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Labeling, biodistribution and evaluation of [^{125}I] gemcitabine: a potential agent for tumor diagnosis and radiotherapy

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In this study, the optimization of gemcitabine labeling with iodine-125 and its biological evaluation are described. Gemcitabine was labeled via direct electrophilic substitution using chloramine-T as an oxidizing agent. The optimum amounts of reactants were 75 μg gemcitabine, 75 μg chloramine-T and 18 MBq carrier-free Na^{125}I. The labeled gemcitabine was stable for more than 20 h. Results of the *in vivo* evaluation revealed that the new tracer, [^{125}I] gemcitabine, tends to localize in tissues with high proliferation rate with preferential accumulation in cancerous tissues. Imaging should be carried at 2-h postinjection. The *in vitro* cell growth inhibition assay showed that the effect of [^{125}I] gemcitabine was stronger than the effect of tenfold cold gemcitabine, which strongly suggested that its cytotoxicity was mainly due to radiotoxicity rather than chemotherapeutic activity. The binding assay revealed that [^{125}I] gemcitabine uptake by the Ehrlich cells was high and that it bound well to DNA where the decay of the radionuclide introduced lethal irreversible double-strand breaks.

Keywords: gemcitabine; Auger-electron emitters; iodine-125; tumor targeting; radionuclides therapy

Introduction

Auger-electron emitters such as ^{125}I are promising agents for cancer diagnosis[1] and therapy assuming that the uptake of the radiopharmaceutical by tumor cells exceeds that of normal dividing cells. If these agents can be placed intracellularly in cancer cells, especially in close proximity to (or within) DNA, their decay would deliver a radiation dose introducing highly toxic double-strand breaks in their genomes. In addition to that the process of ^{125}I decay by electron capture leaves the resulting daughter nuclide ^{125}Te with a mean net charge of +8 interfering with the DNA repair process.[2] In contrast, toxicity is minimal when decay occurs on the cell membranes or extracellular sites.[3] Gemcitabine is an antimetabolite nucleoside analogue that exhibits antineoplastic activity. It is widely used as first-line treatment of patients with metastatic breast cancer, locally advanced (Stage IIIA or IIIB) or metastatic (Stage IV) non-small cell lung cancer and as first-line treatment for patients with adenocarcinoma of the pancreas.[4] The cytotoxic effect of gemcitabine is attributed to a combination of two actions of the diphosphate and the triphosphate nucleosides.[5] Gemcitabine blocks ribonucleic reductase, an enzyme that converts the nucleotides into their deoxy derivatives. In addition, DNA synthesis is further inhibited as gemcitabine blocks the incorporation of thymidine nucleotide into DNA. In CEM T lymphoblastoid cells, gemcitabine induces internucleosomal DNA fragmentation, one of the characteristics of programmed cell death.

In this study, gemcitabine was labeled with ^{125}I and the parameters affecting this labeling reaction were investigated to select the optimum conditions required to produce high labeling yield with high purity. Biological evaluation of the labeled gemcitabine was carried out *in vivo* in normal and tumorized mice. *In vitro* cell growth tests and a DNA binding assay were carried out.

Results and discussion

Iodination of gemcitabine was performed using chloramine-T as an oxidizing agent. The iodinium ion undergoes electrophilic substitution to the heterocyclic ring of gemcitabine, which is activated by the ortho-directing *N*-glucouronid group as in Figure 1. Structure elucidation using ^{1}HNMR and mass spectroscopy confirmed the proposed structure of [^{125}I] gemcitabine. Factors affecting the labeling yield are studied and the results are presented in the following section.

Effect of gemcitabine amount

The quantity of gemcitabine used during this study varied between 25 and 150 μg. The data presented in Figure 3 clearly show that the labeling yield increased as the substrate concentration increased depending on the molar ratio of gemcitabine to iodinium ion. The optimum gemcitabine

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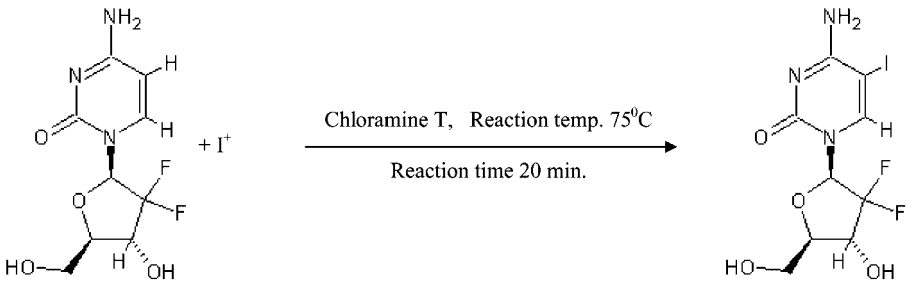

Figure 1. Gemcitabine-labeling reaction with $^{125}I^{+}$.

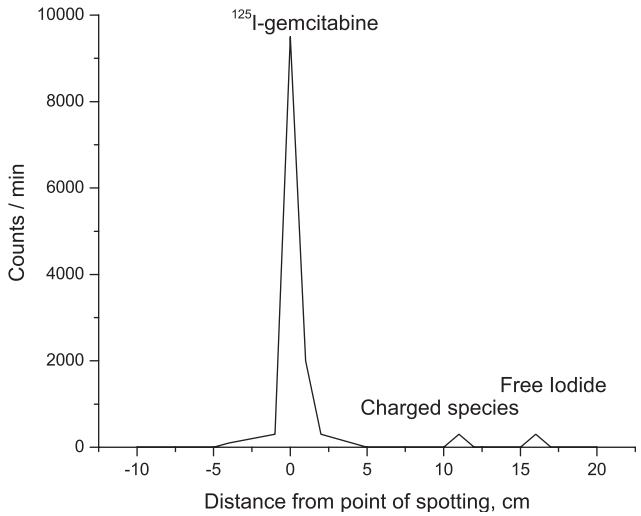

Figure 2. Paper electrophoresis pattern of the radioiodinated gemcitabine ([^{125}I] gemcitabine). Conditions: cellulose acetate strips moistened by 0.05 M phosphate buffer of pH 7, 5 µl of sample set on the strip, standing time: 1.5 h, applied voltage at 300 V.

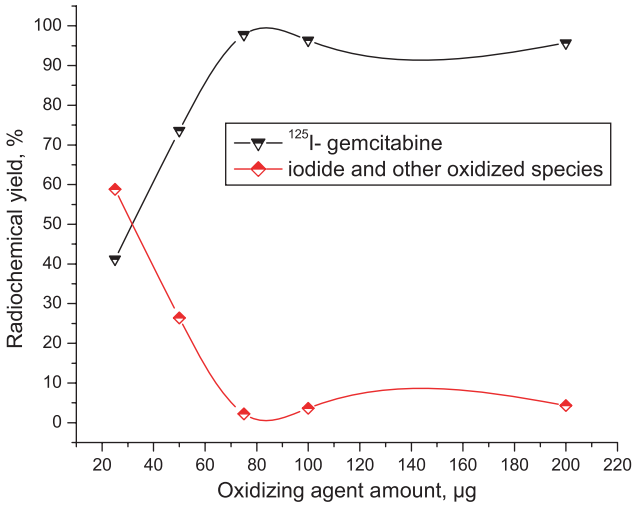

Figure 3. Percent labeling yield of [^{125}I] gemcitabine as a function of oxidizing agent content. Reaction conditions: 75 µg of gemcitabine, 100 µl of 0.5 M phosphate buffer of pH 6, 18 MBq of carrier-free Na^{125}I, X µg of chloramine-T, reaction temperature 75°C, reaction time 20 min, 100 µl of 0.2 N $Na_2S_2O_5$ solution was added.

concentration that gave the highest radiochemical yield, at the experimental conditions, was found to be 75 µg.

Effect of chloramine-T amount

The influence of chloramine-T concentration on the radioiodination of gemcitabine was studied at pH 6. The chloramine-T concentration ranged from 25 to 200 µg. The results of this study are shown in Figure 4. A high radiochemical yield of [^{125}I] gemcitabine (97.8%) was achieved by using 75 µg of chloramine-T. At a concentration of 25 µg of chloramine-T, the yield of [^{125}I] gemcitabine was low (41.2%) and this may be attributed to the insufficiency of chloramine-T to oxidize all iodide ions to iodinium ions, which are the reactive ions.[6] A slight decrease in the radiochemical yield of [^{125}I] gemcitabine was observed when increasing chloramine-T concentration above 75 µg indicating that an excess of chloramine-T is undesirable thus avoiding non-reacted oxidized species that interfere with the labeling reaction.

Effect of pH

The pH of the reaction medium was found to be a critical factor affecting the radiochemical yield of [^{125}I] gemcitabine. The data of this study are presented in Figure 6. At pH 2, the yield was very poor and did not exceed 50%. At pH 6, the yield was high (97.8%). This result was found to be in good agreement with Korde *et al.* on the labeling of UdR.[7] At alkaline pH value equal to 10, the predominant product was the oxidized species that exceeded 52%.

Effect of reaction time

The radiochemical yield of [^{125}I] gemcitabine was determined at different time intervals ranging from 5 to 60 min. The obtained results are illustrated in Figure 5. It is clear from the figure that the radiochemical yield is significantly increased when increasing the reaction time from 5 to 20 min. Increasing the reaction time to 60 min caused no significant change in the radiochemical yield.

Effect of reaction temperature

The radioiodination of gemcitabine with ^{125}I using chloramine-T as an oxidizing agent was found to be temperature dependent, as is clear from Figure 7. The reaction proceeded slowly at room temperature and the yield of [^{125}I] gemcitabine was found to be $<$55% after 20-min reaction time. By increasing the reaction temperature to 75°C, the percent of the labeled compound increased to 97.8%. When the reaction temperature was increased to 100°C, the yield of the labeled compound decreased to 91%, while the percent of the oxidized species increased to 9%.

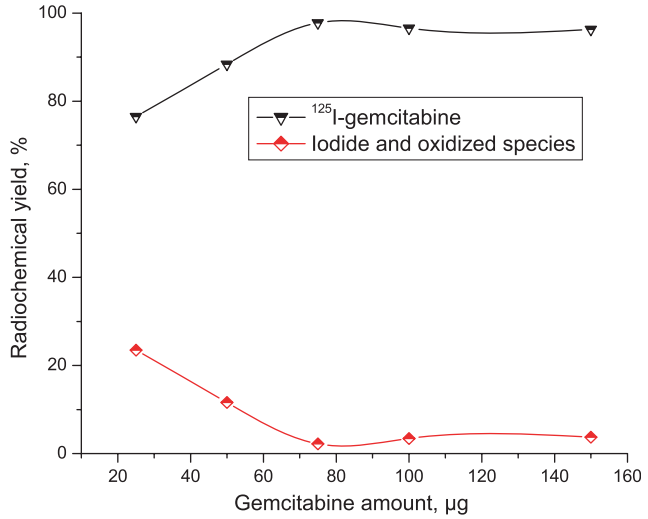

Figure 4. Percent labeling yield of [^{125}I] gemcitabine as a function of gemcitabine concentration. Reaction conditions: X µg of gemcitabine, 100 µl of 0.5 M phosphate buffer of pH 6, 18 MBq of carrier-free Na^{125}I, 75 µg of chloramine-T, reaction temperature 75°C, reaction time 20 min, 100 µl of 0.2 N $Na_2S_2O_5$ solution was added.

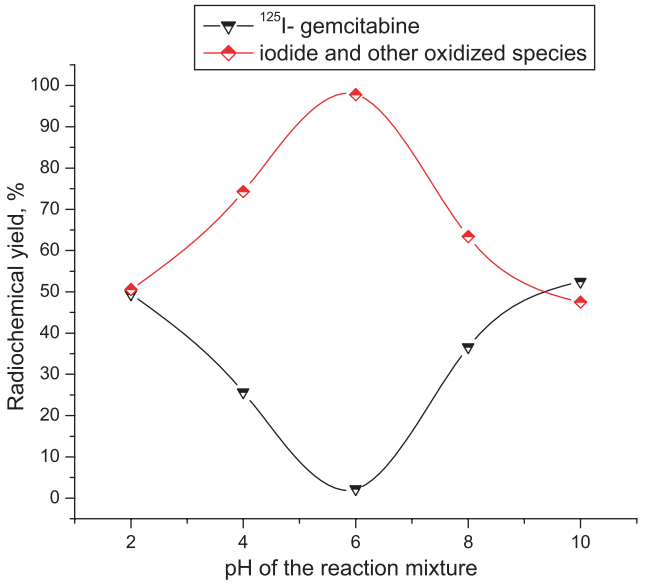

Figure 6. Percent labeling yield of [^{125}I] gemcitabine as a function of oxidizing agent content. Reaction conditions: 75 µg of gemcitabine, 100 µl of buffer of pH = X, 18 MBq of carrier-free Na^{125}I, X µg of chloramine-T, reaction temperature 75°C, reaction time 20 min, 100 µl of 0.2 N $Na_2S_2O_5$ solution was added.

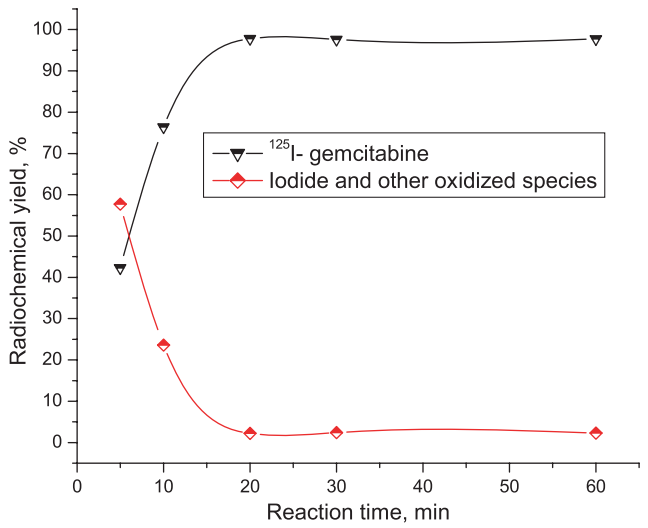

Figure 5. Percent labeling yield of [^{125}I] gemcitabine as a function of reaction time. Reaction conditions: 75 µg of gemcitabine, 100 µl of 0.5 M phosphate buffer of pH 6, 18 MBq of carrier-free Na^{125}I, 75 µg of chloramine-T, reaction temperature 75°C, reaction time X min, 100 µl of 0.2 N $Na_2S_2O_5$ solution was added.

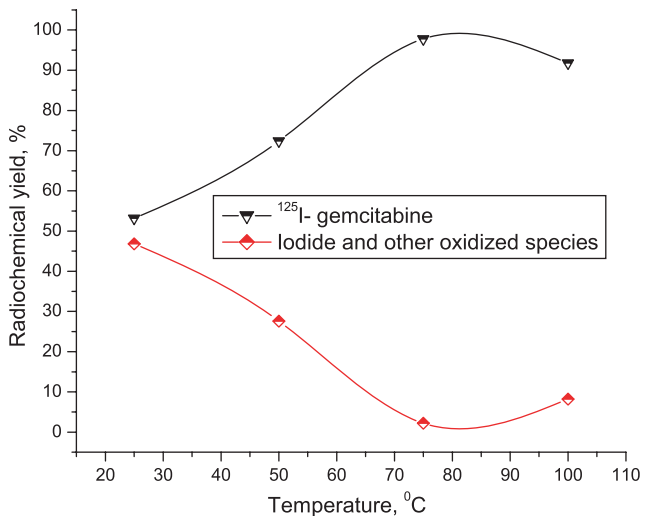

Figure 7. Percent labeling yield of [^{125}I] gemcitabine as a function of reaction temperature. Reaction conditions: 75 µg of gemcitabine, 100 µl of 0.5 M phosphate buffer of pH 6, 18 MBq of carrier-free Na^{125}I, 75 µg of chloramine-T, reaction temperature X°C, reaction time 20 min, 100 µl of 0.2 N $Na_2S_2O_5$ solution was added.

On table lifetime

[^{125}I] gemcitabine prepared by direct iodination using chloramine-T as an oxidizing agent was found to be stable for more than 20 h. The results are given in Table 1.

Biodistribution studies

Biodistribution studies of [^{125}I] gemcitabine in normal and in ascites-bearing mice showed that it was sufficiently stable and there was no sign of *in vivo* degradation as the thyroid uptake was practically insignificant. The results of this study are presented in Tables 2 and 3. The distribution pattern of the tracer in the tumor model was similar to a large extent to that in the normal mice except in the ascitic fluid. It was noted that most of the activity was eliminated via the kidneys and accumulated in the bladder after 30 min of administration reflecting gemcitabine normal metabolic pathway. [^{125}I] gemcitabine was cleared from all body organs 2-h postadministration except for the activities in bone, stomach and intestine due to high proliferation rate of these tissues. The ascitic fluid uptake of the tracer was significant at 7 and 10% after 30 and 120 min, respectively, which clearly indicated the efficacy of [^{125}I] gemcitabine to accumulate and localize specifically in tumor sites.

[^{125}I] gemcitabine binding assay

The cell membrane is impermeable to inorganic radioactive iodine. Gemcitabine effectively acts as a vector to deliver the radioisotope to the nucleus where the [^{125}I] gemcitabine is incorporated into DNA and produces the cytotoxic effect. The results presented in Figure 8 show that more than 77% of the added [^{125}I] gemcitabine was taken up by the Erlich ascite cells after 1-h incubation only. Thirty-one and 70% of the added tracer were incorporated into DNA after 1 and 24 h of incubation, respectively.

Table 1. *In vitro* stability of [^{125}I] gemcitabine as a function of time

Time (h)	% Labeled compound	% Free iodide
1	97.8 ± 0.3	2.0 ± 0.2
2	97.8 ± 0.6	2.2 ± 0.1
4	96.9 ± 0.5	3.1 ± 0.3
8	96.6 ± 0.8*	3.2 ± 0.2
24	95.1 ± 0.7*	3.4 ± 0.4

Mean ± SEM. *Significantly different from the initial value using Student *t*-test ($P < 0.01$).

In vitro cell growth inhibition assay

The MTT [3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H tetrazolium bromide] system is a simple, accurate and reproducible means of measuring the activity of living cells via mitochondrial dehydrogenase activity. The yellow tetrazolium salt (MTT) is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. The color can then be quantified by spectrophotometric means.[8] For each type of cell a linear relationship

Table 2. Percent injected dose per organs and body fluids (in % ID/organs) of [^{125}I] gemcitabine in non-tumor-bearing albino mice

	Time postinjection (min)		
Organ/body fluid	15	30	120
Blood	17.8 ± 0.5	8.4 ± 0.6	3.6 ± 0.5
Bone	6.3 ± 0.8	5.2 ± 0.7	3.9 ± 0.2
Muscle	3.1 ± 0.9	2 ± 0.2	1.2 ± 0.1
Liver	3.9 ± 0.6	4.5 ± 0.5	2.4 ± 0.3
Lung	7.4 ± 0.4	2.5 ± 0.3	1.5 ± 0.1
Heart	2.6 ± 0.3	1.9 ± 0.5	0.8 ± 0.1
Stomach	16.1 ± 0.8	13.4 ± 0.9	5.6 ± 0.6
Intestine	12.1 ± 0.9	9.2 ± 0.8	5.4 ± 0.5
Kidney	22.4 ± 0.9	6.4 ± 0.7	2.5 ± 0.4
Bladder and collected urine	0.4 ± 0.0	35.2 ± 0.7	51.4 ± 0.8
Spleen	0.5 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
Thyroid	0.9 ± 0.1	0.4 ± 0.1	0.3 ± 0.1

Mean ± SE (mean of three mice).

Table 3. Percent injected dose per organs and body fluids (in % ID/organs) of [^{125}I] gemcitabine in tumor-bearing albino mice

	Time postinjection (min)		
Organ/body fluid	15	30	120
Blood	16.4 ± 0.4	9.2 ± 0.6	3.4 ± 0.4
Bone	5.2 ± 0.7	4.9 ± 0.7	3.6 ± 0.3
Muscle	3.0 ± 0.8	1.9 ± 0.3	1.1 ± 0.2
Liver	3.6 ± 0.4	4.1 ± 0.6	2.2 ± 0.2
Lung	7.1 ± 0.6	2.4 ± 0.5	1.4 ± 0.1
Heart	2.3 ± 0.3	1.8 ± 0.3	0.6 ± 0.1
Stomach	15.6 ± 0.7	12.7 ± 0.8	5.2 ± 0.5
Intestine	11.6 ± 0.7	8.9 ± 0.8	4.9 ± 0.6
Kidney	21.6 ± 0.6	5.7 ± 0.7	2.3 ± 0.5
Bladder and collected urine	0.5 ± 0.1	33.4 ± 0.6	48.5 ± 0.7
Spleen	0.5 ± 0.2	0.3 ± 0.1	0.1 ± 0.8
Thyroid	0.8 ± 0.1	0.4 ± 0.2	0.2 ± 0.1
Ascitic fluid	4.2 ± 0.2	6.4 ± 0.3	10.4 ± 0.4

Mean ± SE (mean of three mice).

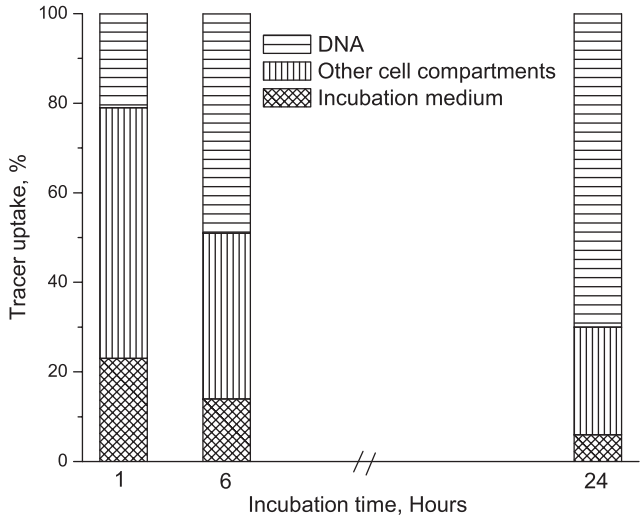

Figure 8. Radiotracer uptake by DNA, other cell compartments and the incubation media after 1, 6 and 24 h expressed as the percent of total added activity. Incubation medium: RPMI 1640 supplied with 10 ng/ml of folic acid, 10% fetal bovine serum and 100 μg/ml of gentamicin to which [^{125}I] gemcitabine (≈210 kBq) was added. The mixture was kept under 5% CO_2 at 37°C.

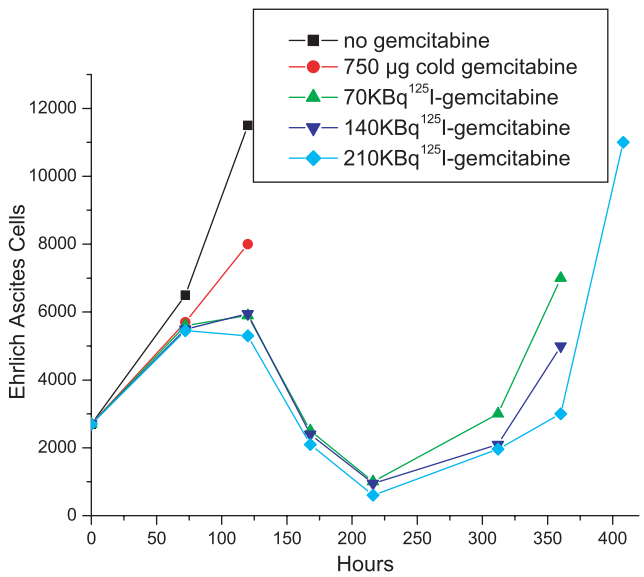

Figure 9. Cold and ^{125}I-labeled gemcitabine effects on the time course of Ehrlich ascite cell growth. Cells were treated with gemcitabine for 24 h. After the removal of gemcitabine, cells were fed with fresh media every 48 h.

between cell number and absorbance is established, enabling accurate, straightforward quantification of changes in proliferation.[9] The time course of cell growth for the Ehrlich cells is shown in Figure 9. In the absence of gemcitabine, the cell line exhibited exponential growth. [^{125}I] gemcitabine induced concentration-dependent inhibition in apparent cell growth. Cell number reached nadir values at 360 and 432 h for the [^{125}I] gemcitabine concentration of 70 and 210 kBq, respectively. However, [^{125}I] gemcitabine effects were significantly delayed relative to the time course of drug exposure. Little change in cell number was observed during the 24-h incubation of the drug (i.e. immediately following [^{125}I] gemcitabine removal, cell number was not significantly different from the untreated cell line. $P > 0.05$ for Ehrlich cell line). The effect of [^{125}I] gemcitabine was stronger than the effect of tenfold cold gemcitabine; the cell number reached nadir values at 360 and 120 h, respectively. This strongly suggested that [^{125}I] gemcitabine cytotoxicity was mainly due to radiotoxicity rather than chemotherapeutic activity.

Experimental

Gemcitabine was obtained from Ely Lilly, Egypt. Na^{125}I was purchased from Amersham, UK, as non-carrier added solution diluted with NaOH. Chloramine-T was purchased from Aldrich Chemical Co. QIAamp DNA Kit was purchased from QIAGEN Ltd., UK. Lysis buffer AL and washing buffers AW1 and AW2 were purchased from QIAGEN Ltd., UK. All other chemicals were purchased from Sigma Chemical Company, USA.

Methods

Gemcitabine labeling

In a 1 ml volume reaction vial, that could be tightly closed by a screw cap, gemcitabine (75 μg) was dissolved in 0.5 M phosphate buffer (100 μl, pH 6) followed by the addition of chloramine-T (75 μg, freshly prepared in water). Then approximately 18 MBq carrier-free Na^{125}I was added. The reaction mixture was kept in a water bath at 75°C for 20 min. The reaction was quenched by the addition of 0.2 N $Na_2S_2O_5$ (100 μl). Labeling efficiencies were determined by paper electrophoresis.

HPLC, NMR and MS analysis

HPLC analysis was carried out using a reversed-phase method as follows. An HPLC system (ThermoQuest Corp., San Jose, CA, USA) equipped with UV and radioisotope detector (Bioscan, Inc., Washington, DC, USA) was used. Analysis was carried out using a Luna C18 reversed-phase column with a 10 mm guard column. The column was eluted at 1.5 ml/min with a linear gradient starting with 100% solution A (0.5 M ammonium acetate, pH = 6.8) and ending with 60% solution B (50% methanol in deionized water). The reaction mixture was transferred to 12 × 75 mm glass tube evaporated to dryness under nitrogen. The dried residue was resuspended in the mobile phase (175 μl) and injected into the HPLC. The peak corresponding to the labeled gemcitabine was obtained at 9-min retention time. ^{1}HNMR of iodo-gemcitabine was performed using Varian Gemini spectrometer (Varian, Mountain View, CA, USA) with DMSO. ^{1}HNMR (in Me_2SO-d_6): δ 3.699 (12, 1H, dt, $J = 6.340$, 7.710), 3.821 (14, 2 H, d, $J = 6.340$), 3.983 (10, 1H, d, $J = 7.710$), 5.801 (13, 1H), 8.286 (15, 1H). Mass spectroscopy was performed using Hewlett Packard 5988 spectrometer: m/z 390 ($M^+ + 1$), 238, 221 and 87.

Electrophoresis was performed using EC 3000P-series 90 programmable (E-C apparatus corporation) power supply and chamber unit. Cellulose acetate strips were moistened with 0.05 M phosphate buffer of pH 7. Samples of 5 μl were applied at a distance of 15 cm from the cathode. Current at 300 V was applied for $1\frac{1}{2}$ h. Developed strips were removed, dried and cut into 1 cm segments. They were counted using well-type NaI scintillation γ-counter. Electrophoresis diagrams were obtained

from these counts:

$$\%\,\text{Radiochemical yield for } [^{125}\text{I}]\text{ gemcitabine} = \frac{\text{Peak activity of } [^{125}\text{I}]\text{ gemcitabine}}{\text{Total activity}} \times 100$$

Three peaks resulted, one corresponding to the free iodide that moved toward the anode with 16 cm distance at the conditions mentioned before. The second peak remained at the point of spotting and the third fraction migrated toward the anode to a lesser extent equal to 11 cm as shown in Figure 3.

On table stability

This experiment was conducted to determine the stability of [^{125}I] gemcitabine after labeling. The yield was measured at different time intervals ranging from 1 to 24 h using paper electrophoresis.

Biodistribution in mice

For animal experiments, we purified the labeled gemcitabine by HPLC. Purification was carried out using an Alltech C18 column with 10 mm guard column and we used the same solvent and detector conditions, which had been used in the analysis procedure. Normal and tumor-bearing Balb/c mice, weighing 20–25 g, were used for the biodistribution study. [^{125}I] gemcitabine (37 kBq in 0.2 ml) was injected intravenously via the tail vein. At appropriate time intervals after injection, mice were sacrificed. The organs as well as the other body parts were dissected. Activity in each organ was counted and expressed as a percent of the injected activity. Blood, bone and muscle were assumed to be 7, 10 and 40% of the total body weight, respectively.[2] Correction was made for background and physical decay during experiment.

Tumor induction

Exponentially growing Ehrlich ascite cells were adjusted to 12.5×10^6 cells/ml in phosphate-buffered saline (PBS). Cells (2.5×10^6 in 0.2 ml) were injected intraperitoneally to produce ascites.[10] Animals were maintained on normal diet for 10–15 days till tumor size reached an estimated size of 0.5–1 g.

Evaluation of [^{125}I] gemcitabine binding to Ehrlich cell line and incorporation into DNA

Sodium phosphate buffer (0.1 M, 0.1 ml, pH = 7) containing 210 kBq of [^{125}I] gemcitabine was added to the incubation media (1 ml, RPMI 1640 media supplied with 10 ng/ml of folic acid, 10% fetal bovine serum and 100 μg/ml gentamicin) containing 1×10^5 cells. The mixture was incubated for 1, 6 and 24 h under 5% CO_2 at 37°C. After incubation, the cells were centrifuged at 300*g* for 5 min and the supernatant was removed. The pellet was washed once with the incubation buffer (1 ml), and the radioactivity in the cell pellet was measured using a γ-counter. The radioactivity associated with cells was expressed as the percent of the added [^{125}I] gemcitabine. The cells were resuspended in PBS to a final volume of 200 μl. Proteinase K (20 μl) and buffer AL (200 μl) were added and mixed by pulse vortexing for 15 s. The mixture was incubated at 56°C for 10 min. Ethanol (200 μl, 96–100%) was added. The mixture was carefully applied to QIAamp spin column in 2 ml collection tube and centrifuged at 6000*g* for 1 min. Then buffer AW1 (500 μl) was added followed by centrifugation at 6000*g* for 1 min. Buffer AW2 (500 μl) was added followed by centrifugation at 20 000*g* for 1 min. Finally, pure DNA was eluted from the spin column by the addition of buffer AE (200 μl), incubation at room temperature (25°C) for 1 min and centrifugation at 6000*g* for another 1 min. The radioactivity associated with DNA was expressed as the percent of the added [^{125}I] gemcitabine.

In vitro cell growth inhibition

Ehrlich ascite cell line was grown within a humidified, 5% CO_2 incubator at 37°C. RPMI 1640 media were prepared to contain approximately 10 ng/ml of folic acid and were supplemented with 10% fetal bovine serum and gentamicin (100 μg/ml). Cell suspensions containing 5000 cells/ml were prepared and 0.1 ml of suspension (i.e. containing 500 cells) was dispensed into well of 12 96-well plates. After allowing the cells to attach for 48 h, media were aspirated through a 25 gauge needle. Following aspiration, RPMI media (100 μl) containing different amounts of gemcitabine (cold and labeled) were added. Each plate was prepared in an identical manner, with four wells used for each concentration of gemcitabine. Cells were incubated with the gemcitabine for 24 h and media were then aspirated. Each well was then washed 4 times with gemcitabine-free media (100 μl). Cell number was determined on days 1, 3, 5, 7, 9, 13, 15 and 17 using one plate per assay via the tetrazolium assay. Briefly, on the day of analysis, media were aspirated from all wells of the assay plate. Fresh media (100 μl) and exactly 25 μl of MTT solution [3-(4,5-Dimethyl-2-thiazol)-2,5-diphenyl-2H tetrazolium bromide; 5 mg/ml in PBS, pH 7.4] were added to each well, and the plate was incubated for 4.5 h at 37°C in the incubator. After incubation, 10% SDS–0.01 M hydrochloric acid (100 μl) was added to each well and the plate was incubated overnight at 37°C. Absorbance in each well was determined at 590 nm using a plate reader. The cell number was determined through the use of the standard curve that relates the absorbance to cell count (linear range 156–12 500 cells); cell number was determined for each treatment.

Conclusion

Gemcitabine has been labeled successfully with ^{125}I. The labeled compound was stable for more than 24 h at room temperature. The biodistribution pattern of the tracer showed that no free radioiodide was observed in the thyroid indicating *in vivo* stability. [^{125}I] gemcitabine accumulated specifically in the tumor sites (ascites) and was quickly cleared from most of the body organs suggesting that it could be used as a tracer for cancer diagnosis. The binding assay and the *in vitro* cell growth inhibition assay indicated that [^{125}I] gemcitabine may be a potential drug for cancer therapy. Further biological evaluation should be done to test the possibilities of achieving therapeutic levels of radiation dose in cancer cells in man and the possibilities of dose fractionation.

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