Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXVI, No. 9

SYNTHESIS AND BIODISTRIBUTION OF ^{14}C-RADIOLABELLED HYPOCRELLIN B

Jixiang Liu[∞], Gerald G. Miller[#], Liren Huang[∞], Zhenjun Diwu[†], J. William Lown[∞*], Kevin Brown[#], Ronald B. Moore[#], John Tulip[§], and Malcolm S. McPhee[#]

∞ Department of Chemistry, University of Alberta, Edmonton, AB, T6G 2G2

Department of Surgery, Cross Cancer Institute, Edmonton, AB, T6G 1Z2

§ Department of Electrical Engineering, University of Alberta, Edmonton, AB, T6G 2G7

† Molecular Probes, Inc., 4849 Pitchford Avenue, Eugene, OR, 97402-9144

SUMMARY

Our research group is currently exploring the use of hypocrellins as potential photosensitizers for photodynamic therapy (PDT). The purpose of this study is to investigate the synthesis and biodistribution of ^{14}C-labelled Hypocrellin B. Radiolabelled hypocrellin-B (HB) with the ^{14}C atom incorporated in either or both the 4- and 13-methoxy groups was synthesized with a specific activity of 2.11 mCi per mmol. The purified, ^{14}C-HB, prototype photodynamic therapy photosensitizer was used to determine the kinetics of uptake in EMT6/Ed cells in monolayer culture, and the kinetics of tumor and tissue distribution in Balb/c mice bearing the EMT6/Ed tumor.

Keywords: Radiolabelling, photodynamic therapy, hypocrellins, cellular uptake, pharmacokinetics.

INTRODUCTION

Current clinical application of photodynamic therapy is based upon the porphryin photosensitizer, Photofrin-II (P-II®). This drug has several limitations, the most significant of which is that it frequently confers prolonged dermal photosensitivity upon the patient.[1] The compound cannot be produced in pure, monomeric form, a feature which has complicated serum, normal tissue, and tumor pharmacokinetics. The absorption

CCC 0362–4803/95/090815–09

Received 1 May 1994
Revised 31 March 1995

spectrum and tissue distribution are markedly influenced by the degree of self-association,[2] and its light absorption maximum, at 400 nm, is suboptimal in terms of light penetration in tissues. These features have resulted in considerable effort to develop a new generation of photosensitizing compounds with more optimal physicochemical and clinical properties. While it is beyond the scope of this paper to review a plethora of new compounds, we report here on preliminary preclinical studies on a prototype photosensitizer of the general class of compounds called perylene quinonoid pigments; specifically, the naturally occurring compound, hypocrellin B. Hypocrellins were first recognized as potential photosensitizers for (PDT) in the early 1980's.[3] More recent reports on its photosensitizing properties in a variety of systems [4,5,6] have fueled our interest in further development of hypocrellins and their derivatives as photosensitizers for PDT.[7] We therefore report on the synthesis of ^{14}C labeled hypocrellin B, its uptake by mammalian cells *in vitro*, and its biodistribution in tumor - bearing mice.

RESULTS AND DISCUSSION

In Vitro Studies

Hypocrellin B uptake by EMT6/Ed monolayer cells is demonstrated in Figure 1.

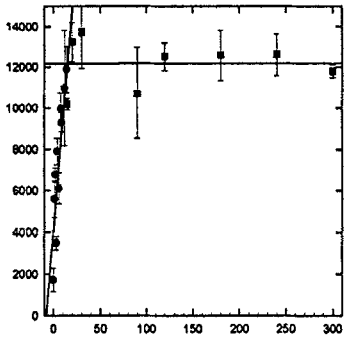


Figure 1. Uptake of ^{14}C-Hypocrellin-B by EMT6/Ed cells in monolayer culture. **Ordinate:** dpm/10^6 cells. **Abscissa:** Time of incubation with labelled compound, minutes. Error bars represent standard deviations of counts from three replicate cell digest samples.

Incorporation occurs rapidly for approximately 15 min, and plateaus for the remainder of the 120 min. observation period. The initial uptake slope corresponds to a rate of 175 pmol of HB per minute per 10^6 cells, to a maximum of 2.60 nmol/10^6 cells at saturation. Such rapid uptake into EMT6/Ed mouse tumor cells *in vitro* was unanticipated, however, this result has since been confirmed by fluorescence confocal microscopy, which

demonstrates rapid uptake *via* the endosomal system (data submitted for publication). Preliminary studies indicate that there is little difference in the efficacy of photosensitization for drug preincubations ranging from 2 - 24 hours.

***In Vivo* Studies**

Two routes of administration were explored to examine biodistribution and pharmacokinetics of ^{14}C-HB in Balb/c mice. Due to lipophilic properties of the drug, and its tendency to aggregate in aqueous solution, intraperitoneal injection of ^{14}C-HB in Balb/c mice did not appear to be a viable route of administration for this photosensitizer (data not shown). On a quantitative basis, tissue distribution was neither reproducible nor predictable, so this method was abandoned in favor of intravenous administration *via* the caudal vein.

The kinetics of incorporation of ^{14}C-HB into various tissues including the EMT6/Ed tumor are shown in Table 1. Plasma concentrations of ^{14}C-HB reached 16.5 +/- 0.03 μM immediately upon administration. This value declined to 390 +/- 48 nM at 2 hours (2.4% of the initial value), 240 +/- 40 nM at 24 hours (1.5%), and 165 +/- 35 nM (1.0%) 48 hours after administration.

Tumor tissue incorporates HB rapidly, with the maximum concentration attained immediately upon administration. The *in vitro* data suggest that maximum uptake by EMT6/Ed cells is not achieved for approximately 15 minutes, so it is likely that the initial radioactivities associated with tumor, (and most other tissues) include a variable contributions from the plasma. Immediately upon administration, all normal tissues contain greater drug concentrations than tumor, a result consistent with impaired vascular flow characteristic of most tumors. Sensitizer levels decline slowly over the 48 hour observation period to approximately 50% of the 2 - hour value.

The majority of normal tissues have peak sensitizer concentration 2 hours following administration. This is comparable to the 5 - 10 hours reported for P-II®.[8] Organs of the reticuloendothelial system, including lung, liver, spleen and kidney, collectively comprise 8 - 85 fold greater than tumor radioactivity immediately upon administration. This may be a function of entrapment of aggregated sensitizer, and is similar to findings reported for Photofrin-II,® [9] and for di- and trisulfonated gallium phthalocyanines.[10] Hypocrellins are amenable to systematic chemical alteration, and

Table 1. Tissue Uptake of ^{14}C-Hypocrellin B (dpm/g)

Tissue	0 Hours	2 Hours	24 Hours	48 Hours
Heart	113,920 ± 3,635	5,135 ± 910	7,835 ± 1,810	2,325 ± 245
Lung	651,100 ± 42,668	8,580 ± 655	3,870 ± 525	2,975 ± 360
Fat	20,550 ± 715	38,570 ± 5,610	19,550 ± 2,210	19,355 ± 2,335
Liver	394,190 ± 7,540	24,620 ± 4,885	22,495 ± 4,440	9,215 ± 720
Spleen	151,870 ± 9,395	58,900 ± 4,205	14,970 ± 3,215	26,700 ± 11,105
Stomach	28,280 ± 145	21,630 ± 3,345	34,385 ± 8,795	12,460 ± 975
Pancreas	32,010 ± 2,165	13,185 ± 12,055	32,390 ± 11,840	16,915 ± 3,845
Ileum	45,400 ± 3,600	20,280 ± 2,850	5,800 ± 645	2,840 ± 595
Kidney	67,344 ± 950	20,855 ± 3,955	12,050 ± 1,845	4,535 ± 765
Skin	14,970 ± 74	3,130 ± 221	2,700 ± 170	1,590 ± 250
Bone	19,825 ± 2,300	3,955 ± 2,070	660 ± 215	1,125 ± 310
Brain	17,560 ± 560	3,855 ± 170	2,840 ± 275	845 ± 90
Muscle	13,665 ± 600	4,050 ± 940	2,875 ± 560	1,015 ± 205
Tumor	7,885 ± 270	3,775 ± 400	2,950 ± 80	2,165 ± 470
Serum	69,975 ± 1,925	1,655 ± 170	1,020 ± 160	700 ± 240

efforts are underway to produce congeners with optimal characteristics of phototoxicity, while minimizing hydrophobicity.

Tissues which bind levels of HB similar to tumor include bone, skin, muscle, heart and brain. This is similar to the pattern reviewed for ^{14}C-P-II® and AlPcS.[11,12]. By 48 hours, tumor ^{14}C-HB levels have declined to approximately 0.5μM, a concentration which demonstrates significant phototoxicity for 630 nm light doses in the 1 J/cm^2 range [7]. Further studies are required to determine the optimal treatment time, as well as drug and light doses commensurate with significant growth delay or control of the EMT6/Ed tumor implanted in Balb/c mice.

The synthetic pathway outlined above for the production of ^{14}C-HB has led to its useful application in studies on the cellular uptake and biodistribution of this prototype hypocrellin photosensitizer. The elegant synthesis of HB by Diwu and Lown was selected since the precursor of HB can be obtained by the demethylation of natural HB, and commercially available ^{14}C-methyl iodide serves as the source of the radiolabel. Physicochemical properties of HB, in addition to its biological properties relevant to its use in photodynamic therapy are reported elsewhere.[7]

The biodistribution of HB is qualitatively similar to that of other lipophilic photosensitizers, including P-II®, although the kinetics are somewhat accelerated in comparison. This finding may be due to hypocrellin's low molecular weight and monomeric form. Rapid clearance of the compound from plasma and full incorporation into murine tumor within 2 hours of intravenous administration, support the development of HB and its congeners[7] as potent photosensitizing compounds. The low levels of HB found in skin 24 hours post-administration suggest that this photosensitizer may avoid the troublesome clinical limitation of prolonged, cutaneous phototoxicity characteristic of P-II®.

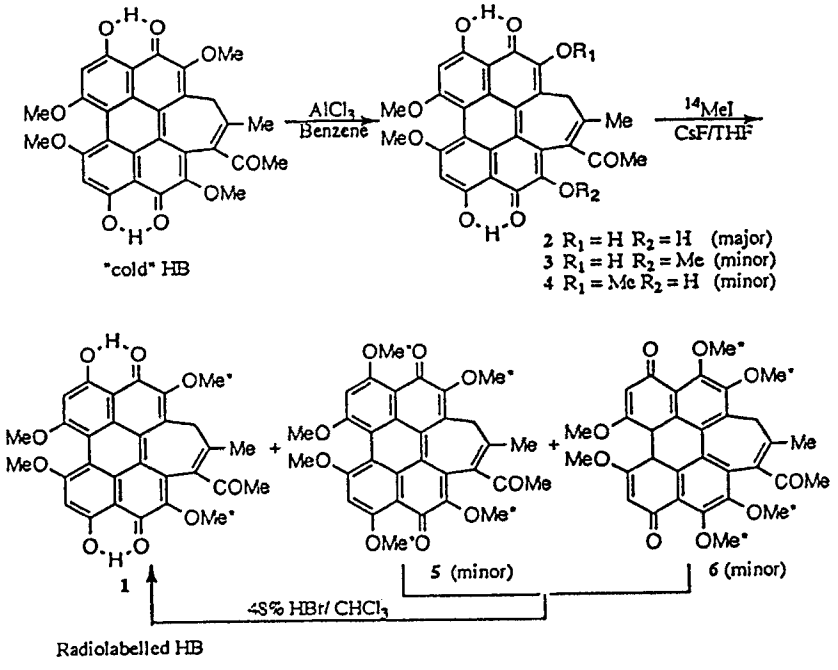
EXPERIMENTAL SECTION

Synthesis of Selectively ^{14}C-Radiolabelled Hypocrellin B

^{14}C-methyl iodide with an activity of 1.0 mCi was purchased from ICN Biomedicals, Inc. The preparation and purification of HB have been described elsewhere.[13] Merck silica gel 60 was used for column chromatography and commercial

Kieselgel 60 F_{254} plates were used for thin layer chromatography (TLC). All solvents were used as received and were reagent grade where available. Radioactivity was quantified with a Beckman Model LS 5801 liquid scintillation counter using the external standard and a carbon-14 quenched standard set (Beckman Instruments, Inc.) for quench correction.

HB (26.0 mg) was dissolved in 6.0 ml of anhydrous benzene containing 130 mg of anhydrous aluminum chloride, and the solution was refluxed for 1 hr under nitrogen. The mixture was poured into 10% aqueous ammonium fluoride, and extracted with chloroform. The chloroform layer was washed with water, dried (Na_2SO_4) and evaporated to afford a red solid that contained compounds **2**, **3** and **4**.[14] Steps in the synthetic pathway are outlined in Scheme 1.



Scheme 1. Me* = ^{14}C-labelled

The red solid was dissolved in 2.0 ml of anhydrous tetrahydrofuran (THF) containing 200 mg of cesium fluoride, and then ^{14}C-methyl iodide (1.0 mCi) in 1.0 ml of anhydrous THF was injected into the sealed flask. The mixture was stirred at room temperature for 24 hr, and stirring was continued another 12 hr at room temperature following the addition of 0.3 ml of methyl iodide. The resulting solution was added to

water and extracted with chloroform. The organic layer that contained compounds **1, 5**, and **6** was concentrated to about 5.0 ml by nitrogen stream evaporation.

A 48% hydrobromic acid solution (2.0 ml) was added to the chloroform solution and the mixture was stirred at room temperature for 30 min. The reaction mixture was poured into water, and extracted with chloroform. Evaporation of the chloroform layer gave a red solid. The solid was chromatographed on silica gel using chloroform as an eluent to afford radiolabelled HB (8.3 mg) with a specific activity of 2.11 mCi per mmol.

In Vitro Studies

In vitro cellular uptake studies were performed using the EMT6/Ed experimental mammary tumor model. EMT6/Ed mouse mammary carcinoma cells were propagated as monolayer cultures in our laboratory as previously reported.[15] Briefly, the cultures were serially passaged in Waymouth's Medium containing 13% fetal calf serum (GIBCO) at 37°C, in a water - saturated atmosphere containing 5% CO_2. At 3 - month intervals the tumor cells were routinely passaged in the flanks of Balb/c mice to maintain vigor of the tumor cell line.

Cellular Uptake of ^{14}C-Radiolabelled Hypocrellin B

Cellular uptake of ^{14}C-HB was determined by plating 5.0 x 10^5 cells in two ml of Waymouth's medium containing 13% fetal calf serum in 35 mm Petri plates and incubating for graded time intervals with ^{14}C-HB at a final concentration of 11.79 μM {(1.0 x 10^5 disintegrations per minute (dpm)}. Free ^{14}C-HB was removed from the plates by three gentle washes with 1.0 ml phosphate - buffered saline (pH 7.4). The cells were lysed with 0.2 ml, 1.0 N NaOH and neutralized with an equal volume of 1.0 N HCl. The lysate was wholly transferred to a scintillation vial and fifteen ml of CytoScint (ICN) were added for counting. Cellular uptake was standardized to dpm/10^6 cells.

In Vivo Uptake Kinetics of ^{14}C-Radiolabelled Hypocrellin B

Tissue and tumor distribution were assessed for 48 hours following administration of ^{14}C-HB, in Balb/c mice bearing EMT6/Ed tumors bilaterally on the flanks. Approximately 7 days before drug administration, 10^5 tumor cells were suspended in 100 μl of Hank's Balanced Salts Solution and injected into both flanks of 10 mice in each of

three, independent experiments. When the tumors reached an approximate volume of 250 µl, ^{14}C-HB was administered either intraperitoneally (i.p.), or intravenously in the caudal vein (i.v.) to a final, "total body" concentration of HB of 11.79 µmolar (10^6 dpm). The ^{14}C-HB was dissolved in a mixture of dimethylsulfoxide, ethyl alcohol and sterile, isotonic saline (10/5/85, v/v) and injected in a maximum volume of 100 µl. At intervals of 0, 2, 24, and 48 hours, the mice were euthanized by i.p. injection of Euthanyl (MTC Pharmaceuticals). Ten - to 80 mg samples of various tissues and tumor were dissected from the mice, weighed, and placed in glass scintillation vials. Blood samples were centrifuged, and the resulting plasma aliquots were treated the same as tissue samples. For each time point, three animals were used, with two replicate tissue/plasma samples per animal. Tissue uptake was expressed as dpm/g +/- the standard error.

ACKNOWLEDGMENTS

We are grateful to the National Cancer Institute of Canada for funding from the Terry Fox Run, as well as to the Alberta Heritage Foundation for Medical Research, and the Alberta Cancer Foundation (Anderson-Acklands) for support. The authors thank Ms. Gail Hipperson and the staff of the Cross Cancer Institute Vivarium for expert maintenance of the animals.

REFERENCES

1. McCaughan, J.S., Jr. in *Photodynamic Therapy of Neoplastic Disease* vol. 1. Ch. 2 (1990) p.22 Clinical Experience With PDT: A 5-Year Study. ed. D. Kessel, CRC Press, Boca Raton, FL.

2. Pantelides, M.L., Moore, J.V., and M.J. Blacklock. A comparison of serum kinetics and tissue distribution of Photofrin II following intravenous and intraperitoneal injection in the mouse. *Photochem. Photobiol.* **49(1):** (1989) 67-70.

3. Wan, X.Y., and Chen, Y.T. Hypocrellin A, a new drug for photochemotherapy. *Kexue Tongbao* (English edition) **26:** (1981) 1040-1042.

4. Fu, N.W., Chu, Y.X., Yuan, L.X., An, J.Y., and Diwu, Z.J. The photodynamic therapy of neoplastic cells by hypocrellin A. *Chinese J. Oncol.* **10:** (1988) 80.

5. Fu, N.W., Chu, Y.X., and An, J.Y. Photodynamic action of hypocrellin A on hepatoma cell mitochondria and microsome. *Act Farm. Sin.* **10:** (1989) 371-373.

6. Dong, C.Y., Jia, HT, and Ma, C.M. The inhibitory effect of the new photosensitizer hypocrellin A on experimental tumors. *Chin. J. Biochem.* **3:** (1987) 468-472.

7. Estey E.P., Brown K., Diwu Z., Liu J., Lown J.W., Miller G.G., Tulip J., and McPhee, M.S. Hypocrellins as photosensitizers for photodynamic therapy: a screening evaluation. In preparation.

8. Bellnier D.A., and Henderson B.W., in *Photodynamic Therapy Basic Principles and Clinical Applications* ch. 9 Determinants for Photodynamic Tissue Destruction p. 118, B.W. Henderson and T.J. Dougherty, ed., Marcel Dekker, Inc., New York (1992).

9. Pantelides M.L., Moore J.V., and Blacklock N.J. A comparison of serum kinetics and tissue distribution of photofrin II following intravenous and intraperitoneal injection in the mouse. Photochem. and Photobiol. 49:1 67-70 (1989).

10. Rousseau J., Langlois H.A., and van Lier J.E. Biological activities of phthalocyanines XII: Synthesis tumor uptake and biodistribution of ^{14}C-labeled disulfonated and trisulfonated gallium phthalocyanine in C3H mice. *J. Photochem. Photobiol. B: Biol.* **6:** 121-132 (1990).

11. Bellnier D.A., and Henderson B.W. *ibid.*

12. Chan W.S., Marshall J.F., Lam G.Y.F., Hart I.R. Tissue uptake, distribution, and potency of the photoactivatable dye chloroaluminum sulfonated phthalocyanine in mice bearing transplantable tumors. *Cancer Res.* **48:** 3040-3044 (1988).

13. Diwu Z.J. and Lown, J.W. Photosensitization with anticancer agents 12. Perylenequinonoid pigments, a novel type of singlet oxygen sensitizer. *J. Photchem. Photobiol. A:Chem.* **64:** 273-287 (1992).

14. Diwu Z.J. and Lown J.W. Photosensitization with anticancer agents 15. Perylenequinonoid pigments as potential photodynamic therapeutic agents: formation of semiquinone radicals and reactive oxygen species on illumination. *J. Photochem. Photobiol. B:Biol.* **18:** 131-143 (1993).

15. Chapman J.D. Baer K, and Lee J. Characteristics of the metabolism-induced binding of misonidazole to hypoxic mammalian cells. *Cancer Res.* **43:** 1523-1528 (1983).