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# The Endogenous Oxindoles 5-Hydroxyoxindole and Isatin Are Antiproliferative and Proapoptotic

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**Oxindole-core synthetic molecules are currently being developed as anticancer drugs that target protein tyrosine kinases associated with growth factor receptors. Oxindole, 5-Hydroxyoxindole, and 2,3-dioxindole [isatin] are natural molecules found in mammalian body fluids and tissues and we addressed the question of similar properties of endogenous oxindoles. 5-Hydroxyoxindole and isatin, but not oxindole, inhibited N1E-115, BALB/c3T3, BBC, PC12, and HL60 proliferation at submicromolar concentrations. Acute treatment with 5-hydroxyoxindole and isatin reduced the activity of extracellular signal regulated protein kinases (ERKs) by 35% at 100 $\mu$M and ERK1 activity was strongly inhibited by 5-Hydroxyoxindole at 10 $\mu$M. Survival of PMA-differentiated HL60 and $FGF_2$-differentiated PC12 cells was not affected by 5-Hydroxyoxindole and isatin treatment, suggesting that endogenous oxindoles interact with growth factors signaling. The physiological implications of these data and the potential utility of 5-Hydroxyoxindole and isatin as antitumor agents are discussed.** 

***Key Words:*** **oxindole; 5-hydroxyoxindole; isatin; endogenous antiproliferative agents; apoptosis.**

Synthetic oxindole-based compounds were developed as potent inhibitors of cell proliferation *in vitro.* They inhibit protein tyrosine kinase (PTK) activity associated with growth factor receptors (1–4) at submicromolar concentrations. Like other small compounds inhibiting PTK (for review 5), these synthetic compounds are useful for studying cellular signaling (6) and have been proposed as anti-tumor (3) and anti-inflammatory (7, 8) agents.

Endogenous oxindoles have been detected in mammal's body fluids raising questions as to their physiological functions and potential effect on cell proliferation *in vivo.* Endogenous oxindoles (oxindole, 5-hydroxyoxindole and 2,3 dioxindole [isatin]) were first identified as urinary compound (9–13). They were recently measured in blood and tissues of mammals, including humans (14–16). Spontaneous increases in the levels of these compounds have been reported in body fluids of patients with rheumatoid arthritis (11, 17), cirrhosis (18), hepatic encephalopathy (18), bulimia nervosa (19) and Parkinson's disease (20).

The aim of this study was to document the effects of oxindole, isatin, and 5-hydroxyoxindole on the proliferation of various cell lines (N1E-115, PC12, BALB/c3T3, BBC, and HL60). We also tested structurally related endogenous compounds (5-methoxytryptamine [melatonin], 5-hydroxytryptamine [serotonin], 5-hydroxyindol acetic acid, 5-hydroxytryptophan, and tryptophan). Effects on cell survival and on activation of extracellular signal regulated protein kinases (ERKs) were also studied.

## MATERIALS AND METHODS

*Materials.* 5-Hydroxyoxindole was a gift from Valbiofrance (Paris, France). Oxindoles and related compounds, bovin serum albumin (BSA), phorbol-12-myristate, 13 acetate (PMA) and (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (Saint Quentin Fallavier, France). Human recombinant basic fibroblastic growth factor (Hr $FGF_2$) produced in procaryotic cells was purified in the laboratory. [*methyl*-$^3$H]Thymidine (35 Ci/mmol) and [$\gamma$-$^{32}$P]ATP (4000 Ci/mmol) were obtained from ICN (Orsay, France).

*Cells.* HL60 (human promyelocytic leukemia), PC12 (rat adrenal pheochromocytoma), and BALB/c3T3 (mouse fibroblast) cell lines were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). Mouse N1E-115 neuroblastoma cells were kindly provided by C. Nahmias (CNRS UPR 415, Paris, France) and bovine brain capillary cells (BBC) were kindly provided by D. Gospodarowicz (UCSF, San Francisco, CA).

*Cell culture.* Cell lines were maintained in high-glucose DMEM supplemented with 10% heat-inactivated fetal calf serum (hiFCS) for N1E-115, 10% fetal calf serum (FCS) for BALB/c3T3, 10% FCS and 2 ng/ml Hr $FGF_2$ for BBC and 10% FCS + 5% horse serum for PC12 in a 7% $CO_2$ atmosphere at 37°C in a humidified incubator. HL60 cells were maintained in RPMI 1640 supplemented with 10% hiFCS in a 5% $CO_2$ atmosphere.

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**TABLE 1**

Effects of Oxindoles and Related Compounds on N1E-115, BALB/c3T3, and BBC Cell Line Proliferation

| Compounds | $EC_{50}$ ($\mu$M) | | |
|---|---|---|---|
| | N1E-115 | BALB/c3T3 | BBC |
| 5-Hydroxyoxindole | 21.2 ± 3.7 | 15.0 ± 3.2 | 19.4 ± 3.5 |
| Isatin | 47.2 ± 4.6[a] | 24.3 ± 11.1 | 37.2 ± 6.6[a] |
| Melatonin | 158.5 ± 21.3[b] | 104.9 ± 73.5[b] | 257.8 ± 52.9[b] |
| 5-Hydroxytryptophan | 184.8 ± 72.9[b] | 133.9 ± 22.9[b] | Linear[b] |
| Serotonin | 400.5 ± 102.2[b] | 395.0 ± 117.3[b] | Linear[b] |
| 5-Hydroxyindole acetic acid | 1332.5 ± 830.1[b] | 1495.5 ± 545.9[b] | 1445.4 ± 122.2[b] |
| Oxindole | Linear[b] | Linear[b] | Linear[b] |
| Tryptophan | Linear[b] | Linear[b] | Linear[b] |

*Note.* $EC_{50}$ values were determined by fitting experimental data to a sigmoid curve with variable slope using GraphPad Prism 2.01 program. Results are means ± SEM of 3 separate experiments performed in triplicate. Italics indicate statistical significance: [a]$P < 0.05$, [b]$P < 0.001$ for 5-hydroxyoxindole versus other compounds.

*Cell treatment.* PC12 and HL60 cell lines were differentiated with Hr $FGF_2$ (5 ng/ml) and PMA (16 nM) respectively, during 2 days. Cell lines were always serum-starved for 18 h before experiments. All compounds were dissolved in ethanol/distilled water (80/20) and filtered through a 0.22-$\mu$m membrane.

*Cell proliferation assay.* Cell lines were seeded in 48-well plates ($10^4$ cells/well/500 $\mu$l DMEM-1% appropriate serum) and proliferation was assessed 20 h after treatment in terms of [$^3$H]thymidine uptake (1 $\mu$Ci/dish). After 4 h of incorporation, dishes were incubated with ice-cold 10% trichloroacetic acid (TCA, 30 min, 4°C). Cultures were then solubilized with NaOH (0.3 M, 30 min, 37°C) and radioactivity was quantified with a liquid scintillation microbeta counter (Wallac, Turku, Finland).

*MTT reduction viability assay.* Experiments were performed in 48-well plates ($10^4$ cells/well/500 $\mu$l DMEM-1% appropriate serum) for 72 h. The number of surviving cells was determined by measurement of the $A_{590\,nm}$ of the dissolved formazan product after addition of MTT (5 ng/ml) for 2 h. We checked that OD correlated with cell numbers by using a cell counter (Coultronics, Margency, France).

*Nuclear staining.* N1E-115 cells were seeded in 24-well plates ($2 \times 10^4$ cells/well/500 $\mu$l DMEM-1% hiFCS). After 72 h of treatment with 5-hydroxyoxindole, cells were fixed with 4% paraformaldehyde (1 h at RT) and stained with 10 $\mu$g/ml Hoechst 33258 (provided by Dr. A. Lombet, INSERM U339, Paris, France) for 10 min at 37°C. After washing in water, the plates were observed under an inverted fluorescence microscope with excitation at 365 nm.

*Western blot analysis of ERKs.* N1E-115 cells ($6 \times 10^4$ cells) were pretreated with oxindoles (5 min, 37°C) and then stimulated with 1% hiFCS (5 min, 37°C). Cells were then washed twice with ice-cold PBS and scraped free in 70 $\mu$l of 2× Laemmli buffer (21). Samples (25 $\mu$g protein) were separated onto 10% SDS polyacrylamide gel. After wet-blotting onto PVDF membranes (Millipore, Saint Quentin en Yvelines, France), filters were blocked (1 h, RT) with BSA (3%) in Tris-buffered saline (TBS, 50 mM Tris pH 7.2, 150 mM NaCl) containing Tween 20 (0.1%). After incubation (overnight, 4°C) with anti-phospho-p42/p44 mitogen activating protein kinase (MAPK) monoclonal antibody (New England Biolabs, Inc., Beverly, MA) and subsequently, peroxidase-conjugated goat anti-mouse secondary antibody, proteins were detected with enhanced chemiluminescence reagents (Boehringer-Mannheim, Germany).

*In vitro ERKs assay.* N1E-115 cells were stimulated as described above for Western blot analysis, then were scraped free in 70 $\mu$l of glycero-phosphate buffer (40 mM pH 7.3) containing EGTA (10 mM), $MgCl_2$ (10 mM), $Na_2VO_3$ (1 mM), $\beta$-mercaptoethanol (1 mM), NP-40 (0.5%), PMSF (1 mM) and leupeptin (1 $\mu$g/ml). Insoluble material was eliminated by centrifugation of the lysate at 12,000*g* for 20 min at 4°C. Supernatant protein (5 $\mu$g) was incubated for 20 min at 37°C with 0.1 $\mu$Ci [$\gamma$-$^{32}$P]ATP (10 $\mu$M), 1 mg/ml protein kinase inhibitor, and 25 $\mu$g of a synthetic peptide (APRTPGGRR) corresponding to amino acids 95–98 of bovine myelin basic protein (Calbiochem, Meudon, France). The reaction was arrested by adding TCA 93%) and centrifuged at 12,000*g* for 15 min at 4°C. The radiolabelled complex was fixed on P81 Whatman paper and washed in a bath of acetic acid (30%) and ATP (3 mM) for 2 h at 4°C. The papers were rinsed in ethanol/ether (50/50) and then in ether, and radioactivity was counted.

## RESULTS

### *Effect of Endogenous Oxindoles and Related Compounds on Cell Proliferation*

The antiproliferative effects of 5-hydroxyoxindole and isatin were compared with those of related endogenous compounds on several cell lines (Table 1). Three classes of compounds emerged on the basis of $EC_{50}$ values: (i) Efficient inhibitors (5-hydroxyoxindole and isatin, $EC_{50}$ 15–50 $\mu$M); (ii) Less efficient inhibitors (melatonin, serotonin, 5-hydroxyindole acetic acid and 5-hydroxytryptophan; $EC_{50}$ 100–1000 $\mu$M); and (iii) Inefficient compounds (oxindole and tryptophan). The $EC_{50}$ of 5-hydroxyoxindole was 15–25 $\mu$M on BALB/c3T3, BBC and N1E-115 cell line proliferation; similar efficiency was obtained with PC12, HL60 and NIH 3T3 cells ($EC_{50}$ 54.7 ± 3.6, 58.2 ± 11.7 and 34.8 ± 3.2 $\mu$M, respectively, data not shown). Except on BALB/c3T3 cells, isatin was significantly less efficient than 5-hydroxyoxindole ($EC_{50}$ 30–50 $\mu$M). The neurotransmitters melatonin and serotonin were less efficient ($EC_{50} > 100$ $\mu$M) and serotonin was found inefficient on BBC cells. 5-hydroxyoxindole and isatin were the most potent inhibitors on all the cell lines used. At a concentration of 0.1 mM they reduced cell proliferation by 80% compared to controls; these two compounds were followed by melatonin (40%), 5-hydroxytryptophan (35%), serotonin (25%) and 5-hydroxyindole acetic acid

**TABLE 2**

Effects of 5-Hydroxyoxindole and Isatin on Differentiated PC12 and HL60 Cell Death

| | Cell death (%) | | | |
|---|---|---|---|---|
| | 5-Hydroxyoxindole | | Isatin | |
| Cell lines | Control cells | Differentiated cells | Control cells | Differentiated cells |
| HL60 | 27.7 ± 3.8 | 5.1 ± 4.4[a] | 31.4 ± 4.0 | 4.8 ± 2.8[b] |
| PC12 | 19.4 ± 5.9 | 2.6 ± 1.8[a] | nd | nd |

*Note.* Results are the percentage of cell death after 72 h of treatment with 5-hydroxyoxindole and isatin (50 $\mu$M). Data are mean ± SEM of 2 separate experiments performed in triplicate. Italics indicate statistical significance: [a]$P < 0.05$, [b]$P < 0.001$ for differentiated versus control proliferative cell lines; nd, not determined.

(12%). Millimolar concentrations could not be tested because of the impact of the vehicle on cells. Vehicle (ethanol/distilled water) had any effect until 0.4% concentration in culture medium.

### *Effects of 5-Hydroxyoxindole and Isatin on Cell Viability*

The two compounds decreased number of viable cells (data not shown). After the first 24 h, compared to control cells, 5-hydroxyoxindole treatment reduced MTT metabolism by 25% whatever the dose (23 ± 7% of cell death up to 100 $\mu$M). Later, 5-hydroxyoxindole induced cell death in a concentration-dependent manner. In contrast, the effect of isatin was always concentration-dependent (14 ± 7 and 76 ± 7% of cell death at 10 and 100 $\mu$M, respectively). The two compounds had similar potency (65 ± 3 and 76 ± 7% of cell death with 100 $\mu$M 5-hydroxyoxindole and isatin, respectively).

As reported in Table 2, survival of non proliferative cells was not affected by 5-hydroxyoxindole and isatin treatment. Macrophages (PMA-treated HL60) and neurons (Hr $FGF_2$-treated PC12) exhibited lower percentage of cell death than their proliferative control cell lines (HL60 and PC12).

### *Effect of 5-Hydroxyoxindole and Isatin on Apoptosis*

Both 5-hydroxyoxindole (Fig. 1B) and isatin (not shown) caused DNA fragmentation and chromatin condensation, as demonstrated by uptake of the DNA-specific fluorescent dye Hoechst 33258. Extrusion of chromatin into the cytosol was also observed. The number of apoptotic cells was time- and concentration-dependent in the MTT reduction assay. Control cells were devoid of nuclear fragmentation and condensation (Fig. 1A).

### *Effects of 5-Hydroxyoxindole and Isatin on ERKs Activation*

ERKs activity was significantly decreased by treatment with 5-hydroxyoxindole and isatin. Basal ERKs activity was 41.3 ± 1.0 fmol/min/mg protein. Activity was inhibited in a concentration-dependent manner by both endogenous compounds (Fig. 2A). Concentrations of 60 $\mu$M were necessary to obtain significant inhibition with 5-hydroxyoxindole and isatin (20% and 25%, respectively). These results were confirmed by Western blot (Fig. 2B): ERK2 phosphorylation was strongly reduced by 10 $\mu$M 5-hydroxyoxindole and >60 $\mu$M isatin. In contrast, ERK1 phosphorylation was little affected by either compound. We found that oxindole had no effect on ERKs phosphorylation (not shown).

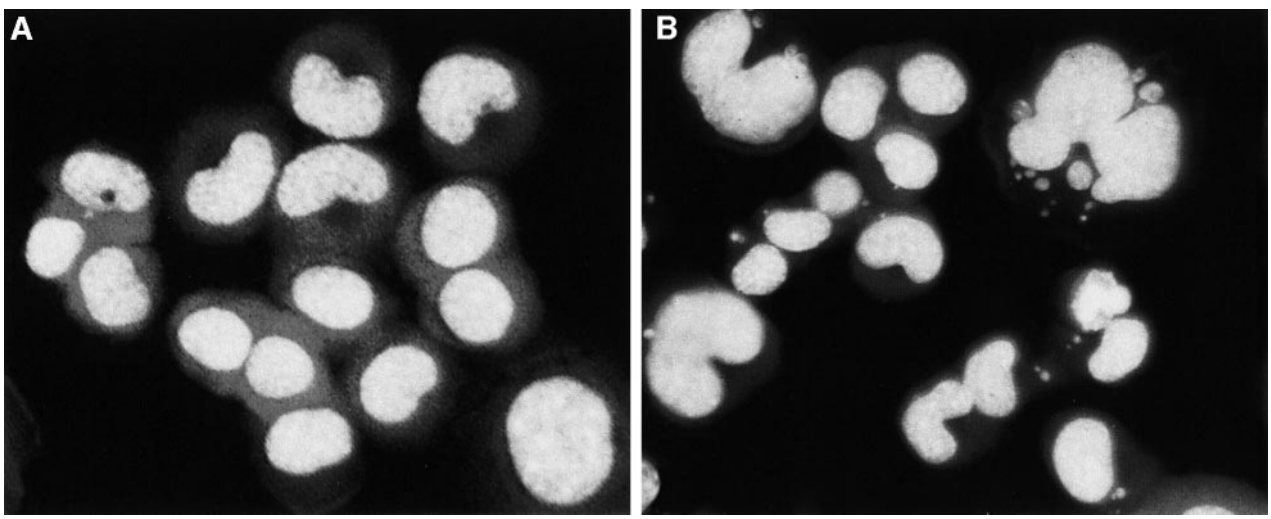


**FIG. 1.** Representative nuclear staining of N1E-115 cells by the DNA-specific dye Hoechst 33258. Cells were cultured without (A) or with 5-hydroxyoxindole (B, 20 $\mu$M) for 3 days. Magnification: 400×.

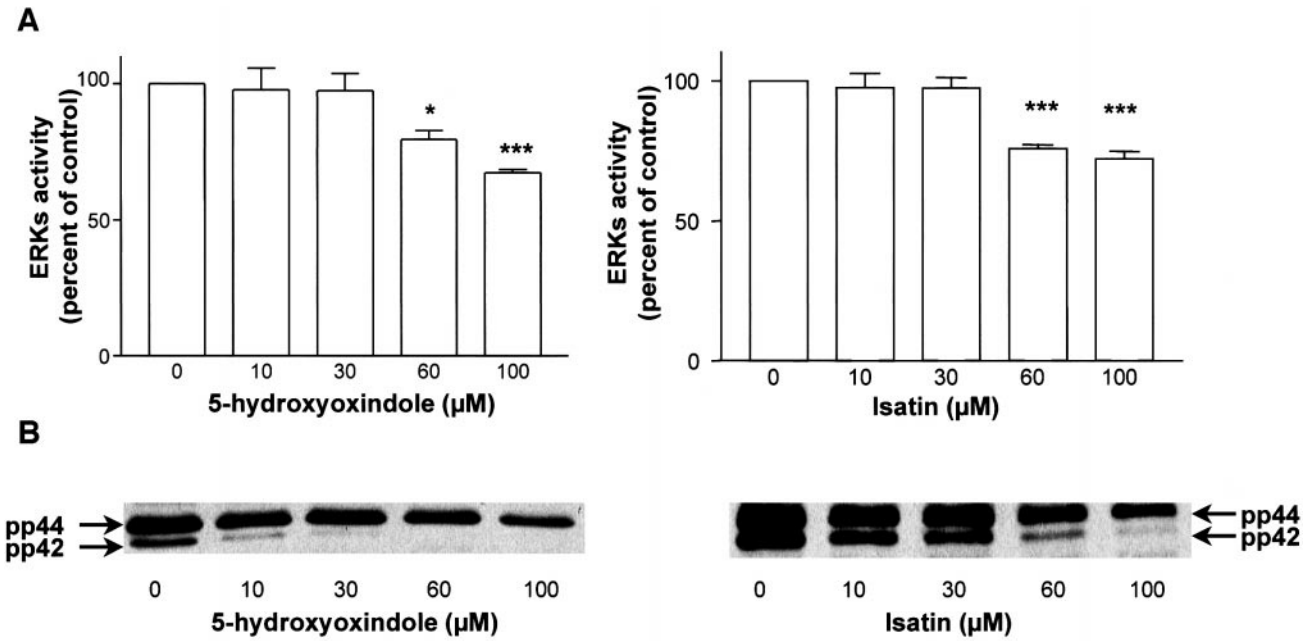


**FIG. 2.** Effects of 5-hydroxyoxindole and isatin on ERK activity in N1E-115 cells. (A) Cells pretreated with 5-hydroxyoxindole and isatin for 5 min and then with 1% hiFCS for 5 min. Cell extracts were tested for ERK activity as described under Materials and Methods. Values are normalized to control cells (cells stimulated with 1% hiFCS for 5 min). Data are means ± SD of one representative experiment performed in triplicate. Statistical significance: $*P < 0.05$, $***P < 0.001$; (B) Experimental conditions as in A. Proteins were resolved by SDS-PAGE followed by immunoblotting with an anti-phospho-p42/p44 MAPK antibody. The phosphorylated activated forms of p42/ERK2 and p44/ERK1 are designated pp42 and pp44.

## DISCUSSION

These results show that the endogenous oxindoles 5-hydroxyoxindole and isatin have antiproliferative actions at submicromolar concentrations *in vitro*, as previously described with synthetic oxindole-core compounds. Moreover, 5-hydroxyoxindole and isatin were proapoptotic but not toxic molecules.

The antiproliferative effect of 5-hydroxyoxindole and isatin was concentration-dependent and also cell line-independent, as neuroblastoma (N1E-115), pheochromocytoma (PC12), endothelial (BBC), fibroblastic (BALB/c3T3) and promyelocytic (HL60) cell lines were responsive. The efficiency ($EC_{50}$ values) of the endogenous compounds tested here on [$^3$H]thymidine uptake inhibition was as follows: 5-hydroxyoxindole (15–20 $\mu$M) > isatin (25–50 $\mu$M) > melatonin (100–268 $\mu$M) > 5-hydroxytryptophan (135–185 $\mu$M) > serotonin (400 $\mu$M) > 5-hydroxyindole acetic acid (1300–1500 $\mu$M). Serotonin and 5-hydroxytryptophan had no effect on BBC cell proliferation, while tryptophan and oxindole had no effect on any of the cell lines. Melatonin and serotonin have previously been reported to stimulate and inhibit cell growth and/or ERKs activity at respectively micromolar (22, 23) and nanomolar concentrations (22, 24). We observed no such biphasic effect of melatonin or serotonin on cell proliferation, even in the picomolar to 0.1 mM concentration range (not shown). The cell lines used here, and the possible presence of cell-membrane 5-HT2B receptors, which are mitogenic (25), could account for these differences, together with the conditions of cell culture and compound dissolution.

The proapoptotic effect of 5-hydroxyoxindole and isatin appeared limited to proliferative cells. When neurons as well as macrophages were exposed to 72 h of treatment, cell death was very limited compare to control proliferative cell lines attesting that 5-hydroxyoxindole and isatin were not cytotoxic. So these endogenous compounds were able to interact with proliferation pathways.

The inhibitory effect of 5-hydroxyoxindole and isatin on cell proliferation is in keeping with the decrease in ERKs activity induced by the two compounds in N1E-115 neuroblastoma cells. Owing to the specific substrate used here, the decrease in activity concerned the ERK1 and ERK2 isoforms. Unexpectedly, ERK2 phosphorylation was inhibited by 30 $\mu$M 5-hydroxyoxindole, while no significant decrease in ERKs activity occurred. This raises two questions: (i) does total ERKs activity mainly reflect the active ERK1 isoform? and (ii) is 5-hydroxyoxindole a selective inhibitor of the ERK2 isoform? As the relative affinity of the anti-phospho-p42/p44 MAPK antibody used here was unknown, together with the respective phosphatase activities, further studies will be necessary to answer to these questions.

Interestingly, the endogenous oxindoles had contrasting effects. Oxindole was inactive, suggesting that radicals other than the oxindole-core may be crucial for inhibition of cell proliferation. The presence of a hydroxyl radical in the C-5 position (5-hydroxyoxindole) appeared to confer greater potency than the C-3 ketone function (isatin). Concerning synthetic oxindoles, C-3-substituted compounds have entered development as specific drugs for inhibition of PTK associated with EGF, PDGF, VEGF and IGF receptors (4). Structure-activity studies have shown that the oxindole core of synthetic compounds SU4984 and SU5402 occupy a site in which the adenine of ATP binds, while the substituents at the C-3 position of the oxindole-core contact residues in the hinge region of FGF receptors (3). Interestingly, 5-hydroxyoxindole and isatin inhibited [$^3$H]thymidine uptake and ERKs phosphorylation in a concentration range (20–100 $\mu$M) similar to that of the SU4984 and SU5402 in NIH 3T3 cells (3). The similar efficiency of endogenous and synthetic oxindoles to inhibit cell proliferation and ERKs phosphorylation suggests that the two categories of compound might share common interactions with ATP pockets associated with receptors of growth factors or other protein kinases as well (26, 27).

It is indeed likely that 5-hydroxyoxindole and isatin compete with ATP *in vitro*. As regards the *in vivo* situation, the intracellular ATP concentration is millimolar, but 100-fold differences have been found in different cell types (28). No information is available on 5-hydroxyoxindole levels in tissues and circulating blood. A wide concentration range of isatin has been reported in tissues (20 ng to 3.4 $\mu$g/g) and plasma (30 to 430 ng/ml), with a marked regional distribution in the brain (14, 15). Such a wide range concentrations (0.1–23.0 $\mu$M) could account for the different reported effects of isatin (29) and also for an antiproliferative property.

Administration of the synthetic oxindole SU5416 (selective for VEGF receptors) reduced tumor volume by 20 to 90% according to the tumor type implanted in mice (30). Growth inhibition of VEGF-responsive tumors required early treatment (one day after tumor cell implantation), lengthy administration (13–38 days) and high doses (25 mg/kg/day). Interestingly, SU5416 was reported to be non toxic at such submillimolar concentration. Isatin is not toxic for rats at millimolar concentrations (300 mg/kg; 31). Toxicity occurs at 2-fold higher concentration (50% lethal dose 800 mg/kg) and neurotoxic effects at 4-fold higher concentrations (1.3 g/kg; 32). No information is available on 5-hydroxyoxindole toxicity. Methods of increasing 5-hydroxyoxindole and isatin endogenous levels might be an alternative to exogenous administration, but better knowledge of their biosynthetic pathways would be required. 5-hydroxyoxindole and isatin result from hydroxylation in the liver (10, 33) and intestinal bacteria also participate in 5-hydroxyoxindole and isatin biosynthesis, as shown by a significant fall in oxindole content in rat blood and brain during antibiotic treatment (34). Interestingly, brain isatin content is normal in germ-free rats, in contrast to urinary levels (35). Availability of isatin and 5-hydroxyoxindole might thus be enhanced by modifying the gut flora or diet.

In conclusion, we observed that the endogenous oxindoles 5-hydroxyoxindole and isatin inhibit cell proliferation *in vitro* through an interaction with the ERKs pathway, and also promote apoptosis. These natural compounds have similar antiproliferative potency to synthetic oxindole-core compounds, and are thus potential new antitumor agents.

A better knowledge of the mechanisms that regulate circulating and tissular levels of 5-hydroxyoxindole and isatin *in vivo* may open new possibilities in the treatment of some proliferative disorders.

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## REFERENCES

1. Shiraishi, T., Domoto, T., Imai, N., Shimada, Y., and Watanabe, K. (1987) *Biochem. Biophys. Res. Commun.* **147,** 322–328.
2. Buzzetti, F., Brasca, M. G., Crugnola, A., Fustinoni, S., Longo, A., Penco, S., Dalla Zonca, P., and Comoglio, P. M. (1993) *Farmaco* **48,** 615–636.
3. Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R., and Schlessinger, J. (1997) *Science* **276,** 955–960.
4. Sun, L., Tran, N., Tang, F., App, H., Hirth, P., McMahon, G., and Tang, C. (1998) *J. Med. Chem.* **41,** 2588–2603.
5. Lawrence, D. S., and Niu, J. (1998) *Pharmacol. Ther.* **77,** 81–114.
6. Wang, Z., Canagarajah, B. J., Boehm, J. C., Kassisa, S., Cobb, M. H., Young, P. R., Abdel-Meguid, S., Adams, J. L., and Golsmith, E. J. (1998) *Structure* **6,** 1117–1128.
7. Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. R., Strickler, J. E., McLaughlin, M. M., Siemens, I. R., Fisher, S. M., Lici, G. P., White, J. R., Adams, J. L., and Young, P. R. (1994) *Nature* **372,** 739–746.
8. Levitzki, A., and Gazit, A. (1995) *Science* **267,** 1782–1787.
9. Böhm, F. (1940) *Hoppe Seylers Z Physiol. Chem.* **265,** 210–221.
10. King, L. J., Parke, D. V., and Williams, R. T. (1966) *Biochem. J.* **98,** 266–277.
11. Yamaguchi, M., and Matsukawa, S. (1971) *Wakayama Med. Rept.* **15,** 127–134.
12. Voeltler, W., Jung, G., Breitmaier, E., Konig, W., Gupta, D., and Breitmaier, G. (1971) *Z. Naturforsch B.* **26,** 1380–1381.
13. Glover, V., Halket, J. M., Watkins, P. J., Clow, A., Goodwin, B. L., and Sandler, M. (1988) *J. Neurochem.* **51,** 656–659.
14. Watkins, P., Clow, A., Glover, V., Halket, J., Przyborowska, A., and Sandler, M. (1990) *Neurochem. Int.* **17,** 321–323.

15. Manabe, S., Gao, Q., Yuan, J., Takahashi, T., and Ueki, A. (1997) *J. Chromatogr. B Biomed. Sci. Appl.* **691,** 197–202.
16. Carpenedo, R., Carla, V., Moneti, G., Chiarugi, A., and Moroni, F. (1997) *Anal. Biochem.* **244,** 74–79.
17. Nishimura, N., Mori, Y., Matsunaka, M., Matoba, S., Kawasaki, H., and Isobe, S. (1966) *Wakayama Med. Rept.* **11,** 1–14.
18. Moroni, F., Carpenedo, R., Venturini, I., Baraldi, M., and Zeneroli, M. L. (1998) *Lancet* **351,** 1861.
19. Brewerton, T. D., Zealberg, J. J., Lyliard, R. B., Glover, V., Sandler, M., and Ballenger, J. C. (1995) *Biol. Psychiatry* **37,** 481–483.
20. Minami, M., Hamaue, N., Endo, T., Hirafuji, M., Terado, M., Ide, H., Yamazaki, N., Yoshioka, M., Ogata, A., and Tashiro, K. (1999) *Folia Pharmacol. Jpn.* **114**(Suppl. 1), 186P–191P.
21. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
22. Roth, J. A., Rabin, R., and Agnello, K. (1997) *Brain Res.* **768,** 63–70.
23. Kribben, A., Herget-Rosenthal, S., Lange, B., Michel, M. C., and Phillipp, T. (1998) *Ren. Fail.* **20,** 229–234.
24. Slominski, A., and Pruski, D. (1993) *Exp. Cell Res.* **206,** 189–194.
25. Launay, J. M., Birraux, G., Bondoux, D., Callebert, J., Choi, D. S., Loric, S., and Maroteaux, L. (1996) *J. Biol. Chem.* **271,** 3141–3147.
26. Fox, T., Coll, J. T., Xie, X., Ford, P. J., Germann, U. A., Porter, M. D., Pazhanisamy, S., Fleming, M. A., Galullo, V., Su, M. S. S., and Wilson, K. P. (1998) *Protein Sci.* **7,** 2249–2255.
27. Kent, L. L., Hull-Campbell, N. E., Lau, T., Wu, J. C., Thompson, S. A., and Nori, M. (1999) *Biochem. Biophys. Res. Commun.* **260,** 768–774.
28. Cane, A., Breton, M., Koumanov, K., Béréziat, G., and Colard, O. (1998) *Am. J. Physiol.* **274,** C1040–C1046.
29. Medvedev, A. E., Clow, A., Sandler, M., and Glover, V. (1996) *Biochem. Pharmacol.* **52,** 385–391.
30. Fong, T. A. T., Shawver, L. K., Sun, L., Tang, C., App, H., Powell, T. J., Kim, Y. H., Schreck, R., Wang, X., Risau, W., Ullrich, A., Hirth, K. P., and McMahon, G. (1999) *Cancer Res.* **59,** 99–106.
31. Kumar, R., Bansal, R. C., and Mahmood, A. (1994) *Indian J. Med. Res.* **100,** 246–250.
32. Kohli, R. P., Sareen, K., Amma, M. K. P., and Gujral, M. L. (1962) *Indian J. Physiol. Pharmacol.* **6,** 145.
33. Beckett, A. H., and Morton, D. M. (1966) *Biochem. Pharmacol.* **15,** 937–946.
34. Carpenedo, R., Mannaioni, G., and Moroni, F. (1998) *J. Neurochem.* **70,** 1998–2003.
35. Sandler, M., Przyborowska, A., Halket, J., Watkins, P., Glover, V., and Coates, E. (1991) *J. Neurochem.* **57,** 1074–1075.