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Research Article

Preparation, analysis and biodistribution in mice of iodine-123 labelled derivatives of hypericin

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Summary

The synthesis of iodine-123 labelled mono- and di-iodo derivatives of hypericin, an hydroxylated phenanthroperylenequinone which is used for photodynamic therapy and has been shown to accumulate in several tumour types, is reported. Labelling was performed by electrophilic substitution on hypericin or iodohypericin with [^{123}I]iodide in the presence of peracetic acid and provided both mono-[^{123}I]iodohypericin and di-[^{123}I]iodohypericin in good radiochemical yields (>80%). Mono-[^{123}I]iodohypericin was obtained with a high specific activity (925 GBq/μmol) whereas di-[^{123}I]iodohypericin was obtained in low specific activity (4 GBq/μmol) due to deiodination and subsequent iodination of iodohypericin which occurred during the labelling reaction. Biodistribution studies in mice showed a slow blood clearance and extensive hepatobiliary clearance as well as faecal excretion for both compounds. They will be further evaluated with regard to their tumor seeking properties. Copyright © 2004 John Wiley & Sons, Ltd.

Key Words: mono-[^{123}I]iodohypericin; radio-LC-MS; biodistribution; iodine-123; hypericin

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Introduction

During the last two decades photodynamic detection and therapy (PDT) has been proposed and developed as a promising new modality for the diagnosis and treatment of cancer.

One of the compounds which can be used for these purposes is hypericin, a hydroxylated phenanthroperylenequinone derivative and constituent of a number of plants of the genus *Hypericum*.[1] Recent studies have shown that hypericin is an outstanding tool for the fluorescence detection of urothelial carcinoma.[2] In addition, *in vivo* studies in mice[3] have revealed that hypericin has a specific affinity for non-bladder malignant tissue as well. For instance, in a mouse P388 lymphoma tumour model a 16-fold higher concentration of hypericin was found in tumour tissue vs surrounding healthy tissue. Furthermore, hypericin has proven to be a promising agent for photodynamic therapy (PDT), both *in vitro*[4] and *in vivo*.[5]

As limited structural modifications of hypericin do not alter its tumour-seeking properties,[6] radiolabelled hypericin derivatives could be useful for the scintigraphic detection and treatment of tumours that are not easily accessible to fluorescence detection or photodynamic therapy. Therefore, we have prepared and analysed mono- and di-iodohypericin and their iodine-123 labelled homologues, and investigated their biodistribution in mice.

Results and discussion

Both non-radioactive and iodine-123 labelled iodohypericin and di-iodohypericin were synthesized by a standard electrophilic substitution with sodium iodide or sodium [^{123}I]iodide in the presence of peracetic acid on respectively hypericin and iodohypericin (Figure 1).

During the synthesis of carrier amounts of iodohypericin, di-iodohypericin was always obtained as a by-product which was separated from hypericin using medium pressure reversed phase chromatography. ^{1}H-NMR served to confirm that for mono-iodohypericin the iodine atom is located at the 2-position whereas for di-iodohypericin the iodine atoms are located at the 2- and 5-positions.

In the preparation of iodine-123 labelled iodohypericin in no-carrier-added conditions, the presence of di-iodohypericin was never observed and [^{123}I]iodohypericin was obtained with a high specific activity ($>$925 GBq/μmol, UV-detection limit for hypericin). In contrast, di-[^{123}I]iodohypericin could not be obtained with a high specific activity due to de-iodination of the iodohypericin precursor and subsequent electrophilic substitution of the formed (non-radioactive) iodide on iodohypericin resulting in the formation of carrier di-iodohypericin and a specific activity of about 4 GBq/μmol. This hypothesis was confirmed by an experiment in which mono-iodohypericin was incubated in the presence of peracetic acid, but in the absence of iodide.

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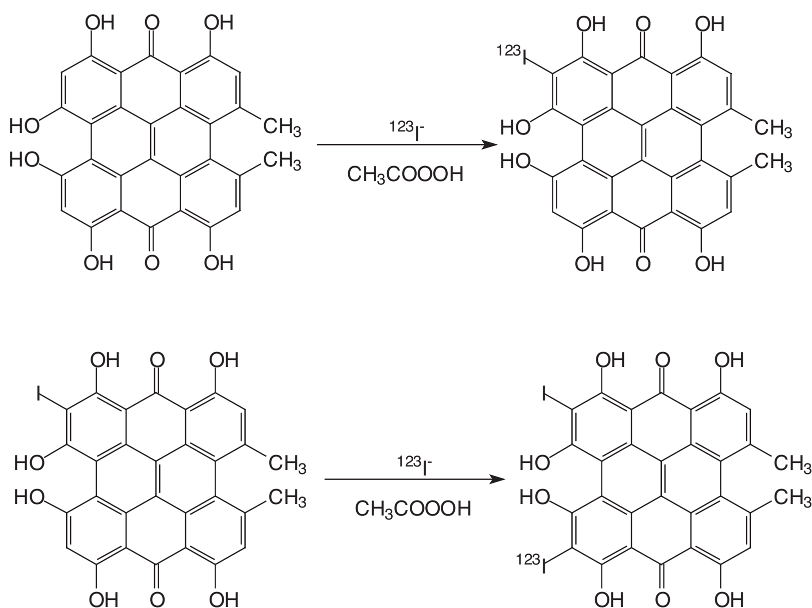

Figure 1. Synthesis of mono- and di-[^{123}I]iodohypericin

RP-HPLC analysis of the incubation mixture indeed showed the presence of di-iodohypericin (20% of the amount of starting mono-iodohypericin) and an equivalent amount of hypericin.

As expected, upon co-injection of the iodine-123 labelled compounds with the authentic non-radioactive compounds during LC-MS-HPLC analysis, the peaks observed in the radiometric channel, the UV channel (254 nm) and the corresponding single mass MS-channel had identical retention times.

Purification of both ^{123}I-labelled iodohypericin and ^{123}I-labelled di-iodo-hypericin was achieved by reversed phase HPLC, using mixtures of buffer and ethanol as the mobile phase. To obtain a preparation which is suitable for intravenous administration, the peak isolated with HPLC can be either diluted to a sufficiently low concentration of ethanol, or ethanol can be removed by evaporation with a stream of nitrogen. Re-analysis of the solution of radioiodinated hypericin prepared in this way showed that both mono- and di-[^{123}I]iodohypericin are stable as the radiochemical purity was higher than 99% 24 h after formulation.

Tumor accumulation of hypericin for PDT applications is observed after administration of relatively large amounts of hypericin (5 mg/kg).[7] In order to investigate the influence of the injected mass on the biodistribution of iodohypericin, we have studied the biodistribution of both no carrier added (nca) mono-[^{123}I]iodohypericin and carrier added (ca) mono-[^{123}I]iodohyper-icin (5 mg mono-iodohypericin per kg body mass). Due to the low solubility of

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the hypericin derivatives in water, the mono-[^{123}I]iodohypericin preparations were formulated in water/polyethylene glycol 400 (80:20, V/V) mixtures.

Table 1 shows the results of the biodistribution experiments in NMRI mice of no carrier added and carrier added mono-[^{123}I]iodohypericin at 10 min, 4 h and 24 h post injection. The biodistributions of the nca and ca formulations at the three studied time points are almost identical. Mono-[^{123}I]iodohypericin is slowly cleared from blood as about 30% of the injected dose is still present in the blood at 4 h p.i. Hypericin is known to bind extensively to both high and low density lipoproteins in plasma[1] and this may explain the slow blood clearance.

Mono-[^{123}I]iodohypericin is highly lipophilic ($\log P = 3.08 \pm 0.03$; $n = 3$) and is accordingly cleared from plasma mainly by the hepatobiliary system, resulting in high amounts accumulating in the liver and intestines at 10 min and 4 h p.i. and in a large fraction which is excreted with the faeces (about 70% of the injected dose after 24 h). Less than 10% of the injected dose is excreted in the urine. As expected for a compound with a large size (628 Da), mono-[^{123}I]iodo-hypericin does not show any substantial brain uptake. The slow blood clearance implies that potential tumor imaging with mono-[^{123}I]iodohypericin will only be possible starting at least after 12 h post injection to obtain an acceptable blood concentration.

The biodistribution of di-[^{123}I]iodohypericin, which is even more lipophilic than mono-[^{123}I]iodohypericin, shows a blood concentration which is lower at 10 min p.i., equal at 4 h p.i. and higher at 24 h p.i. compared to mono-[^{123}I]iodohypericin (Table 2). The liver uptake was higher for

Table 1. Biodistribution in mice of no carrier added (nca) mono-[^{123}I]iodohypericin and carrier added (about 5 mg/kg body mass) mono-[^{123}I]iodohypericin. Results are expressed as % of injected dose

	10 min p.i. (mean $\pm$ sd, $n = 3$)		4 h p.i. (mean $\pm$ sd, $n = 3$)		24 h p.i. (mean $\pm$ sd, $n = 3$)	
	NCA	CA	NCA	CA	NCA	CA
Urine + bladder	0.0 ± 0.0	0.1 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	3.2 ± 0.3	9.0 ± 4.6
Kidneys	2.9 ± 0.4	2.4 ± 0.3	2.4 ± 0.3	2.5 ± 0.5	1.3 ± 0.1	1.4 ± 0.1
Liver	28.7 ± 1.7	33.9 ± 4.9	23.1 ± 4.2	32.1 ± 2.2	5.2 ± 5.2	2.5 ± 0.2
Spleen	2.5 ± 0.3	1.7 ± 0.4	1.4 ± 0.1	1.9 ± 0.1	0.3 ± 0.2	0.2 ± 0.1
Lungs	5.2 ± 0.9	4.6 ± 1.0	2.4 ± 0.3	2.6 ± 0.7	0.6 ± 0.1	0.7 ± 0.1
Heart	1.1 ± 0.1	1.5 ± 0.7	0.8 ± 0.2	0.7 ± 0.2	0.1 ± 0.0	0.1 ± 0.1
Intestines	2.9 ± 0.9	3.7 ± 0.6	30.4 ± 4.3	25.4 ± 2.2	7.3 ± 2.7	6.0 ± 0.1
Stomach	0.4 ± 0.1	0.3 ± 0.1	1.3 ± 0.4	2.7 ± 1.2	1.7 ± 1.1	0.6 ± 0.1
Brain	0.1 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Cerebellum	0.2 ± 0.2	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Blood	68.9 ± 8.4	66.7 ± 12.0	29.8 ± 1.1	32.7 ± 2.1	2.1 ± 0.8	2.3 ± 1.1
Faeces	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	68.0 ± 9.8	68.5 ± 5.3
Carcass	30.7 ± 4.2	26.0 ± 4.5	20.3 ± 1.5	18.2 ± 1.3	10.5 ± 1.1	9.9 ± 1.7

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Table 2. Biodistribution in mice of di-[^{123}I]iodohypericin. Results are expressed as % of injected dose

	10 min p.i. (mean $\pm$ sd, $n = 3$)	4 h p.i. (mean $\pm$ sd, $n = 3$)	24 h p.i. (mean $\pm$ sd, $n = 3$)
Urine + bladder	0.0 $\pm$ 0.0	0.1 $\pm$ 0.0	2.8 $\pm$ 1.5
Kidneys	1.7 $\pm$ 0.3	2.1 $\pm$ 0.4	2.0 $\pm$ 0.1
Liver	49.5 $\pm$ 1.3	47.3 $\pm$ 1.9	7.1 $\pm$ 0.5
Spleen	2.2 $\pm$ 0.2	1.9 $\pm$ 1.0	0.6 $\pm$ 0.2
Lungs	1.4 $\pm$ 0.2	1.2 $\pm$ 0.4	1.5 $\pm$ 0.3
Heart	1.2 $\pm$ 0.2	1.2 $\pm$ 0.5	0.5 $\pm$ 0.1
Intestines	1.7 $\pm$ 0.1	6.4 $\pm$ 3.2	9.2 $\pm$ 1.7
Stomach	0.4 $\pm$ 0.0	0.9 $\pm$ 0.2	0.8 $\pm$ 0.2
Brain	0.1 $\pm$ 0.0	0.1 $\pm$ 0.1	0.1 $\pm$ 0.0
Cerebellum	0.1 $\pm$ 0.1	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0
Blood	30.1 $\pm$ 0.5	30.5 $\pm$ 8.1	16.1 $\pm$ 1.3
Faeces	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	42.6 $\pm$ 4.7
Carcass	25.4 $\pm$ 1.0	24.2 $\pm$ 4.1	27.2 $\pm$ 2.6

di-[^{123}I]iodohypericin at all studied time points. The slow blood clearance makes the di-[^{123}I]iodohypericin less attractive than the mono-[^{123}I]iodohypericin as a tracer agent for tumor visualization.

Experimental

Reagents and solvents were purchased from Merck, Acros, Aldrich, UCB or BDH. Iodine-123 was purchased from Bristol-Myers Squibb Pharma Belgium (Brussels, Belgium). ^{1}H-NMR spectra were recorded on a Gemini 200 MHz spectrometer (Varian, Palo Alto, CA). Chemical shifts are reported in ppm relative to TMS ($\delta = 0$). For liquid chromatography in series with mass spectrometry (LC-MS) analysis, a RP C18 column (Xterra™ MS C18 3.5 μ 2.1 mm × 50 mm, Waters, Milford MA, USA) was used which was eluted at a flow rate of 300 μl/min with an acetone-0.05 M NH_4OAc (70:30, V/V) mixture. The eluate was analysed using a UV detector (595 nm) in series with a radiometric detector (2-in NaI(Tl) detector coupled to a single channel analyser) and a time of flight mass spectrometer equipped with an orthogonal ESI probe (Micromass LCT, Manchester, UK) in negative ion mode. For accurate mass determinations, the column eluate was mixed with *S*-benzoyl-mercaptoacetyltriglycine (0.1 mg/ml), prepared as described in the literature[8], at a flow rate of 10 μl/min. Aliquots of 10 μl of a 50 μg/ml solution of the investigated compound were applied on the column. Identity confirmation between radiolabelled and authentic non-radioactive compounds was performed by co-injection of 400 kBq of the labelled compound with 10 μl of the 50 μg/ml solution of the authentic compound and comparison of the retention times of the peaks in the UV-channel and radiometric channel.

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Synthesis and preparation of the compounds

Hypericin. Hypericin (Figure 1) was synthesized as reported previously[9] (yield: 27%). ^{1}H-NMR (DMSO) δ 2.70 (s, $2 \times CH_3$), 6.50 (s, 2-*H*, 5-*H*), 7.39 (s, 9-*H*, 12-*H*). HR-MS-ES$^-$: 503.0729 Da (theoretical $[M–H]^-$: 503.0772 Da).

Mono- and di-iodohypericin. To 150 ml of a 0.5 mg/ml solution of hypericin in EtOH were successively added 25 ml 0.5 M H_3PO_4, 50 ml 0.2 M peracetic acid and 50 ml EtOH. Subsequently, 11 ml of a 1 mg/ml solution of NaI in 0.01 M NaOH was added dropwise to the reaction mixture which then was stirred for a further 30 min.

Purification of the reaction mixture was carried out using medium pressure liquid chromatography (MPLC) with acetone-0.05 M NH_4OAc (70:30, V/V) as the eluent. The MPLC system consisted of a pump (model B-688, Büchi, Flawil, Switzerland) and a 45 cm × 2.5 cm column filled with octadecylsilyl silica gel (LiChroprep®, Merck, Darmstadt, Germany). The relevant fractions containing mono-iodohypericin or di-iodohypericin were diluted with an equal volume of water and applied on a high capacity C18 SPE column (Alltech® Extract Clean, bed weight 10 g, mean particle size 50 μm, mean pore diameter 60 Å, Alltech, Deerfield IL, USA). The column was rinsed with 100 ml of water and mono- or di-iodohypericin was eluted with 100 ml of methanol. The resulting eluates were evaporated on a rotary evaporator to yield solid mono- or di-iodohypericin.

Mono-iodohypericin: ^{1}H-NMR (DMSO) δ 2.72 (s, $2 \times CH_3$), 6.63 (s, 5-*H*), 7.43 (s, 9-*H*, 12-*H*). HR-MS ES$^-$: 628.9760 Da (theoretical $[M–H]^-$: 628.9739 Da).

Di-iodohypericin: ^{1}H-NMR (DMSO) δ 2.70 (s, $2 \times CH_3$), 7.36 (s, 9-*H*, 12-*H*). HR-MS ES$^-$: 754.8654 Da (theoretical $[M–H]^-$: 754.8705 Da).

Mono-[^{123}I]iodohypericin. To a labelling vial containing 37 MBq [^{123}I]NaI in 6 μl 0.01 M NaOH were successively added: 150 μl of a 0.5 mg/ml solution of hypericin in EtOH, 25 μl 0.5 M H_3PO_4, 50 μl 0.2 M peracetic acid and 50 μl EtOH. After incubation for 30 min at room temperature, the reaction mixture was purified by RP-HPLC. The HPLC system consisted of a 4.6 mm × 250 mm RP C18 column (Xterra™ C18, mean particle size 5 μm, Waters) eluted with EtOH-0.05 M NH_4OAc (85:15, V/V) at a flow rate of 1 ml/min. Detection was carried out with a radiometric detector (3-in. NaI(Tl) crystal coupled to a single channel analyser) and a UV-detector at 595 nm. (Yield: 70–97% relative to starting ^{123}I activity.) The collected fraction containing mono-[^{123}I]iodohypericin was concentrated with a flow of nitrogen.

Di-[^{123}I]iodohypericin. Synthesis, purification and sample preparation of this compound was done as described for mono-[^{123}I]-iodohypericin, except

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for the fact that a 0.5 mg/ml solution of mono-iodohypericin in EtOH was used instead of a solution of hypericin. (Yield: 64–88% relative to starting ^{123}I activity.)

Synthesis of di-iodohypericin by incubation of iodohypericin. The procedure for this 'blank' synthesis was the same as for the synthesis of di-iodohypericin, except for the fact that no iodide was added.

Log P determination

To an Eppendorf tube was added 2 ml of 0.025 M phosphate buffer pH 7.4 and 2 ml 1-octanol. 2 kBq of the ^{123}I-labelled compound was added to the tube which was closed and vortexed for 5 min. The tube was then centrifuged at 1500*g* for 10 min. About 50 μl of octanol and 500 μl of phosphate buffer were pipetted from the tube, weighed and the radioactivity counted in a gamma counter. Log *P* was calculated as the logarithm of the ratio of the radioactivity concentration in the octanol and phosphate buffer.

Animals and biodistribution

Normal male NMRI mice (weight range 30–40 g) were used throughout this study. All aspects of the animal experiments were carried out in compliance with national and European regulations and were approved by the Animal Care and Use Committee of Katholieke Universiteit Leuven. Injection was done via a tail vein with either 15 kBq mono-[^{123}I]-iodohypericin (*no-carrier-added form*) dissolved in 0.1 ml water/polyethylene glycol 400 (80:20, V/V) or with a mixture of 15 kBq mono-[^{123}I]-iodohypericin and 5 mg/kg carrier mono-iodohypericin (*carrier-added form*) dissolved in 0.1 ml water/polyethylene glycol 400 (80:20, V/V). For the di-iodinated derivative of hypericin, preparation of a no-carrier-added formulation was not possible (see above). As a consequence, di-[^{123}I]-iodohypericin was prepared in one formulation only (cf. supra), resulting in injection of 15 kBq di-[^{123}I]-iodohypericin—and an unknown amount of carrier di-iodohypericin—dissolved in 0.1 ml water/polyethylene glycol 400 (80:20, V/V). The animals were sacrificed by decapitation at 10 min, 4 h or 24 h after injection ($n = 3$ at each time point). The organs and other body parts were dissected and weighed in tared tubes. Blood was collected in a tared tube and weighed. The radioactivity in all the organs and other body parts was counted in a gamma sample changer (Wallac Wizard, Turku Finland). Corrections were made for background radiation and physical decay during counting. Results are expressed as percentage of injected dose (%ID), equal to the sum of the net counts in all body parts. For calculation of total blood radioactivity, blood mass was assumed to be 7% of the body mass.

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Conclusion

We have prepared mono- and di-iodohypericin in good yields and purified both compounds using RP-HPLC. Mono-[^{123}I]iodohypericin was obtained with a high specific activity whereas di-[^{123}I]iodohypericin always contained relatively large amounts of carrier due to deiodination and subsequent iodination of the precursor occurring during the labelling reaction. Biodistribution in mice shows a slow clearance of both mono- and di-iodohypericin from the blood by mainly the hepatobiliary system.

The compounds will be further evaluated with regard to their potential usefulness as tumor tracers.

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References

1. Lavie G, Mazur Y, Lavie D, Meruelo D. *Med Res Rev* 1995; **15**: 111–119.
2. D'Hallewin MA, de Witte PA, Waelkens E, Merlevede W, Baert L. *J Urol* 2000; **164**: 349–351.
3. Chen B, de Witte PA. *Cancer Lett* 2000; **150**: 111–117.
4. Vandenbogaerde AL, Kamuhabwa AR, Delaey EM, Himpens BE, Merlevede WJ, de Witte PA. *J Photochem Photobiol* 1998; **5**: 87–94.
5. Kamuhabwa AR, Agostinis P, D'Hallewin MA, Kasran A, de Witte PA. *Anticancer Res* 2000; **20**: 2579–2584.
6. Vandenbogaerde AL, Delaey EM, Vantieghem AM, Himpens BE, Merlevede WJ, de Witte PA. *Photochem Photobiol* 1998; **67**: 119–125.
7. Chen B, Roskams T, de Witte PAM, *Photochem Photobiol* 2002; **76**: 509–513.
8. Brandau W, Bubeck B, Eisenhut M, Taylor DM. *Appl Radiat Isot* 1988; **39**: 121–129.
9. Falk H, Meyer J, Oberreiter M. *Monatsh Chem* 1993; **124**: 339–341.

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