Cytotoxicity of an ^{125}I-Labelled DNA Ligand

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Acta Oncologica Vol. 39, No. 6, pp. 681–685, 2000

The subcellular distribution and cytotoxicity of a DNA-binding ligand [^{125}I]-Hoechst 33258 following incubation of K562 cells with the drug was investigated. The ability of a radical scavenger, dimethyl sulphoxide, to protect cells from the ^{125}I-decay induced cell death was also studied. Three different concentrations and specific activities of the drug were used to provide different ligand : DNA binding ratios. The results demonstrated a trend toward improved delivery of the ligand to the nucleus and to chromatin at higher ligand concentrations, with concomitant increased sensitivity to ^{125}I-decay induced cytotoxicity and decreased protection by dimethyl sulphoxide. This correlation of radiobiological parameters with subcellular drug distribution is consistent with the classical dogma that attributes cytotoxicity to DNA double-stranded breakage in the vicinity of the site of decay, where the high LET nature of the damage confers minimal sensitivity to radical scavenging.

Received 25 October 1999
Accepted 31 May 2000

The cytotoxicity of incorporated ^{125}I is generally attributed to the induction of DNA double-stranded breaks in the vicinity of the sites of decay (1–4). Cell culture studies with a variety of cell lines have shown that a lethal event corresponds to 30–100 decays per cell (4, 5). Moreover, studies with purified DNA have also confirmed the highly focused nature of strand breakage as a result of decay of incorporated ^{125}I, and the relative insensitivity of the breakage at the site of decay to the hydroxyl radical scavenger dimethyl sulphoxide (DMSO) (6, 7).

The implied nexus between the cytotoxicity and DNA double-stranded breaks has been called into question by recent reports that cytotoxicity is substantially more susceptible to protection by DMSO than the yield of DNA double-stranded breaks (5, 8). Although the DNA double-stranded breaks in cells can only be measured indirectly by neutral elution, the reports undoubtedly underline the need to reconsider the classic dogma, invoking DNA double-stranded breaks at the site of decay as a principle source of lethal lesions. Such a review was foreshadowed by earlier observations showing that the high LET nature of ^{125}I-induced cytotoxicity was dependent on the labelling protocol (9, 10).

While most studies on the radiobiology of ^{125}I have involved the use of DNA precursors and covalent incorporation of the radioactive atom into DNA (3–5, 8–10), ^{125}I-labelled DNA ligands have been shown to be effective in terms of both DNA double-stranded breaks and cytotoxicity (11, 12). In this study, an analogue of the synthetic DNA minor groove binding bisbenzimidazole, Hoechst 33258, which can be iodinated directly by electrophilic aromatic substitution, was used to affect nuclear localization of ^{125}I.

Hence, the aim of this study was to investigate the subcellular distribution of ^{125}I-iodoHoechst 33258 following incubation of K562 human myeloid erythroleukemic cells (13) with various concentrations of the ligand, and to determine the effect of this distribution on the sensitivity of the cells to ^{125}I-decays. The extent of protection of ^{125}I-decay induced cytotoxicity by DMSO was also examined.

MATERIAL AND METHODS

Drugs and chemicals

Hoechst 33258 was obtained from the Sigma Chemical Co., 5 mM stock solutions were prepared in 45% ethanol/55% distilled water containing 0.1% trifluoroacetic acid. The bisbenzimidazole analogue, iodoHoechst 33258, was prepared as described previously (14) and purified (> 97%) by high-performance liquid chromatography (HPLC). Purified iodoHoechst 33258 was lyophilized and stored as pellets. Carrier-free [^{125}I]-iodoHoechst 33258 was prepared as described previously (15), using carrier-free Na^{125}I (100 mCi/ml; NEN™ Life Science Products). The stable and radiolabelled forms of iodoHoechst 33258 were used to prepare stock solutions with the desired specific activities. Bisbenzimidazole stock solutions were stored at

 ISSN 0284-186X

4°C. Other chemicals were of spectroscopic grade and solvents were of HPLC grade.

Cells

Human chronic myelogenous leukemia K562 cells (13) were grown in suspension in a humidified atmosphere of 95% air and 5% CO_2 (v/v) at 37°C in RPMI-1640 medium supplemented with 20 mM HEPES, pH 7.4, 10% (v/v) fetal bovine serum (Commonwealth Serum Laboratories), 2 mM L-glutamine, and 80 μg/ml gentamicin. Cells were maintained in exponential growth phase and all experiments were performed with cells that were collected at a density of $4-7\times 10^5$ cells/ml. The doubling time for K562 cells was determined to be approximately 19 h. Cells were routinely tested for mycoplasma contamination.

Cell fractionation studies

Cells (10^6/ml) were incubated with 1 nM, 5 nM or 5 μM [^{125}I]-iodoHoechst 33258 (specific activities, 5.2 Ci/mmole for 1 nM and 5 nM and 9.5 mCi/mmole for 5 μM) for 1 h at 37°C. Following incubation, cells were separated from the medium by centrifugation at 700× g for 5 min, and the extracellular and cell-associated ^{125}I-activity was determined in gamma-counter (1282 Compugamma CS). In parallel assays, the washed cell pellets, following incubation with [^{125}I]-iodoHoechst 33258, were suspended in ice-cold 10 mM Tris-Cl, pH 7.4, 3 mM $CaCl_2$, 2 mM mg acetate (hypotonic buffer). After incubation for 5 min at 0°C, an equal volume of hypotonic buffer containing 1% Triton X-100 was added while vortexing, and the cells were sheared by passage through a 22-gauge needle. The nuclei were separated from the cytosol by centrifugation at 1000× g for 10 min. The nuclear preparations were washed in hypotonic buffer and pipetted to avoid clumping. The concentration of nuclei isolated from each sample was determined by counting with a hemocytometer and the nuclear- and cytoplasm-associated ^{125}I-activity was determined by gamma-counting. The nuclear fractions (0.14 M NaCl residual fraction, nucleoplasm and nuclear membranes and chromatin) were isolated by washing the nuclear pellet with 10 mM Tris-HCl, pH 8.3, 140 mM NaCl (isotonic buffer) and by ultra-centrifugation using previously published procedures (16). The ^{125}I-activity associated with each fraction was determined by gamma-counting. Assays were performed in triplicate.

Extraction of [^{125}I]-iodoHoechst 33258 and thin layer chromatography

Cells (10^6/ml) were incubated with 5 μM [^{125}I]-iodoHoechst 33258 (specific activity, 18.5 mCi/mmole) for 1 h at 37°C. Cell-associated and free [^{125}I]-iodoHoechst 33258 was separated as described above. The cells were resuspended in RPMI-1640 medium supplemented with 20 mM HEPES, pH 7.4, 20% (v/v) fetal bovine serum, 2 mM L-glutamine, and 80 μg/ml gentamicin (complete medium), and an equal volume of ice-cold complete medium, either with or without 20% (v/v) DMSO was added dropwise. The cells were incubated for a further 24, 48 or 72 h at approximately 3–5°C. Following incubation, cell-associated and free [^{125}I]-iodoHoechst 33258 was separated, and the drug was extracted from each fraction by addition of two volumes of acetonitrile. The samples were centrifuged at 2000× g for 10 min and aliquots of the resulting supernatant containing the drug were lyophilized to dryness. The lyophilized pellets were dissolved in 2% tri-*n*-butylamine in methanol and analyzed by thin-layer chromatography using silica gel TLC plates and a methanol : ethanol: tri-*n*-butylamine (200 : 100 : 3) solvent system. The ^{125}I-activity of the developed TLC plates was obtained by the PhosphorImager™ technique (Molecular Dynamics). This involved exposure of a PhosphorImager screen to the TLC plate for 24 h and subsequent scanning of the screen.

Clonogenic survival assays

Cloning was performed by serial dilution of an appropriate number of viable K562 cells in the alpha modification of Eagle's medium (AMEM) supplemented with 20 mM HEPES, 20% (v/v) fetal bovine serum, 2 mM L-glutamine, 80 μg/ml gentamicin and 0.33% noble agar (Difco Laboratories). Aliquots (1.5 ml) of the cell suspension were overlaid onto 2 ml of a 0.5% agar layer in 6-well microtiter plates (Greiner). The plates were incubated in a humidified atmosphere of 5% oxygen and 5% carbon dioxide in nitrogen, at 37°C for 14 days to allow for colony formation. After incubation, individual colonies containing more than 50 cells were counted. The control plating efficiency of K562 cells was estimated to be $52\% \pm 5\%$.

In the representative experiment, cells (10^6/ml) were incubated with 1 nM, 5 nM or 5 μM [^{125}I]-iodoHoechst 33258 (specific activities, 174.3 Ci/mmole, 46.0 Ci/mmole and 22.3 mCi/mmole, respectively) for 1 h at 37°C. The cells were centrifuged and resuspended in 4 ml of AMEM, supplemented with 20 mM HEPES, 20% (v/v) fetal bovine serum, 2 mM L-glutamine, and 80 μg/ml gentamicin. The cell concentration was determined by counting with a hemacytometer, and the cell-associated ^{125}I-activity was measured in a gamma-counter. The average number of ^{125}I-disintegrations per cell per hour and therefore the time interval to accumulate the required number of ^{125}I-decays per cell were calculated. An equal volume of ice-cold AMEM either with 20% (v/v) DMSO or without DMSO was added slowly to each 4 ml sample and the cells were dispensed into ampoules and stored at approximately 3–5°C to accumulate ^{125}I-decays. Following incubation, the ampoules were diluted appropriately with a dropwise addition of pre-warmed AMEM containing 20% (v/v) fetal bovine serum and six replicates were cloned. In parallel assays the appropriate control plating efficiency of K562 cells after storage at 3–5°C with and without 10% DMSO

was determined to allow calculation of the surviving fraction of K562 cells after ^{125}I-decay using the equation:

$$SF = [\text{number of colonies/number cells plated} \times (PE/100)] \quad [1]$$

where, SF is the surviving fraction and PE is the plating efficiency of the appropriate control.

Analysis of survival assay data

Data derived from the clonogenic survival assays were analyzed by non-linear regression using the following model:

$$S(D) = 1 - (1 - \exp(-D/D_o))^n \quad [2]$$

where D is the dose (decays per cell), S is the surviving fraction, D_o is the dose required to produce on average 1 lethal event per cell and n is the extrapolation number. Values for D_o and n were determined from the regression analysis. The dose modification factor (DMF) was calculated as a ratio of the corresponding D_o values.

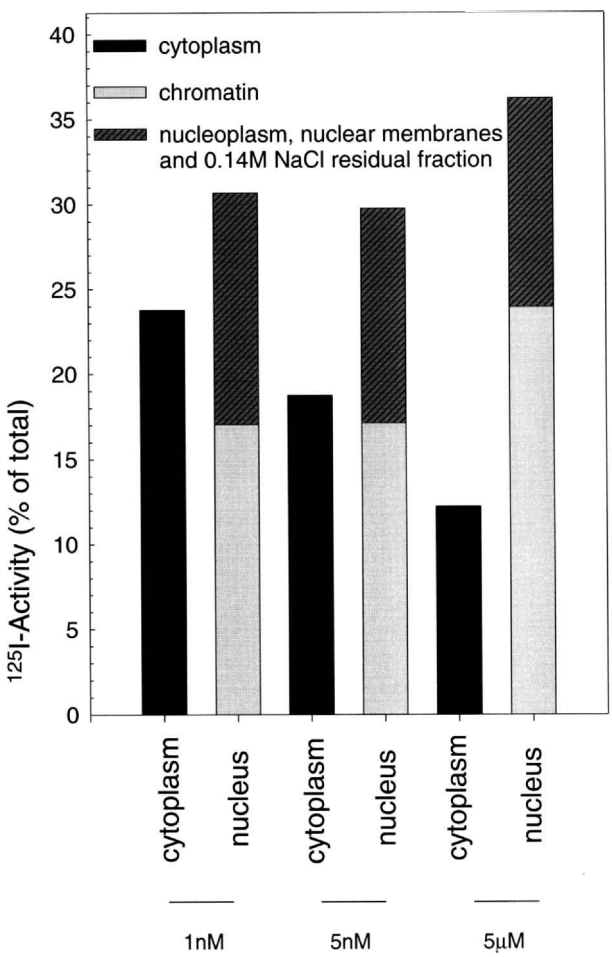

Fig. 1. Subcellular distribution of [^{125}I]-iodoHoechst 33258 in K562 cells. Cells were incubated with 1 nM, 5 nM or 5 μM [^{125}I]-iodoHoechst 33258 for 1 h at 37°C. The cells were fractionated and the ^{125}I-activity in the cytoplasm and in the indicated nuclear compartments was determined by gamma-counting.

RESULTS AND DISCUSSION

Subcellular distribution and stability of [^{125}I] iodoHoechst 33258 in K562 cells

An important consideration in the design of cytotoxicity experiments with ^{125}I-labelled DNA ligands is to ensure that the ligand-specific activity and concentration are sufficient to provide an adequate number of accumulated decays over a practical incubation period. For [^{125}I]-iodoHoechst 33258 a sufficient amount of the isotope can be delivered using ligand concentrations of just a few nM in a 10^6 cells/ml suspension. Use of low concentrations (such as 1–5 nM) may be an advantage, since it would minimize the chemical cytotoxicity of the ligand. However, at these concentrations the number of potential binding sites for the ligand exceeds the available amount of the radiolabelled drug, so the ligand could be incorporated quite heterogeneously. In contrast, at a concentration of 5 μM in a suspension of 10^6 cells/ml, the ligand is in excess compared to the number of minor groove-binding sites.

Another consideration in these experiments is to ensure that the labelled ligand is primarily localized in nuclear DNA. In this context, although the fluorescence of DNA-bound ligand provides a convenient means to investigate intracellular location, the enhanced fluorescence yield of the DNA-bound ligand compared to free ligand (17) would exaggerate the extent of nuclear localization. Thus we have used a more conventional biochemical fractionation technique, which is applicable given the relatively slow efflux of the drug from cells and nuclei (unpublished observations).

The subcellular distribution of [^{125}I]-iodoHoechst 33258 following incubation of K562 cells with the ligand is shown in Fig. 1. The results demonstrate an apparent trend toward improve delivery of the ligand to chromatin at the higher ligand concentrations. This suggests that there is a limited number of high-affinity binding sites that are not associated with DNA.

It has been noted that [^{125}I]-iodoHoechst 33258, may be prone to deiodination both in vitro and in vivo, given the presence of the orthoiodophenol moiety which is analogous to that in thyroxins. This consideration has prompted other investigators to synthesize iodoHoechst 33342 which contains the ethoxy substitution on the 4-phenyl ring (18, 19). Accordingly, we investigated the stability of [^{125}I]-iodoHoechst 33258 following incubation with K562 cells and the results are presented in Fig. 2. The data indicate that no significant deiodination occurs during incubation of the [^{125}I]-iodoHoechst 33258 with the cells for an initial 1 h at 37°C, followed by a further 24, 48 or 72 h at 3–5°C. This study confirmed that intracellular

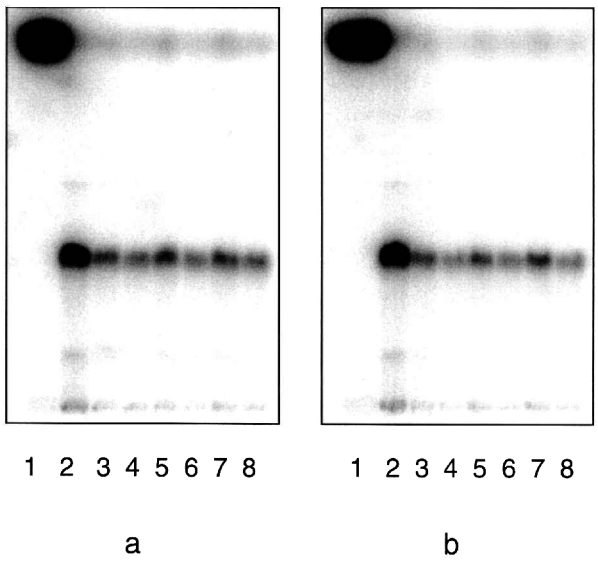

Fig. 2. Stability of [^{125}I]-iodoHoechst 33258 following incubation with K562 cells. Cells were incubated with 5 μM [^{125}I]-iodoHoechst 33258 for 1 h at 37°C. Following incubation, the cell pellets were resuspended in ice-cold media (a) with or (b) without 10% (v/v) DMSO and incubated further at 3–5°C. At the required time intervals the cell-associated and extracellular ligand was extracted with 67% acetonitrile an analyzed by thin layer chromatography. The chromatogram was visualized using a PhosphorImager technique. Lanes: 1. free ^{125}I; 2. [^{125}I]-iodoHoechst 33258 control; 3, 5, 7-cellular ligand, 4, 6, 8-extracellular ligand; 3, 4–24 h incubation; 5, 6–48 h incubation; 6, 8–72 h incubation.

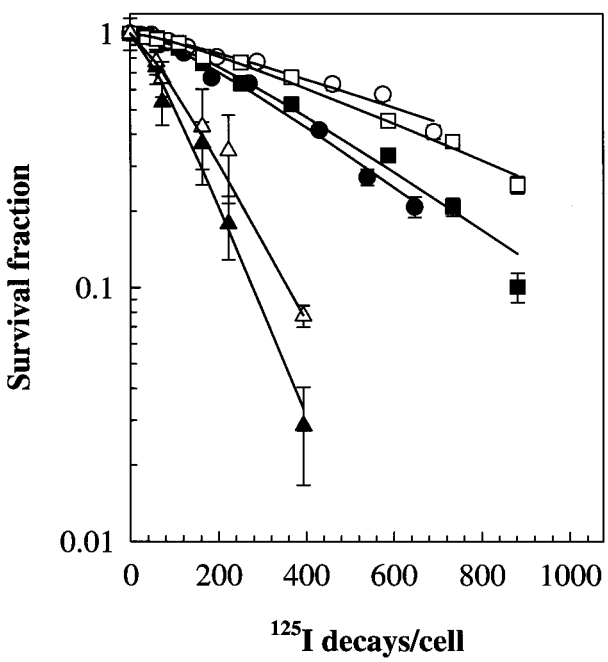

Fig. 3. Clonogenic survival of K562 cells following incubation with 1 nM (circles), 5 nM (squares) or 5 μM (triangles) [^{125}I]-iodoHoechst 33258 and accumulation of ^{125}I-decays with (filled symbols) and without (open symbols) 10% (v/v) DMSO.

Table 1

Summary of clonogenic survival of K562 cells following incubation with ^{125}I-iodoHoechst 33258 and accumulation of ^{125}I-decays with and without DMSO

[(^{125}I)-iodoHoechst 33258]	n*	D_o†		DMF‡
		− DMSO	+ DMSO	
1 nM	2	365 ± 44	676 ± 93	1.85 ± 0.09
5 nM	2	348 ± 47	529 ± 74	1.52 ± 0.12
5 μM	3	132 ± 33	163 ± 33	1.36 ± 0.11

distribution results reflect the distribution of the ligand per se, rather than some other chemical form of the ^{125}I derived from dehalogenation of the ligand. Hence, we concluded that [^{125}I]-iodoHoechst 33258 was suitable for the proposed study.

Clonogenic survival of K562 cells following accumulation of ^{125}I-decays

The survival curves for K562 cells incubated with three different concentrations of [^{125}I]-iodoHoechst 33258 with or without DMSO are shown in Fig. 3. Table 1 summarizes the radiosensitivity parameters obtained by regression analysis of survival curves from several such experiments. Two observations are evident from this data. There is an increase of radiosensitivity (or decrease of D_o) and a decrease of DMSO protection factor as the ligand concentration rises from 1 nM to 5 μM. These changes correlate with the parallel trend of improved nuclear delivery, and the higher fraction of the chromatin-associated ligand.

More generally, the modest DMF obtained for chromatin-associated ^{125}I-decay, in contrast to the recent reports (5, 8, 19), is consistent with the classical dogma which attributes cytotoxicity to DNA double-stranded breakage in the vicinity of the site of decay, where the high LET nature of the damage confers minimal sensitivity to radical scavenging.

ACKNOWLEDGEMENTS

We acknowledge the support of the Australian Institute of Nuclear Science and Engineering (AINSE). T. C. K. is the recipient of an AINSE Postgraduate Research Award.

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