**7a**–**e**) and *N*-benzyl-5-(*p*-fluoro)phenyl (**7f**–**j**) indole derivatives, the imine compounds **7a**–**e**, that did not have any tyrosine kinase inhibition potential, exhibited higher antioxidant capacity than the amine congeners, except **7f**–**i** with fluoro substitution at 5-position of the phenyl ring. Al-

though most of the amine derivatives from the series **8a**–**j**, namely **8c**, **8f**, **8g**, and **8h**, were potential inhibitors of tyrosine kinase, they exhibited slight inhibition on both SOD and LP. These results revealed that fluoro substitution at 5-position of the phenyl ring do not have any positive impact on both tyrosine kinase inhibitory activity and antioxidant capacity of the compounds. However, these findings may suggest that compounds having inhibitory activities on both tyrosine kinases and lipid peroxidation, and so having higher antioxidant capacity, can provide protection against redox stimulation of cellular protein kinases.

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