[^{125}I/^{127}I]IodoHoechst 33342: Synthesis, DNA Binding, and Biodistribution

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An iodinated analog of the DNA-minor-groove-binding agent Hoechst 33342 has been synthesized and evaluated for DNA binding and tumor targeting. The bis-benzimidazole ring system of the title compound was constructed from the piperazinyl terminus via a Pinner-type cyclization followed by oxidative cyclization of the diamine Schiff base. To synthesize radioiodoHoechst 33342, (trimethylstannyl)Hoechst 33342 was prepared by the same strategy and subjected to mild radioiododestannylation in the presence of lactoperoxidase. After purification by HPLC, the radiochemical was separated in carrier-free form with >85% radiochemical yield and >99% chemical and radiochemical purity. Fluorescence spectrometric analysis of the binding of iodoHoechst 33342 to calf thymus DNA gave an equilibrium association constant (K_a) of 2.57×10^7 M^{-1} comparable to the K_a value of Hoechst 33342. Fluorescence microscopy of viable V79 cells demonstrated that the iodinated dye stained the nuclei with avidity similar to that of the noniodinated dye. The biodistribution of [^{125}I]-iodoHoechst 33342 in LS174T tumor-bearing athymic mice 4 h postadministration showed a tumor uptake of 3−4% injected dose per gram (ID/g), tumor/blood ratio of 6−8, and tumor/nontumor ratios above unity for most organs. A low thyroid uptake (∼2% ID/g) indicated that the radiochemical did not deiodinate and was stable *in vivo*.

Introduction

The bis-benzimidazole dye Hoechst 33342, 2-[2-(4-ethoxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazinyl)benzimidazole (**1**) (Scheme 1), is a DNA-binding, fluorescent stain used in flow cytometry studies to quantify DNA content in live cells[1] and to differentiate cells at various locations in multicell spheroids[2] and is also used as a therapeutic agent for solid tumors.[3−5] The minor groove of the B-form of DNA offers ideal sites for the binding of Hoechst dyes as well as other ligands such as netropsin,[6,7] distamycin,[8] and CC-1065,[9] and often such binding occurs preferentially at dAdT-rich sequences. Chen et al.[10] have demonstrated that Hoechst 33342 (**1**) and its parent compound Hoechst 33258 (**2**) specifically interrupt the breakage/reunion reactions of mammalian DNA topoisomerase I and nonspecifically inhibit the catalytic activities of many DNA enzymes. Furthermore, Durand and Olive[2] have shown that Hoechst 33342 is superior to Hoechst 33258 in staining the nuclei of viable cells. Recent investigations by Denison et al.[11] have confirmed these findings and demonstrated that the uptake of Hoechst 33342 is nearly 3-fold greater than that of its parent Hoechst 33258 under comparable conditions and that its efflux from cells is very slow. In their study Chen et al.[10] found that Hoechst 33342 possessed enhanced membrane permeability and was about 2 orders of magnitude more cytotoxic than Hoechst 33258 which they ascribed to the ethoxy substitution on the 4-phenyl ring of the parent compound. These authors also detected a large number of protein−DNA cross-links and DNA strand breaks in the cells treated with Hoechst 33342 but not in those treated with Hoechst 33258. Hence, although the two dyes are structurally similar, they differ significantly in their biologic properties.

Hoechst 33258 (pibenzimol) has shown antitumor activity *in vivo* against intraperitoneal murine L1210 and P388 leukemia and is undergoing preclinical evaluation by the National Cancer Institute.[12] Martin et al.[13,14] reported its radioiodination with sodium [^{125}I]-iodide by direct electrophilic reaction and utilized the radiochemical in studying the radiobiologic effects of the decaying radioiodine in the minor groove of plasmid DNA. Our studies, however, have indicated that as a consequence of the presence of the *o*-iodophenol grouping, ^{125}I-labeled Hoechst 33258 is prone to excessive deiodination both *in vitro* and *in vivo* (unpublished data). This observation and the superior cellular uptake and DNA binding of Hoechst 33342 prompted us to synthesize iodoHoechst 33342 in radioactive as well as stable form. We report herein the synthesis of ^{127}I-labeled iodoHoechst 33342 (**3**) and (trimethylstannyl)-Hoechst 33342 (**4**) from the piperazinyl terminus and the no-carrier-added synthesis of radioiodoHoechst 33342 (^{125}I/^{131}I) by destannylation of the tin precursor. Due to the absence of phenolic activation of its aromatic ring, Hoechst 33342 could not be iodinated by direct electrophilic reaction. DNA binding, cellular localization, and biodistribution of [^{125}I]iodoHoechst 33342 in tumor-bearing nude mice have been examined.

Results and Discussion

Loewe et al.[15] reported the first synthesis of bis-benzimidazole Hoechst 33258 and a wide variety of its congeners. The synthetic strategy consisted of construction of the bis-benzimidazole ring system from the piperazinyl terminus via two consecutive Pinner-type

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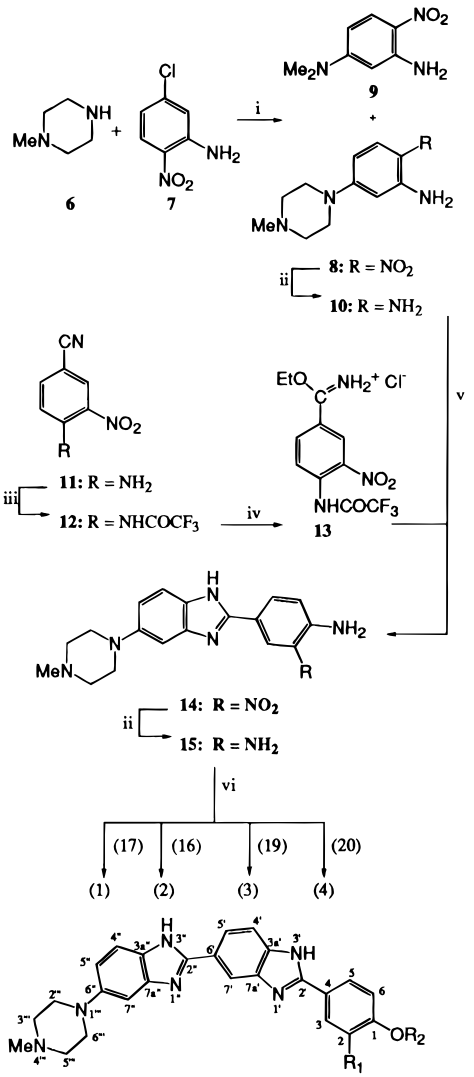
Scheme 1. Synthesis of Bis-benzimidazoles by Pinner-Type Cyclization Followed by Oxidative Cyclization of Diamine Schiff Base[a]

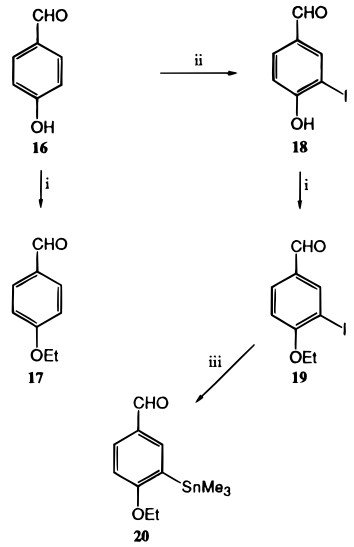


1: Hoechst 33342 (R_1 = H, R_2 = Et)
2: Hoechst 33258 (R_1, R_2 = H)
3: IodoHoechst 33342 (R_1 = I, R_2 = Et)
4: TrimethylstannylHoechst 33342 (R_1 = $SnMe_3$, R_2 = Et)
5: IodoHoechst 33258 (R_1 = I, R_2 = H)

[a] (i) Anhydrous K_2CO_3, DMF, reflux, 4.5 h; (ii) H_2, 10% Pd/C, room temperature, 1.25 h; (iii) $(CF_3CO)_2O$, Py, −20 °C, 0.5 h; (iv) 10% ethanol−ether, dry HCl, −20 °C, 2 h; (v) acetic acid, 55 °C, stirring, 12 h; (vi) nitrobenzene−toluene (4:1), 60 °C, 0.5 h; then 98−100 °C, 24−36 h.

cyclizations. In the present approach (Scheme 1), we adapted the above procedure to construct the monoimidazole ring system with some improvements and prepared the diamine **15**. In brief, treatment of 5-chloro-2-nitroaniline (**7**) with 1-methylpiperazine (**6**) in the presence of anhydrous potassium carbonate in DMF afforded the required piperazinylaniline **8** in nearly 60% yield together with a byproduct, 5-(dimethylamino)-2-nitroaniline (**9**). The required diamine **10** was obtained by the catalytic hydrogenation of **8** using palladized carbon in a Parr reactor at ambient temperature. In our modified synthesis of ethyl benzimidate **13**, amino nitrile **11** was converted to its trifluoroacetyl derivative **12** by trifluoroacetic anhydride treatment and reacted with hydrogen chloride to obtain ethyl benzimidate **13** in 80% yield. The latter (**13**) was condensed with diamine **10** in acetic acid and the imidazole **14**, obtained

Scheme 2. Synthesis of Substituted Benzaldehydes for Construction of the Second Imidazole Ring[a]



[a] (i) Acetone/anhydrous K_2CO_3, stirring, room temperature, 30 h; (ii) ICl, CH_2Cl_2, room temperature, stirring, 17 h; (iii) $(Me_3Sn)_2$, dioxane, $(Ph_3P)_4Pd(0)$, reflux, 1 h.

in 63% yield, was reduced further to diamine **15** under Parr hydrogenation conditions.

The second imidazole ring was constructed by heating the diamine **15** with appropriately functionalized benzaldehyde (Scheme 2) in a mixture of nitrobenzene and toluene at elevated temperature (95−100 °C) to afford the bis-benzimidazole **1**. In their ^{1}H NMR spectra, iodoHoechst and (trimethylstannyl)Hoechst 33342 displayed a total of nine aromatic protons and two highly deshielded imidazole protons ($\sim\delta = 13$ ppm) in addition to the other expected signals. The (trimethylstannyl)-Hoechst 33342 also displayed the most shielded signal due to the trimethylstannyl group at $\delta = 0.4$ ppm with the characteristic tin satellites 55 Hz apart integrating for nine protons. Ethoxybenzaldehyde **17** was obtained by the Williamson reaction of **16** with ethyl iodide in acetone and anhydrous potassium carbonate (Scheme 2). The iodination of **16** with iodine monochloride in dichloromethane afforded the monoiodo aldehyde **18** (75% yield), which was converted to ethoxy aldehyde **19** in 90% yield. Trimethylstannyl aldehyde **20**, synthesized from **19** using the tin exchange procedure of Azizian et al.,[16] was obtained as white crystals. The crucial step in this synthetic methodology, however, was to radioiodinate the tin precursor **4** via a radioiodine−tin exchange reaction. Based on the successful iododestannylation using stable iodine, radioiododestannylation of the tin precursor was carried out using sodium [^{125}I]/[^{131}I]iodide and lactoperoxidase in the presence of small amounts of hydrogen peroxide. A representative radio/UV HPLC profile of the radioiodination mixture spiked with iodoHoechst 33342 (Figure 1) shows that nearly 90% of the radioactivity eluted at a retention time of 45 min, corresponding to the peak of iodoHoechst 33342, in chemically and radiochemically pure form.

Fluorescence spectroscopy was used to determine the equilibrium association constant (K_a) of the binding of iodoHoechst 33342 and parent dyes to calf thymus DNA. Whereas the fluorescence enhancement for the two noniodinated bis-benzimidazoles reached the maximum of nearly 900 units following the addition of increasing

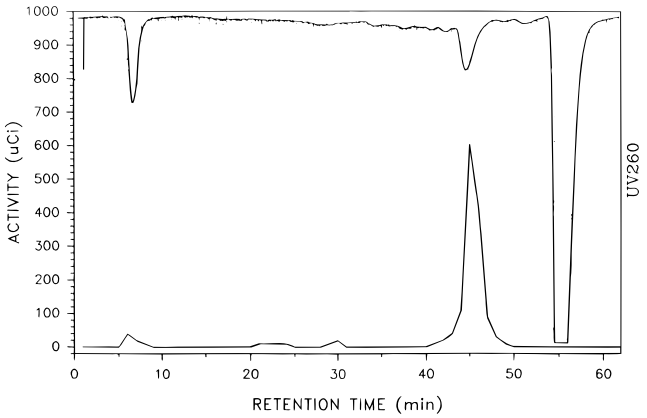


Figure 1. Radio/UV HPLC profile of the radioiododestannylation reaction mixture in the synthesis of [^{125}I]iodoHoechst 33342 spiked with stable iodoHoechst 33342: (top) UV profile and (bottom) radioactivity profile. Note the difference in retention times of the two chemicals. Lactoperoxidase and buffer salts eluted out in void volume.

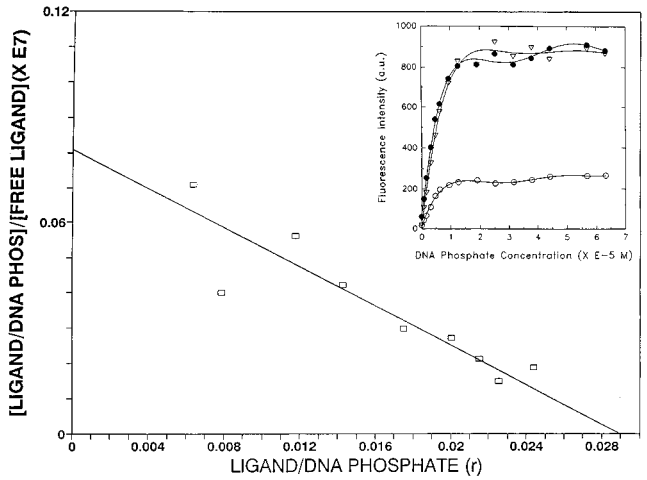


Figure 2. Scatchard plot of binding data for iodoHoechst 33342 and calf thymus DNA obtained from figure shown in insert. Data were plotted as ratio of bound/free (abscissa) versus bound fraction (ordinate) and yielded straight line with slope (=association constant, K_a) of 2.57×10^7 M^{-1}. Insert: Fluorescence titration curves for bis-benzimidazoles with increasing calf thymus DNA concentration in phosphate-buffered saline (pH 7.4). Fluorescence maximum for all dyes was achieved at a DNA concentration of 20 μM. Note that enhancement in fluorescence for noniodinated dyes was identical and nearly 3 times that observed for iodoHoechst 33342: (○) iodoHoechst 33342, (●) Hoechst 33342, and (▽) Hoechst 33258. Ligand concentration was 1.69×10^{-7} M.

amounts of DNA (Figure 2, insert), the corresponding increase for the iodoHoechst 33342 was only 240 units, although the saturation concentration of DNA was the same (20 mM). The low fluorescence quantum yield (~one-third) observed for iodoHoechst 33342 compared to noniodinated Hoechst 33258 and Hoechst 33342 appears to be due to the quenching effects of the iodine atom present on this molecule. A plot of bound/free ratio of ligand (abscissa) versus bound ligand (ordinate) furnished a straight line with a slope equal to $-K_a$, indicating that this ligand binds to calf thymus DNA by only one mode of binding interaction (Figure 2). The results summarized in Table 1 show that the K_a for all three ligands is similar ($2.6-2.8 \times 10^7$ M^{-1}); the presence of the bulky iodine on Hoechst 33342 does not affect the strength of binding of this molecule within

Table 1. Summary of Binding Studies

chemical	K_a ($\times 10^7$ M^{-1})	n[a]	nucleotide/ ligand
Hoechst 33258	2.64	0.0275	36
Hoechst 33342	2.84	0.0282	36
iodoHoechst 33342	2.57	0.0280	36

[a] Number of ligands per nucleotide phosphate.

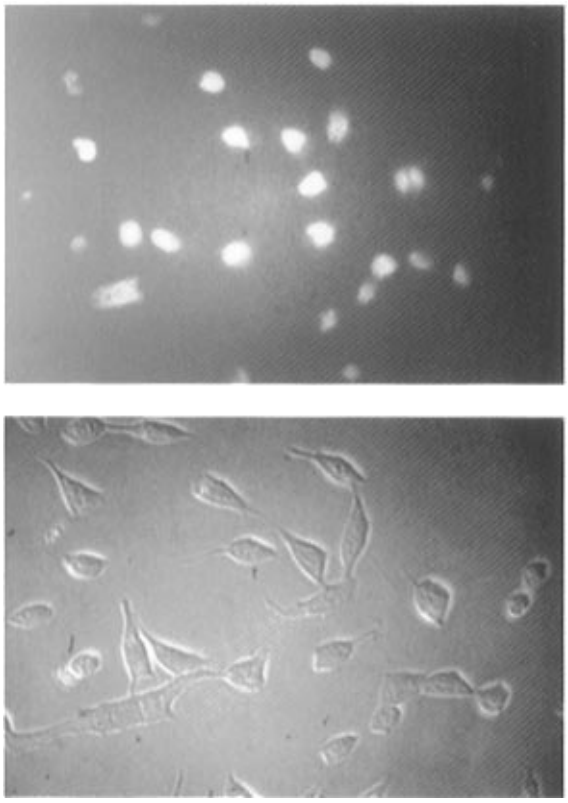


Figure 3. Top: Fluorescence micrograph of viable V79 cells stained with [^{127}I]iodoHoechst 33342. Brightly lit areas in the center are nuclei stained with dye. Bottom: Photomicrograph of fluorescence image processed and converted by Spex cation measurement system indicating that cytoplasm is devoid of dye.

the DNA minor groove. The fluorescence microscopy data (Figure 3) of viable V79 cells stained with iodoHoechst 33342 demonstrated cellular uptake and localization within the nucleus. Together with the DNA-binding data, these results indicate that iodoHoechst 33342 permeates live mammalian cells and binds avidly to their DNA. RadioiodoHoechst 33342 may, therefore, play an important role in examining the radiobiologic effects of radioiodine bound to the DNA minor groove. Our results with plasmid-bound ^{125}I-labeled iodoHoechst 33342, in which the effects of Auger electron emissions from within the minor groove have been examined, may be found elsewhere.[17]

Prompted by the reported antitumor activity of the parent bis-benzimidazole Hoechst 33258 (pibenzimol) against intraperitoneal murine L1210 and P388 leukemia,[12] the tumor-targeting potential of [^{125}I]iodoHoechst 33342 was assessed in LS174T tumor-bearing athymic mice 4 h after its administration (Figure 4), at which point this molecule would be expected to have cleared from the systemic circulation. Our results corroborate this expectation (0.5% ID/g in blood) and indicate that the greatest amount of the injected radioactivity was taken up by the kidneys (22% ID/g) followed by the liver

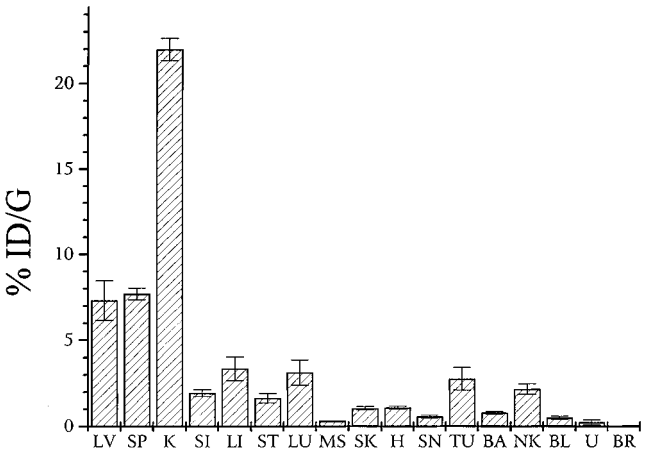


Figure 4. Biodistribution (% ID/g) of [^{125}I]iodoHoechst 33342 in LS174T tumor-bearing athymic mice following intravenous injection via lateral tail vein: liver (LV), spleen (SP), kidney (K), small intestine (SI), large intestine (LI), stomach (ST), lungs (LU), muscle (MS), skeleton (SK), heart (H), skin (SN), tumor (TU), bladder (BA), neck contents (NK), blood (BL), urine (U), and brain (BR).

and spleen (~7% each), while the subcutaneous tumor had 3−4% ID/g. This led to tumor-to-blood ratios of 6−8 and a tumor-to-muscle ratio of ~10. The tumor-to-nontumor ratios for most of the organs remained above 1 except for kidneys, liver, and spleen. The radiochemical did not cross the blood−brain barrier (brain uptake 0.04%). Interestingly the activity in neck contents was only ~2% ID/g, reflecting the *in vivo* stability of this radiochemical due to the *o*-iodoethoxy moiety. If this chemical can be conjugated to a suitable carrier such as an antibody, it may be targeted to tumors effectively. In view of the fact that the parent Hoechst 33258 is already undergoing clinical trials,[12] it is worthwhile to examine the antitumor activity of the newly synthesized iodoHoechst 33342. The pursuit of such studies is also justified since the substitution by stable iodine (^{127}I) may sometimes lead to improvements in the biologic action of certain drugs, as exemplified by 4′-iodo-4′-deoxydoxorubicin, a chemical that has shown greater anticancer activity and less cardiac toxicity than doxorubicin.[18]

Experimental Section

The reagents and chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI). Calf thymus DNA and lactoperoxidase (EC 1.11.1.7) were purchased from Sigma Chemical Co. (St. Louis, MO). Carrier-free sodium [^{125}I]iodide (0.63 TBq/mg) and sodium [^{131}I]iodide (0.44 TBq/mg) were obtained from Amersham Corp. (Arlington Heights, IL). Elemental analyses were carried out by Atlantic Microlabs (Atlanta, GA). Melting points were determined on a Fisher-Johns melting point apparatus (Pittsburgh, PA) and are uncorrected. NMR spectra were obtained on either a Varian XL-300 or a Varian XL-500 instrument. Electron impact (EI) and direct chemical ionization fast atom bombardment (DCI/FAB; H) mass spectra were recorded on a ZAB 7070 mass spectrometer at the University of Illinois, Urbana Campaigna. The UV spectra were recorded on a Hitachi UV/vis spectrophotometer (Lambda 3B), and fluorescence spectra were obtained from a Perkin-Elmer fluorescence spectrophotometer (LS-50B). TLC analyses were carried out on either silica gel plates (Baker-flex, IB2-F; J. T. Baker Inc., Phillipsburg, NJ) or reversed phase TLC plates (Uniplate RPSF; Analtech, Newark, DE). Silica gel plates were run in a mobile phase consisting of methanol: ethanol:tri-*n*-butylamine (20:10:0.3), whereas the reversed phase plates were run in 40% methanol in aqueous ammonia (2.2%). The plates were examined under UV and exposed to X-ray film, and autoradiographs were developed for the visualization of radioactivity.

2-Nitro-5-(1-methyl-4-piperazinyl)aniline (8). This compound was prepared per Loewe et al.:[15] analysis by silica gel TLC plates revealed a single spot, $R_f = 0.48$; mp 153−154 °C (lit.[15] mp 155 °C); ^{1}H NMR (DMSO-d_6) δ 2.18 (s, 3 H, N-CH_3), 2.375 (m, 4 H, piperazinyl methylenes), 3.26 (m, 4 H, piperazinyl methylenes), 6.18 (d, $J = 3$ Hz, 1 H, H-6), 6.38 (dd, $J = 12$, 3 Hz, 1 H, H-4), 7.24 (bs, 2 H, -NH_2), 7.78 (d, $J = 12$ Hz, 1 H, H-3). Anal. ($C_{11}H_{16}N_4O_2$) C, H, N.

A residue from acetic acid treatment was purified on a preparative TLC run in 0.5% methanol in dichloromethane, and **9** was isolated: mp 105−107 °C; ^{1}H NMR (DMSO-d_6) δ 4.50 (s, 3 H, N-CH_3), 4.59 (s, 3 H, N-CH_3), 7.65−7.70 (m, 4 H, H-4, H-6, -NH_2), 9.50 (d, $J = 12$ Hz, 1 H, H-3).

5-(1-Methyl-4-piperazinyl)-1,2-diaminobenzene (10). A solution of **8** in methanol (4 g, 17 mmol) was hydrogenated in a Parr reactor. The dry gray product (3.95 g) was crystallized in aqueous ethanol: mp 65−67 °C; ^{1}H NMR (DMSO-d_6) δ 2.18 (s, 3 H, N-CH_3), 2.38 (m, 4 H, piperazinyl methylenes), 2.83 (m, 4 H, piperazinyl methylenes), 3.96 (bs, 2 H, -NH_2), 4.38 (bs, 2 H, -NH_2), 6.02 (dd, $J = 3$, 12 Hz, 1 H, H-5), 6.21 (d, $J = 3$ Hz, 1 H, H-3), 6.40 (d, $J = 12$ Hz, 1 H, H-6). Anal. ($C_{11}H_{18}N_4$) C, H, N.

4-(Trifluoroacetamido)-3-nitrobenzonitrile (12). To a stirred and chilled (−20 °C) solution of amino nitrile **11** in 50 mL of dry dichloromethane (2.283 g, 14 mmol) under an argon atmosphere was added 3.54 mL of dry pyridine (42 mmol) followed by 4.487 mL of trifluoroacetic anhydride (31 mmol) added dropwise over 10 min. The solution was stirred for 30 min and the reaction quenched by addition of 1.3 mL of methanol (31 mmol) while still at −20 °C. The reaction mixture was allowed to warm to room temperature, and the solvent was evaporated to dryness. The residue was stirred with 50 mL of dry diethyl ether and filtered, and the pyridinium-trifluoroacetate-containing residue thoroughly washed with ether. The combined filtrate was evaporated and then coevaporated with toluene to remove all pyridine. The pale fluffy product (yield 2.1 g) was crystallized in benzene−hexane, and colorless needles were obtained, mp 96−98 °C.

Ethyl 4-(Trifluoroacetamido)-3-nitrobenzimidate Hydrochloride (13). To a solution of the trifluoro derivative **12** in 50 mL of anhydrous ether (3 g, 105 mmol) were added 10 mL of dry ethanol and 4A molecular sieves (15−20). Through this solution at −20 °C was bubbled dry hydrogen chloride gently until saturation (2 h), and the solution was kept in the freezer overnight. The crystallized imidate **13** was filtered and thoroughly washed with chilled ether followed by hexane and dried *in vacuo* (2.75 g yield). The crude amidate was used as such for the cyclization reaction described below: $_1$H NMR δ 1.5 (t, $J = 6$ Hz, 3 H, -CH_3), 4.65 (q, $J = 6$ Hz, 2 H, -CH_2-), 7.95 (d, $J = 12$ Hz, 1 H, H-5), 8.08 (dd, $J = 3$, 12 Hz, 1 H, H-6), 8.78 (d, $J = 3$ Hz, 1 H, H-2).

2-(4-Amino-3-nitrophenyl)-6-(1-methyl-4-piperazinyl)-benzimidazole (14). Acetic acid (20 mL) in an RB flask was purged with dry argon for 15 min. The diamine **10** (1.01 g, 4.9 mmol) was added followed by the imidate **13** (1.46 g, 4.68 mmol). The stirred solution was heated to 55 °C in an oil bath for 12 h with intermittent addition of 0.55 g of imidate **13** in three portions. Acetic acid was evaporated, the crude material was purified by ammonia−acetic acid treatment, and the bright-red nitro amine **14** was filtered and washed thoroughly with 2% methanolic ether followed by ether and dried *in vacuo* (yield 1.171 g): TLC $R_f = 0.35$; mp 182−183 °C (lit.[15] mp 183−185 °C); ^{1}H NMR δ 2.2 (s, 3 H, N-CH_3), 2.42 (m, 4 H, piperazinyl methylenes), 3.15 (m, 4 H, piperazinyl methylenes), 6.9 (d, $J = 12$, 1 H, H-6), 7.01 (dd, $J = 12$, 14 Hz, 1 H, H-7′), 7.15 (d, $J = 12$ Hz, 1 H, H-6′), 7.38 (dd, $J = 12$, 16 Hz, 1 H, H-5), 7.78 (bs, 2 H, D_2O exchangeable, -NH_2), 8.15 (d, $J = 12$ Hz, 1 H, H-4′), 8.75 (d, $J = 12$ Hz, 1 H, H-3), 13.01 (bs, 1 H, imidazole N-H). Anal. ($C_{18}H_{20}N_6O_2 \cdot H_2O$) C, H, N.

2-(3,4-Diaminophenyl)-6-(1-methyl-4-piperazinyl)-benzimidazole (15). The nitro amine **14** (1.1 g) was reduced as above, and a dark gray solid (**15**) was obtained: mp 264−266 °C (lit.[15] mp 268 °C); ^{1}H NMR δ 2.22 (s, 3 H, N-CH_3),

3.04−3.18 (m, 8 H, piperazinyl methylenes), 4.62 (bs, 2 H, D_2O exchangeable, -NH_2), 4.90 (bs, 2 H, D_2O exchangeable, -NH_2), 6.58 (d, J = 12.5 Hz, 1 H, H-6), 6.84 (dd, J = 12.5, 3 Hz, 1 H, H-5), 6.93 (bs, 1 H, H-3), 7.15 (dd, J = 3, 12.5 Hz, 1 H, H-6′), 7.28 (dd, J = 12.5, 14 Hz, 1 H, H-7′), 7.33 (bd, J = 12.5 Hz, 1 H, H-4′). Anal. ($C_{18}H_{22}N_6 \cdot H_2O$) C, H, N.

4-Ethoxybenzaldehyde (17). Anhydrous potassium carbonate was added to a stirred solution of hydroxybenzaldehyde **16** (1 g, 8.2 mmol) and iodoethane (1.56 g, 10 mmol) in 25 mL of dry acetone. The mixture was filtered, and the residue was thoroughly washed with acetone. The combined acetone solution was evaporated to dryness; the residue was extracted with chloroform, worked up as usual, and purified on a short silica gel column to obtain an oil (yield 2.1 g): ^{1}H NMR δ 1.38 (t, J = 6 Hz, 3 H, -CH_3), 4.15 (q, J = 6 Hz, 2 H, -CH_2-), 7.13 (d, J = 12 Hz, 2 H, H-3, H-5), 7.88 (d, J = 12 Hz, 2 H, H-2, H-6). Anal. ($C_9H_{10}O_2$) C, H.

3-Iodo-4-hydroxybenzaldehyde (18). To a stirred solution of hydroxybenzaldehyde **16** (1.22 g, 10 mmol) in 20 mL of dichloromethane at ambient temperature was added a solution of iodine monochloride (1.8 g, 11 mmol) in 1 mL of acetic acid (17 h). The product was worked up as usual, and the residue was purified on a silica gel column (yield 1.8 g): mp 128−130 °C; ^{1}H NMR δ 7.15 (d, J = 13 Hz, 1 H, H-5), 7.89 (d, J = 13 Hz, 1 H, H-6), 8.25 (bs, 1 H, H-2), 9.68 (s, 1 H, -CHO). Anal. ($C_7H_5O_2I$) C, H, I.

3-Iodo-4-ethoxybenzaldehyde (19). The above procedure was followed with iodobenzaldehyde **18** (1 g, 4 mmol), and the crude product was purified on a silica gel column to afford white crystals (yield 0.45 g): mp 81 °C; ^{1}H NMR δ 1.38 (t, J = 6 Hz, 3 H, -CH_3), 4.21 (q, J = 6 Hz, 2 H, -CH_2-), 7.20 (d, J = 12 Hz, 1 H, H-5), 7.91 (d, J = 12 Hz, 1 H, H-6), 8.30 (bs, 1 H, H-2), 9.82 (s, 1 H, -CHO); EI-HRMS calcd for $C_9H_9O_2I$ 275.964 732, found 275.964 670.

3-(Trimethylstannyl)-4-ethoxybenzaldehyde (20). A solution of iodo aldehyde **19** (138 mg, 0.5 mmol), hexamethylditin (250 mg, 0.76 mmol), and tetrakis(triphenylphosphine)-palladium(0) (25 mg, 0.022 mmol) in 15 mL of anhydrous dioxane was heated under gentle reflux in a nitrogen atmosphere (1 h). The product was worked up and purified on a silica gel column conditioned in hexane. The chemical eluted in hexane−2% ethyl acetate (yield 130 mg): mp 104 °C; ^{1}H NMR δ 0.28 (s, with Sn satellites, $^2J_{Sn-CH}$ = 55 Hz, 9 H, $SnMe_3$ with tin satellites), 1.38 (t, J = 6 Hz, 3 H, -CH_3), 4.18 (q, J = 6 Hz, 2 H, -CH_2-), 7.12 (d, J = 12 Hz, 1 H, H-5), 7.90 (s, 1 H, H-2), 7.95 (d, J = 12 Hz, 1 H, H-6), 9.90 (s, 1 H, -CHO); ^{119}Sn NMR ($CDCl_3$/Me_3Sn) δ −25.2; EI-HRMS calcd for $C_{12}H_{18}O_2Sn$ 310.032 424, found 310.032 049.

2-[2-(3-Iodo-4-ethoxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazinyl)benzimidazole (IodoHoechst 33342, 3). A suspension of diaminobenzimidazole **15** (250 mg, 0.78 mmol) and iodo aldehyde **19** (218 mg, 0.79 mmol) in 20 mL of nitrobenzene containing 10−15 molecular sieves was stirred at 60 °C for 0.5 h and then at 98−100 °C (36 h). Initially a clear dark-red solution was formed and then, by the end of 24 h, a yellow suspension. A small amount of the aldehyde **19** (20 mg) was added and the reaction continued for 12 h. The yellow precipitate was filtered and washed thoroughly with benzene, acetone, and hexane. The crude material was purified on a silica gel column preconditioned in ethyl acetate. The product was eluted in a mixture of 15% methanol and 0.4% tri-*n*-butylamine in ethyl acetate. Following evaporation, the residue was dissolved in 2 mL of methanol and cooled in an ice bath. A methanolic solution of hydrogen chloride (2 M) was added, and the precipitate was filtered and washed with methanol:acetone, 1:1, followed by acetone and dried *in vacuo*: mp 233 °C dec; ^{1}H NMR δ 1.42 (t, J = 6 Hz, 3 H, -CH_3), 2.21 (s, 3 H, N-CH_3), 3.10−3.50 (m, 8 H, H-2‴, H-3‴, H-5‴, H-6‴), 4.20 (q, J = 6 Hz, 2 H, -CH_2- of ethoxy), 6.9−7.0 (m, 2 H, H-7″, H-6), 7.20 (d, J = 12 Hz, 1 H, H-7′), 7.40 (bd, J = 12 Hz, 1 H, H-6″), 7.70 (bd, J = 12 Hz, 1 H, H-6′), 8.05 (bs, 1 H, H-4″), 8.20 (d, J = 12 Hz, 1 H, H-5), 8.30 (bs, 1 H, H-4′), 8.64 (s, 1 H, H-3); FAB-HRMS calcd for $C_{27}H_{28}N_6OI$ (M^+ + H) 579.136 937, found 579.137 700. Anal. ($C_{27}H_{27}N_6OI \cdot H_2O$) C, H, N.

2-[2-(4-Ethoxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazinyl)benzimidazole (Hoechst 33342, 1). The above procedure was followed with ethoxy aldehyde **17**, and pure Hoechst 33342 (**1**) was isolated in 80% yield.

2-[2-[3-(Trimethylstannyl)-4-ethoxyphenyl]-6-benzimidazolyl]-6-(1-methyl-4-piperazinyl)benzimidazole ((Trimethylstannyl)Hoechst 33342, 4). The above procedure was followed with diaminobenzimidazole **15** (16.1 mg, 0.05 mmol) and stannyl aldehyde **20** (15.9 mg, 0.0507 mmol), and the crude material was purified by column chromatography (yield 21 mg): mp 203 °C dec; ^{1}H NMR δ 0.4 (s, with Sn satellites, $^2J_{Sn-CH}$ = 55 Hz, 9 H, -$SnMe_3$), 1.40 (t, J = 6 Hz, 3 H, -CH_3), 2.21 (s, 3 H, N-CH_3), 3.20−3.50 (m, 8 H, H-2‴, H-3‴, H-5‴, H-6‴), 4.10 (q, J = 6 Hz, 2 H, -CH_2- of ethoxy), 6.88−6.98 (m, 2 H, H-7″, H-6), 7.13 (d, J = 12 Hz, 1 H, H-7′), 7.45 (m, 1 H, H-6″), 7.67 (dd, J = 12, 14 Hz, 1 H, H-6′), 8.01 (t, J = 3, 14 Hz, 1 H, H-4″), 8.15−8.17 (m, 2 H, H-3, H-5), 8.30 (d, J = 14 Hz, 1 H, H-4′); FAB-HRMS calcd for $C_{30}H_{37}N_6OSn$ (M^+ + H) 617.205 084, found 617.204 200. Anal. ($C_{30}H_{37}N_6OSn$) C, H, N.

2-[2-(3-[^{125}I]Iodo-4-ethoxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazinyl)benzimidazole (Radioiodo-Hoechst 33342, 3). In a vial were placed 20 μL of a solution of 1.5 mg/mL (trimethylstannyl)Hoechst 33342 (**4**) in methanol, 2 mCi of sodium [^{125}I]iodide (or sodium [^{131}I]iodide) in 20 μL of 0.1 M sodium hydroxide, 40 μL of 0.2 M acetate buffer (pH 4.9), 1 μL of lactoperoxidase solution (4 mg/mL), and 10 μL of dilute hydrogen peroxide (30% solution diluted 500× in water). The vial was vortex-mixed and incubated for 5 min, and 10 μL of dilute hydrogen peroxide was added. After a 5-min incubation, the reaction was terminated by addition of 100 μL of 2% tri-*n*-butylamine in methanol, and the mixture was purified by HPLC on a C_{18} column. Mobile phase A was 10% methanol in potassium phosphate buffer (20 mM, pH 7.0), and phase B was 20% potassium phosphate buffer in methanol. A linear gradient from 100% A to 100% B in 30 min at a flow rate of 1 mL/min was employed. A radiochemical yield of 70−80% and radiochemical purity of 99.8% were obtained.

Equilibrium Association Constants of Hoechst 33258, Hoechst 33342, and IodoHoechst 33342. Calf thymus DNA (Sigma Chemical Co.) was dissolved in phosphate-buffered saline (PBS), pH 7.4, and the solution was filtered through a 0.22-μm filter. Based on the molecular weight (1.9×10^{12}) and the ϵ/phosphate of 6490,[19,20] the concentration of DNA was calibrated to 50 μg/mL (2.63×10^{-14} M ≡ 1.6×10^{-4} M phosphate) for an OD_{280} of 1 unit. From this stock solution, dilutions were prepared ((7.9×10^{-7}) − (6.312×10^{-5}) M). To cuvettes containing 2 mL each of these solutions was added either Hoechst 33258, Hoechst 33342, or iodoHoechst 33342 in 25 μL of methanol to a final ligand concentration of 1.69×10^{-7} M. Following mixing and a 5-min equilibration, the fluorescence intensity (FI) in each cuvette was measured in a thermostated (25 °C) fluorescence spectrophotometer (Perkin-Elmer, LS-50B) at 335 nm excitation and 450 nm emission with slit widths of 10 nm. The data were prepared as a Scatchard plot (Figure 2) in the form of FI versus DNA phosphate concentration by adapting the procedure of Peacocke[21] developed for UV absorption spectroscopy. Using the ratio of fluorescence in the presence of excess DNA to that in the absence of DNA (36.2 for Hoechst 33258, 14.63 for Hoechst 33342, and 14.85 for iodoHoechst 33342), the concentration of unbound ligand was calculated.

Cellular Localization of IodoHoechst 33342 in V79 Cells by Fluorescence Microscopy. V79 cells in monolayers were grown in tissue culture dishes containing circular cover slips in 5 mL of Dulbecco's modified minimum essential medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 000 units/L penicillin−streptomycin, and 100 mM nonessential amino acids. A solution of 0.1 mg/mL iodoHoechst was prepared in DMEM, 100 μL of this solution was added, and the culture dishes were incubated for 1 h in an incubator at 37 °C. The medium was decanted, and the cells were washed three times with 2 mL of cold PBS. The coverslips containing cells were transferred to a DSC 200 Duorak-Stotler culture chamber

(Nicholson Precision Instruments, Gaithersburg, MD) mounted on a Nikon Diaphot fluorescence microscope.

Biodistribution of [^{125}I]IodoHoechst 33342 in LS174T Tumor-Bearing Athymic Mice. Human colonic adenocarcinomas, LS174T cells (#F-11130 from ATCC), were maintained as monolayers in DMEM. To provide single-cell suspensions for injection, the cells were trypsinized, gently pipetted, and repeatedly passed through a syringe with a 22-gauge needle. Athymic Nu/Nu female mice (4−5 weeks old; Harlan Sprague−Dawley, Indianapolis, IN) were injected subcutaneously in the flank with 10^6 cells/0.1 mL of PBS/mouse. Each mouse received an intravenous injection (lateral tail vain) of 5 μCi of carrier-free radioiodoHoechst 33342 in 0.1 mL of PBS. The mice were sacrificed at 4 h, and organs were dissected for counting. Based on the injected activity and its fraction found in each organ or tissue, the percent activity per gram of tumor, organ, or tissue and the tumor-to-nontumor (T/NT) ratios were determined.

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