Pergamon

SCIENCE DIRECT

Bioorganic & Medicinal Chemistry 11 (2003) 5007–5012

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Pharmacokinetic Profile of a Quaternary Ammonium Derivative of Chlorambucil, a Potential Anticancer Drug for the Chemotherapy of Chondrosarcoma

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Received 16 May 2003; accepted 1 September 2003

Abstract—As a part of our targeting program based on the affinity of the quaternary ammonium moiety for cartilage, our objective was to develop more selective anticancer drugs towards chondrosarcoma that would concentrate in this malignant cartilaginous tissue and so improve the therapeutic index through a reduction of side effects. For this purpose we have synthesized and labeled with ^{14}C a quaternary ammonium (QA) derivative of chlorambucil. Biological studies performed in rats showed that [^{14}C]-CQA and [^{14}C]-chlorambucil exhibited different pharmacokinetic profiles. The blood elimination of [^{14}C]-CQA was faster than that of parent compound. [^{14}C]-CQA was principally excreted by the fecal way. However, until 15 min after administration, levels of radioactivity were measured in cartilaginous tissues of rats given [^{14}C]-CQA which was not the case for the rats which had received [^{14}C]-chlorambucil. Although rates of radioactivity were quantified only during 15 min, these results prove that the functionalization of chlorambucil by a quaternary ammonium group allows the molecule to be carried selectively to cartilaginous tissues.

Introduction

Chondrosarcoma is a malignant cartilaginous tumor of bad prognosis, representing the second most common bone tumor after osteosarcoma.[1–3] The only effective therapy of this neoplasm remains adequate surgery. Radiotherapy does not appear to affect the course of the disease, as well as chemotherapy, which is furthermore highly toxic.[3–6]

An alternative way to potentially improve the chemotherapy of chondrosarcoma may consist in the development of anticancer agents possessing an enhanced affinity for the cartilaginous tumors. Precisely, the targeting of cartilage is a research program investigated by our laboratory,[7–15] since we have discovered that the introduction of a quaternary ammonium (QA) entity in the structure of compounds could be a means to increase their affinity for cartilaginous tissues.[16–18] In the context of the improvement of the chemotherapy of chondrosarcoma, our strategy consists of the binding of anticancer agents to the QA carrier in order to strengthen the selectivity of the drugs for the cartilaginous tumors and consequently to increase the therapeutic benefit.

To initiate this project, we have decided to conjugate the QA moiety with chlorambucil (**1**) and melphalan (**2**) (Chart 1).[14,15] We were interested in the selection of these two drugs because their chemical structure lent itself well to a binding with our QA carrier through the carboxylic acid function, and also to a convenient [^{14}C]-radiolabeling thanks to the chloroethyl alkylating moieties: in fact, radiolabeled analogues were required to perform the in vivo biodistribution studies aimed at the checking of the cartilage targeting. Thus, we have developed a program based on the design of QA derivatives of chlorambucil and melphalan, CQA (**3**) and MQA (**4**) respectively (Chart 1), and on the evaluation of their pharmacological activity as well as their biodistribution.[14,15] We have shown that not only the QA functionalization does not alter the cytotoxic potency of the QA derivatives, which is even improved in the chondrosarcoma cell line, but also allows the derivative of melphalan to be carried selectively to cartilaginous tissues.[14,15]

In this paper, we report the syntheses explored for the preparation of [^{14}C]-labeled CQA and the pharmaco-

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doi:10.1016/j.bmc.2003.09.006

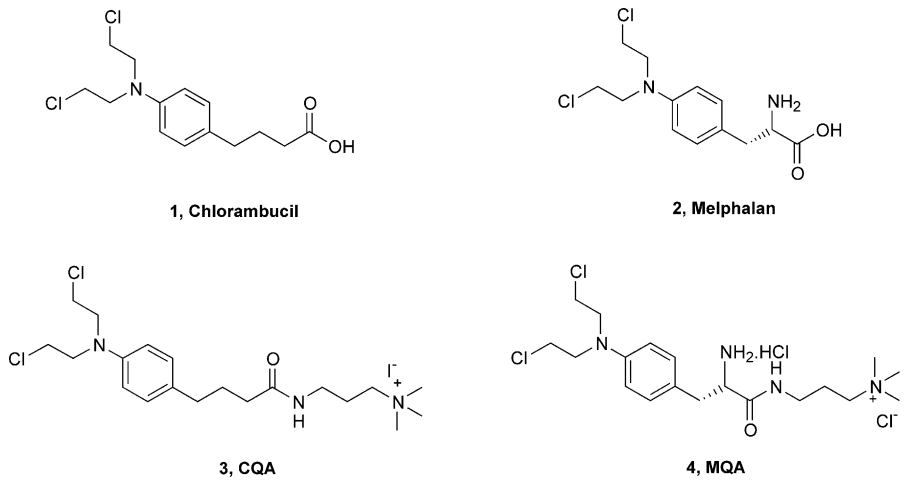


Chart 1.

kinetic study in rats of [^{14}C]-CQA in comparison with [^{14}C]-chlorambucil.

Chemistry

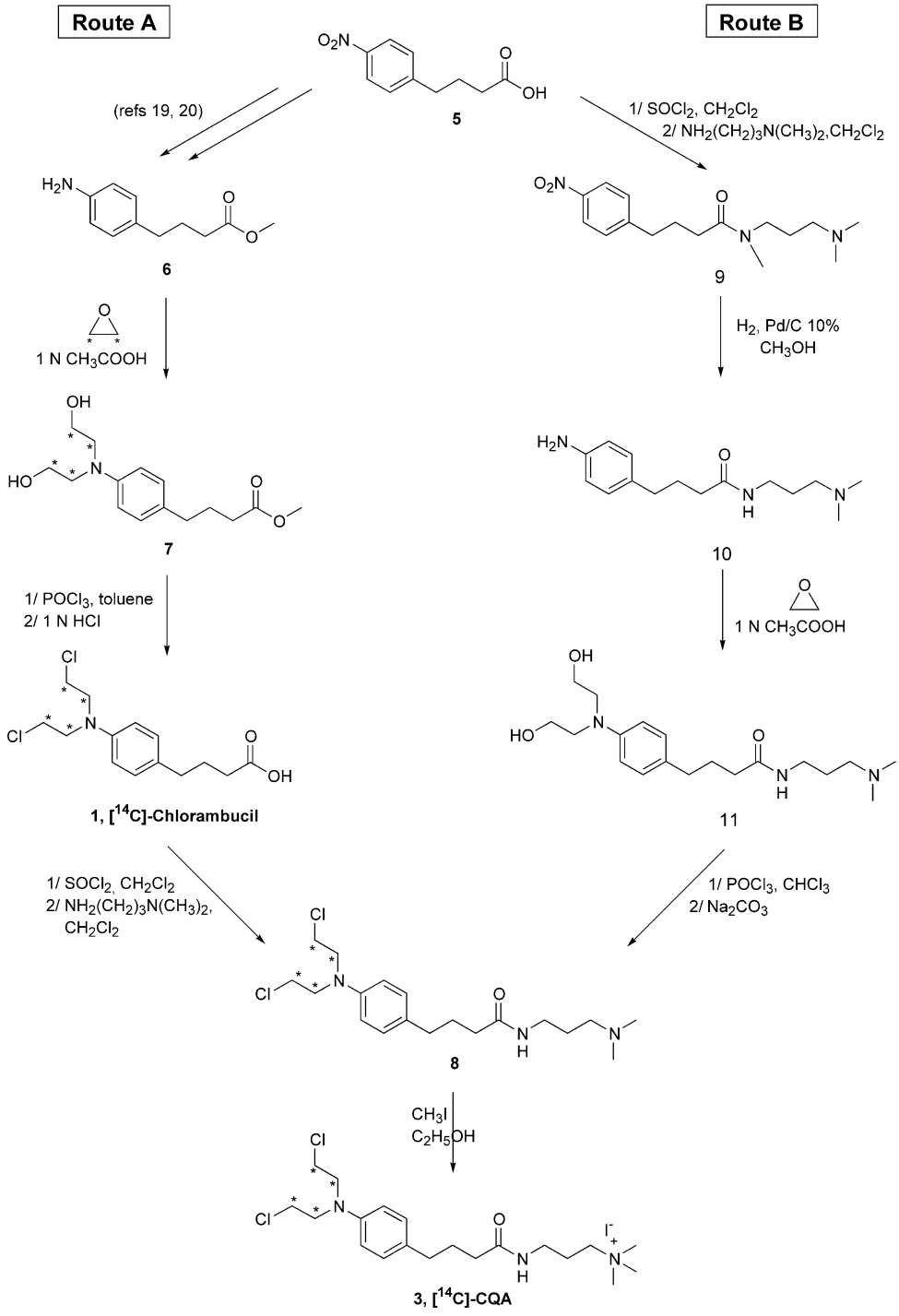
The radiolabeling was introduced in the chloroethyl groups by treatment of the appropriate arylamine with [1,2-^{14}C]ethylene oxide. This radiolabeling position was chosen because it offered the possibility to follow the becoming of the biological active entities of the compound and to compare the biological results between [^{14}C]-CQA and [^{14}C]-chlorambucil. With the aim to optimize the radiochemical preparation of [^{14}C]-CQA, two reversed reaction paths, differing in the order of introduction of the nitrogen mustard group and of the spacer carrying the QA moiety, were explored with unlabelled materials (route A and route B—Scheme 1). As these two chemical sequences were found to provide similar overall yield, route A was finally chosen for the [^{14}C]-radiosynthesis of CQA. Route A is one radiolabeled step shorter for the preparation of [^{14}C]-chlorambucil and [^{14}C]-CQA as compared to route B (Table 1).

According to route A, the two first steps consisted of the preparation of methyl 4-(4-aminophenyl)butyrate (**6**) from commercially available 4-(4-nitrophenyl)butyric acid (**5**) and were carried out with a quantitative yield as usually described.[19,20] Then, the route used for the preparation of [^{14}C]-chlorambucil (**1**) from compound **6** relied on an adaptation of the syntheses reported in the literature for the use of radioactivity.[19,20] First, compound 6 was reacted with [1,2-^{14}C]ethylene oxide in 1 N acetic acid to provide, after a purification on silica gel, the corresponding 4-[bis(2-hydroxy-[1,2-^{14}C]ethyl)]aminophenyl derivative (**7**) in 46% radiochemical yield. The treatment of compound **7** with phosphorus oxychloride in toluene and subsequently with 1 N hydrochloric acid gave [^{14}C]-chlorambucil (**1**) in 90% yield. According to these conditions, [^{14}C]-chlorambucil was isolated with a specific activity of 329 MBq/mmol (8.9 mCi/mmol) and a radiochemical purity of 97.3% as determined by thin layer chromatography analysis.

For the preparation of [^{14}C]-CQA from [^{14}C]-chlorambucil (**1**), the synthetic sequence was based on the syntheses developed for cold compounds.[14] Thus, [^{14}C]-chlorambucil (**1**) was conjugated with 3-(dimethylamino)propylamine after its preliminary conversion to acid chloride by treatment with thionyl chloride to afford the corresponding butyramide derivative (**8**) in 85% yield after chromatographic purification. The last step consisted of quaternarization of the free tertiary amine of **8** by methyl iodide in ethanol to provide the target quaternary ammonium derivative (**3**) in 91% yield. Finally, [^{14}C]-CQA (**3**) was isolated with a specific activity of 329 MBq/mmol (8.9 mCi/mmol) and a radiochemical purity determined to be better than 99% by thin layer chromatography analysis. The overall radiochemical yield of the labelling sequence was 32% based on [1,2-^{14}C]ethylene oxide.

Results and Discussion

Results of the in vivo biodistribution experiments with [^{14}C]-CQA and the parent molecule chlorambucil in rats are shown in Table 2. For both compounds radioactivity was distributed in many tissues especially in the metabolizing and eliminating organs, liver and kidney. Radioactivity rates in blood of rats given [^{14}C]-chlorambucil were higher than those given [^{14}C]-CQA. From 15 min after injection, no measurable radioactivity level was found in blood of rats receiving [^{14}C]-CQA which indicates a rapid blood clearance. The high radioactivity levels measured in the kidney and liver proves an important and fast biliary elimination. Until 15 min after administration, significant levels of radioactivity were detected in cartilaginous tissues of rats given [^{14}C]-CQA when no measurable radioactivity was found in rats which had received [^{14}C]-chlorambucil. Figure 1 shows an example of a whole-body autoradiograph performed 5 min after administration of [^{14}C]-CQA to rat. This image shows a radioactivity level in articular and intercostal cartilages, and in the intervertebral disks, higher than one of surrounding tissues, bone and muscle, that confirms the affinity of CQA for cartilaginous tissues. As in previous studies done in our laboratory



Scheme 1.

Table 1.

	[^{14}C]-Radiolabeling of CQA	[^{14}C]-Radiolabeling of chlorambucil	Total of radiolabeled steps
Route A	Four steps from compound **6**	Achieved in the preparation of [^{14}C]-CQA	4
Route B	Three steps from compound **10**	Two additional steps from compound **6**	5

on other QA derivatives,[7,8,10,12,15] these results also show that the QA derivative of chlorambucil exhibits an affinity for cartilaginous tissues. However, [^{14}C]-CQA is more quickly eliminated from cartilage than the QA derivative of the melphalan, for which rates of radioactivity having been detected in the cartilaginous tissues up to 1 h after injection.[15] Table 3 presents the radioactivity excreted in urine, feces and expired air after injection to rats of [^{14}C]-chlorambucil and [^{14}C]-CQA. Few radioactivity was found in expired air for the two compounds. The

Table 2. Comparative biodistribution of [^{14}C]-chlorambucil (CHLO) and [^{14}C]-CQA (CQA) in male Sprague–Dawley rats following intravenous injection[a]

Time	Compd	Tissue					
		Liver	Kidney	Cartilage	Blood	Spleen	Lung
2 min	CHLO	98.15±8.42	238.14±51.22	[b]	101.13±7.92	21.42±2.89	84.24±4.68
	CQA	131.80±14.29	374.22	22.90±5.86	41.06	13.37±1.49	31.56±2.12
5 min	CHLO	68.75±7.50	95.55±24.32	[b]	67.10±12.07	12.86±2.51	53.95±10.66
	CQA	111.58±18.51	284.61±116.12	14.32±3.54	12.59	10.08±0.93	12.61±5.73
10 min	CHLO	57.95±6.34	102.17	[b]	48.16±1.31	11.28±3.55	31.61±7.41
	CQA	83.43±6.56	133.28±19.73	10.40±1.86	6.28	9.81±1.73	10.26±1.73
15 min	CHLO	62.51±2.35	65.95±6.58	[b]	46.22±2.36	9.69±0.34	30.42±3.18
	CQA	41.04±2.65	53.62±1.91	7.04±0.62	[b]	15.58±1.50	8.93±0.70
30 min	CHLO	51.20±7.22	73.33±7.22	[b]	40.91±6.48	8.56±1.43	29.74±6.20
	CQA	27.14±2.83	48.42±5.90	[b]	[b]	10.48±1.03	8.45±0.71
1 h	CHLO	54.43±1.93	85.17±15.48	[b]	26.76±0.99	5.30	27.01±1.96
	CQA	19.9±1.79	46.33±4.89	[b]	[b]	16.70±2.52	7.95
2 h	CHLO	28.67±2.02	58.24±7.51	[b]	16.81±0.63	5.13	13.49±1.58
	CQA	11.46±1.59	34.53±6.75	[b]	[b]	7.09±1.26	8.38±1.29
6 h	CHLO	16.84±1.35	41.56±12.16	[b]	10.18±0.79	4.41	8.03±0.81
	CQA	8.78±1.71	24.22±3.97	[b]	[b]	4.97±0.94	4.78±1.17
24 h	CHLO	5.08±0.30	14.07±1.37	[b]	[b]	[b]	b
	CQA	5.29±0.76	20.18±3.74	[b]	[b]	4.27±0.79	3.27±0.83

[a]Results are expressed as nmol drug equivalent per gram of tissue and correspond to the mean±standard deviation for the quantification of six whole-body sections of one animal for each point.
[b]Non detectable.

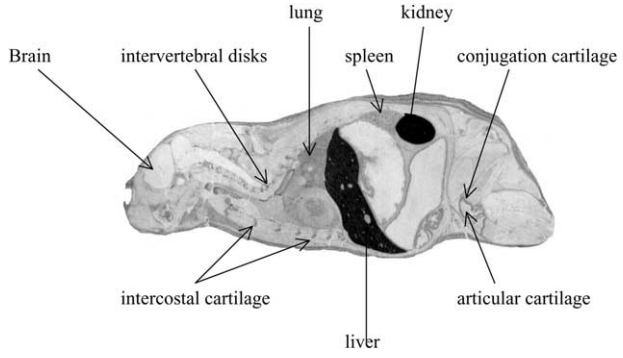


Figure 1. An example of whole-body autoradiography of serial longitudinal section of rat 5 min after injection of [^{14}C]-CQA.

Table 3. Cumulative radioactivity excreted in urine feces and expired air after intravenous administration of [^{14}C]-chlorambucil (CHLO) and [^{14}C]-CQA (CQA) in male Sprague–Dawley Rats[a]

	Time (h)	Urine	Feces	$^{14}CO_2$	Total excretion
CHLO	0–24	56.94±2.26	21.86±1.70	2.37	
	0–48	58.41±2.11	26.15±1.02	2.59	
	0–72	59.90±1.95	26.57±1.04	2.74	89.21
CQA	0–24	25.75±4.96	41.68±6.38	1.25	
	0–48	27.47±5.37	42.53±6.25	1.49	
	0–72	28.33±5.39	43.34±5.69		73.16

[a]Results are expressed as the percentage of the injected dose and correspond to the mean±standard deviation for five animals for each point, except for $^{14}CO_2$ (one rat studied).

amounts of radioactivity measured in lung can be explained by the strong vascularization of this organ. Whereas 56% of the radioactivity was excreted in the urine of rats given [^{14}C]-chlorambucil in the first 24 h, only 26% of the injected dose was recovered in the urine for [^{14}C]-CQA. Though mainly urinary, the excretion of the QA conjugate of melphalan was only 55% at 72 h.[15] Our previous works showed that QA compounds were mainly eliminated by urinary route (more than 85%) as unchanged molecule, the introduction of the polar quaternary ammonium function leading to a decrease of the lipophilicity.[10,12] The difference of urinary excretion of the QA conjugates of chlorambucil and melphalan could be related to their partition coefficient, which is strongly negative for MQA (log P = −1.12±0.14) which explains why MQA is a more hydrophilic compound than CQA.[14] Moreover, higher percentage of radioactivity was found in the feces of rats given [^{14}C]-CQA than in those of rats given [^{14}C]-chlorambucil. These results let us assume different metabolic pathways for [^{14}C]-CQA, [^{14}C]-MQA and their parent compounds. It is possible that [^{14}C]-CQA was more metabolized than [^{14}C]-MQA and especially more quickly, explaining a weaker concentration in blood and cartilaginous tissues than for the QA conjugate of melphalan.

Conclusion

The binding of the QA carrier to chlorambucil was part of our cartilage targeting program based of the known

affinity of the quaternary ammonium moiety for cartilage. We already used this property to specifically carry different active principles towards cartilaginous tissues.[8,10,14,15] Previous works carried out with the melphalan series showed the affinity of the QA conjugate of melphalan towards cartilaginous tissue. Cartilage targeting by [^{14}C]-CQA was obtained as expected but unfortunately weaker than that of MQA. We also demonstrated in cell culture that the cytotoxic activity of chlorambucil was not affected by the QA binding, and even enhanced on chondrosarcoma line.[14] Studies were undertaken concerning the evaluation of the in vivo antitumor activity of the QA conjugate of chlorambucil and chiefly with that of melphalan on animals bearing chondrosarcoma. The results will be reported elsewhere.

Experimental

General comments

4-(4-Nitrophenyl)butyric acid (**5**) was purchased from Aldrich and [1,2-^{14}C]ethylene oxide from NEN. All solvents and reagents obtained from commercial sources were used without further purification. Nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR) were performed on a Bruker AM 200 (4.5 T) spectrometer. Analytical thin layer chromatography (TLC) was conducted on precoated silica gel plates (SDS, 60 F_{254}, 0.25 mm thick) or on precoated aluminium oxide plates (Merck, 60 F_{254}, neutral type E, 0.20 mm thick) with both detection by ultraviolet light and visualisation with iodine. Silica gel 60 (Chromagel, 35–60 μm, SDS) was used for medium pressure chromatography using the indicated solvent mixture expressed as volume/volume ratios. Radiochemical purity was determined by scanning the TLC plates with an Ambis 4000 detector. Specific activity of compounds was measured in a Wallac Winspectral 1414 liquid scintillation counter.

Methyl 4-{4-[bis(2-hydroxy-[1,2-^{14}C]ethyl)amino]phenyl}butyrate (7). The reaction was performed in a vacuum manifold by the transfer of [1,2-^{14}C]ethylene oxide (1850 MBq (50.0 mCi), specific activity: 503 MBq/mmol (13.6 mCi/mmol)) on a solution of methyl 4-(4-aminophenyl)butyrate (**6**)[19,20] (775 mg, 4.01 mmol) in 1 N acetic acid (7 mL) cooled into liquid nitrogen. After completing the transfer, the reaction mixture was progressively warmed to 5 °C, stirred at this temperature for 1 h and then for another 16 h at room temperature. After this time, the solution was cooled again to 5 °C, treated with cold ethylene oxide (1.8 mL, 36 mmol), stirred for 5 h at room temperature and then evaporated under reduced pressure. The resulting oil was dissolved in diethyl ether and this organic phase was washed with 1 N sodium carbonate and with water, dried over magnesium sulfate and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (eluent: gradient of ethyl acetate in cyclohexane from 0 to 20%). Compound **7** was isolated as a clear oil with a radiochemical yield of 46% [733 mg, 858 MBq (23.2 mCi)] and with a specific activity of 329 MBq/mmol (8.9 mCi/mmol). The radiochemical purity was 97.4% as determined by TLC analysis (R_f 0.30, TLC silica, ethyl acetate). Compound 7 was found by NMR and TLC to be identical to the unlabeled reference compound.[20]

4-{4-[Bis(2-chloro-[1,2-^{14}C]ethyl)amino]phenyl}butyric acid ([^{14}C]-chlorambucil, 1). To a stirred solution of **7** (710 mg, 2.52 mmol) in toluene (5 mL) at 0 °C, was added, under an inert atmosphere, phosphorus oxychloride (2 mL). The reaction mixture was refluxed for 2 h and then evaporated under reduced pressure. The residue was treated with 37% hydrochloric acid (5 mL) and heated to reflux for 2 h. The mixture was then diluted and extracted with diethyl ether. The organic fractions were combined, dried over magnesium sulfate, filtered and evaporated under reduced pressure. [^{14}C]-Chlorambucil (**1**) was isolated as a white solid in 90% yield (691 mg, 830 MBq (22.4 mCi)) and with a specific activity of 329 MBq/mmol (8.9 mCi/mmol). The radiochemical purity was 97.3% as determined by TLC analysis (R_f 0.45, TLC silica, cyclohexane/ethyl acetate 2/1; R_f 0.80, TLC silica, dichloromethane/ethanol 9/1). Compound **1** was found by NMR and TLC to be identical to the unlabeled reference compound.[20]

***N*-[3-(Dimethylamino)propyl]-4-{4-[bis(2-chloro-[1,2-^{14}C]ethyl)amino]phenyl}-butyramide (8).** To a solution of [^{14}C]-chlorambucil (**1**) (350 mg, 1.15 mmol) in dichloromethane (5 mL), at 0 °C, was added, under an inert atmosphere, thionyl chloride (0.88 mL). The mixture was stirred at 4 °C for 16 h and then the excess of thionyl chloride was evaporated under reduced pressure. To the resulting oil dissolved in dichloromethane (5 mL) was added, under an inert atmosphere, a solution of 3-(dimethylamino)propylamine (0.16 mL, 1.27 mmol) in the same solvent (5 mL). This mixture was stirred for 1 h at room temperature, then treated with a second amount of diamine (0.16 mL, 1.27 mmol) and stirred for another 4 h at room temperature. After washing with 1 N sodium hydrogen carbonate and then with water, the organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (eluent: gradient of ethanol in dichloromethane from 0 to 50% and then dichloromethane/ethanol/ammonium hydroxide 50/49/1). Compound **8** was obtained in 85% yield [381 mg, 322 MBq (8.7 mCi)] with a specific activity of 329 MBq/mmol (8.9 mCi/mmol). The radiochemical purity was 99.5% as determined by TLC analysis (R_f 0.70, TLC silica, dichloromethane/ethanol/ammonium hydroxide 50/47/3; R_f 0.77, TLC aluminium oxide, dichloromethane/ethanol 9/1). Compound **8** was found by NMR and TLC to be identical to the unlabeled reference compound.[14]

{3-[(4-{4-[Bis(2-chloro-[1,2-^{14}C]ethyl)amino]phenyl}butanoyl)amino]propyl}trimethyl-ammonium iodide ([^{14}C]-CQA, 3). To a solution of **8** (260 mg, 0.67 mmol) in ethanol (7 mL), was added, under an inert atmosphere, methyl iodide (63 μL, 1.01 mmol). The mixture was stirred at room temperature for 3 h and then evaporated under reduced pressure. The resulting oil was dissolved

in the minimum amount of methanol and poured into diethyl ether (150 mL). The resulting precipitate was then filtered, washed with diethyl ether and dried to afford 3 as a white solid [323 mg, 201 MBq (5.4 mCi), 91%] with a specific activity of 329 MBq/mmol (8.9 mCi/mmol). The radiochemical purity was 99.5% as determined by TLC analysis (R_f 0.05, TLC silica, dichloromethane/ethanol/ammonium hydroxide 50/47/3; R_f 0.30, TLC aluminium oxide, dichloromethane/ethanol 9/1). Compound **3** was found by NMR and TLC to be identical to the unlabeled reference compound.[14]

Tissue distribution study

Tissue distribution analyses were performed in 5-week-old male Sprague–Dawley rats weighing 100–120 g (Iffa-Credo, L'Arbresle, France) after intravenous administration of each [^{14}C]-labeled test compound [22.5 μmol/kg, 890 kBq (24 μCi) in 200 μL of physiological saline for CQA or 0.1 M *N*-methyl-D-glucamin (Meglumin) solution for chlorambucil]. Animals were euthanasied by carbon dioxide inhalation 2, 5, 10, 15 and 30 min and 1, 2, 6 and 24 h after administration and rapidly frozen by immersion in liquid nitrogen. They were then embedded in a 2% carboxymethyl cellulose gel. The resulting carboxyl methylcellulose block was sagittally sectioned at −22 °C with a Reichert-Jung Cryopolycut cryomicrotome (Heidelberg, Germany). When section surfaces of interest appeared, the corresponding 40-μm-thick slices were taken using no. 810 Scotch band tape (3M, Saint Paul, MN, USA) and dried for 48 h at −22 °C. These selected slices ($n=6$ for each time studied) were subsequently analyzed with the Ambis 4000 detector (B. Braun Sciencetec), which allows visualization and quantification of the radioactivity distribution in whole-body sections. The quantification leads to a surface activity (cpm/mm^2) which, because of a calibration, is directly converted into nCi per gram of tissue.[21] The final results are expressed as nanomoles drug equivalent per gram of tissue. To obtain more defined images for identification of zones of interest, tissue slices were also applied to hyperfilm βmax (Amersham).[22]

Excretion study

The excretion study was conducted in 5-week-old male Sprague–Dawley rats weighing 100–120 g given each [^{14}C]-labeled test compound as described above and housed in metabolic cages (Iffa-Credo, l'Arbresle, France) enabling separate collection of feces and urine. Urine and feces were collected 24, 48, 72 h after administration. For the collection of expired [^{14}C] CO_2, animals ($n=1$ for each test compound) were housed in an apparatus designed by us to trap [^{14}C]CO_2 with a flask containing ethanolamine/methanol (20:80 v/v). Radioactivity of urine and ethanolamine/methanol samples was directly measured after addition of Packard Ultima Gold cocktail in the Wallac Winspectral 1414 liquid scintillation spectrometer. Radioactivity of dried feces was measured after combustion in a Packard 306 Oxidizer (Packard Instrument SA, Rungis, France).

Acknowledgements

This work was supported by Adir, Servier (France).

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