Annals of Nuclear Medicine Vol. 11, No. 2, 95–99, 1997

Accumulation of ^{99m}Tc-HMPAO and ^{99m}Tc-ECD in rodent and human breast tumor cell lines *in vitro*

James R. BALLINGER, Jill DUNCAN, H. Annie HUA and Masanori ICHISE

Departments of Oncologic Imaging and Experimental Therapeutics, Ontario Cancer Institute; Division of Nuclear Medicine, Mount Sinai Hospital; and Faculties of Pharmacy and Medicine, University of Toronto, Canada

The accumulation of ^{99m}Tc-HMPAO and ^{99m}Tc-ECD was studied in rat (MatB) and human (MCF-7) breast tumor cell lines *in vitro* as a function of incubation time. The general pattern was the same for both tracers and both cell lines: the tracer rapidly and extensively accumulated in the cells but a plateau was reached in 15–30 minutes. Accumulation of HMPAO was higher than that of ECD, did not show a difference between rat and human cells, and correction of HMPAO data for intracellular sequestration and extracellular metabolism resulted in a linear increase in accumulation with time. In contrast, accumulation of ECD was ~2-fold higher in human cells than in rat cells but after correction for sequestration and metabolism a plateau remained. These experiments show differences between HMPAO and ECD in their accumulation and retention in breast cancer cells *in vitro* and support that the need for further work on the potential clinical role for HMPAO in tumor characterization.

Key words: technetium-99m-HMPAO; technetium-99m-ECD; tumor imaging; retention mechanism

INTRODUCTION

^{99m}Tc-d,l-HMPAO (Exametazime, Ceretec™, Amersham Healthcare) and ^{99m}Tc-ECD (Bicisate, Neurolite®, DuPont Pharma) are neutral, lipophilic complexes which cross the blood-brain barrier by passive diffusion and are used for imaging regional cerebral perfusion with SPECT.[1,2] Once in the brain, both are rapidly converted to hydrophilic species which cannot exit the brain, but their mechanisms of retention are different. HMPAO rapidly decomposes into hydrophilic species, possibly due to interaction with glutathione (GSH).[3–5] In contrast, the ethyl ester groups of ECD are sequentially hydrolyzed by esterase enzymes, generating the increasingly polar mono-acid and di-acid metabolites.[6–8]

Received September 4, 1996, revision accepted February 13, 1997.

For reprint contact: James R. Ballinger, Ph.D., Ontario Cancer Institute, 610 University Avenue, Toronto, Ontario, CANADA, M5G 2M9.

e-mail: jim_ballinger@pmh.toronto.on.ca

Despite its utility for cerebral perfusion imaging, HMPAO is of limited value in screening for brain tumors. As reviewed recently by Grunwald et al.,[9] the extent of accumulation in tumors can be less than, equal to, or greater than that in normal brain, dependent on tumor type, although a correlation has been shown between HMPAO accumulation and GSH content in brain tumors.[10] However, HMPAO also accumulates in a variety of non-cerebral tumors, as reviewed by Shih et al.,[11] and there may be a role for HMPAO in biological characterization of tumors. There is very limited information on accumulation of ECD in tumors.[12,13]

In order to better understand and assess the utility of these two tracers for tumor characterization, we have compared their accumulation in cultured tumor cells *in vitro*. Moreover, because of the reported species differences in trapping of ECD in the brain,[6] we felt it was important to study human as well as rodent cell lines.

MATERIALS AND METHODS

HMPAO and ECD were prepared by addition of ^{99m}Tc-

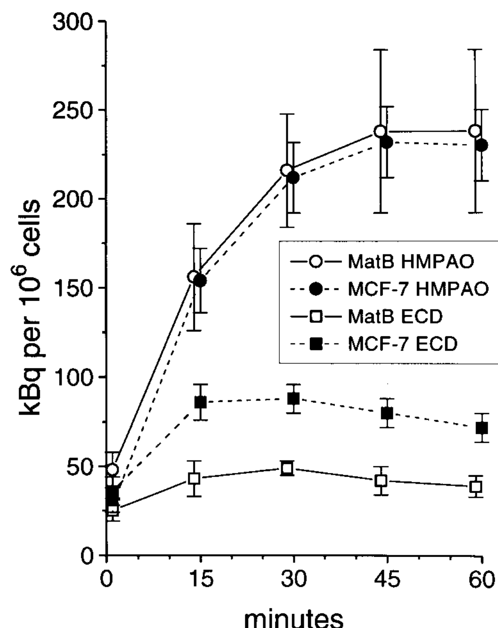

Fig. 1 Accumulation of HMPAO (circles) and ECD (squares) in MatB (open symbols) and MCF-7 (closed symbols) cells as a function of time. Each point is mean ± s.d. for 4 experiments performed at 1×10^6 cells/m*l* and normalized to an initial tracer concentration of 1 MBq/m*l*.

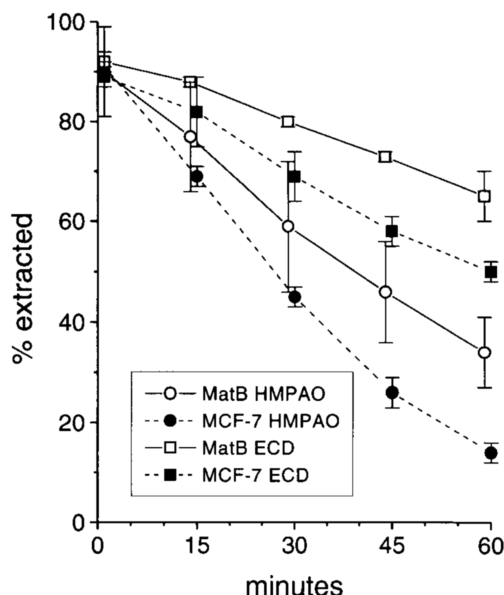

Fig. 2 Effect of incubation time on recovery of unmetabolized HMPAO (circles) and ECD (squares) by extraction into ethyl acetate from supernatant of MatB (open symbols) and MCF-7 (closed symbols) cell suspensions. Each point is mean ± s.d. for 4 experiments.

pertechnetate to nonradioactive kits and dilution with saline to a radioactivity concentration of 20 MBq/m*l*. Radiochemical purity was > 85% for HMPAO and > 90% for ECD as determined by chromatography.

The cell lines used were the rat breast adenocarcinoma, MatB 13762,[14] and human breast carcinoma, MCF-7 (HTB 22, American Tissue Type Culture Collection, Rockville, MD), which were grown in polystyrene flasks as a monolayer in alpha modification minimum-essential medium (MEM). Cells were released from the surface of the flask by incubation with trypsin, washed and centrifuged, then resuspended in fresh MEM at a concentration of 1×10^6 cells/m*l*. We have demonstrated previously that gentle trypsinization does not damage cells. Accumulation studies were performed as described previously.[15–17] Briefly, 10-m*l* aliquots of cell suspension were incubated with stirring at 37°C under room air. At 1, 15, 30, 45, and 60 min after addition of the tracer (2 MBq in 100 *μl*), duplicate aliquots of 500 *μl* were removed from the cell suspension and transferred to 1.5-m*l* microcentrifuge tubes which contained 500 *μl* ice-cold saline. After centrifugation for 1 min at 14,000 *g*, 100-*μl* aliquots of the supernatant were transferred to 10-m*l* tubes for extraction as described below. The remainder of the supernatant was aspirated and the pellet was gently washed with 500 *μl* cold saline, which was also aspirated. The tip of the tube containing the pellet was then clipped off and placed in a gamma counting tube. To the tubes containing 100-*μl* aliquots of supernatant were added 2 m*l* ethyl acetate and 2 m*l* phosphate-buffered saline (PBS). The tubes were capped, vortex mixed for 30 sec, then centrifuged for 30 sec at 250 *g* to separate the phases. The phases were transferred to separate gamma counting tubes. The tubes, along with a standard dilution of the dose, were assayed in a gamma well counter with an energy window of 90–190 keV.

Values for the cellular accumulation of tracer were calculated from the counts in the cell pellet and expressed as radioactivity per 10^6 cells as a function of time. For each of the extractions, the total counts (which represented the activity in 50 *μl* of supernatant diluted with 50 *μl* saline) and percent extracted into ethyl acetate (which represented the lipophilic parent drug) were tabulated. Finally, the concentration ratio of radioactivity inside the cells (calculated using an independent measurement of intracellular space: 10^6 cells = 2.0 *μl*) to that in an equal volume of supernatant (C_{in}/C_{out}) was calculated using as the denominator the actual concentration of parent drug in the supernatant at each time point (i.e. corrected for depletion and metabolism) as determined in the extraction experiments.

The stability of each tracer in saline and in cell-free MEM over the course of the experiment was verified. Finally, the plateau of ECD accumulation was studied further by performing experiments at 10-fold higher and 10-fold lower concentrations of ECD with MatB cells.

RESULTS

Within 1 min after addition of HMPAO to the MatB cell suspension, the concentration of radioactivity accumulated within the cells was much greater than that in the

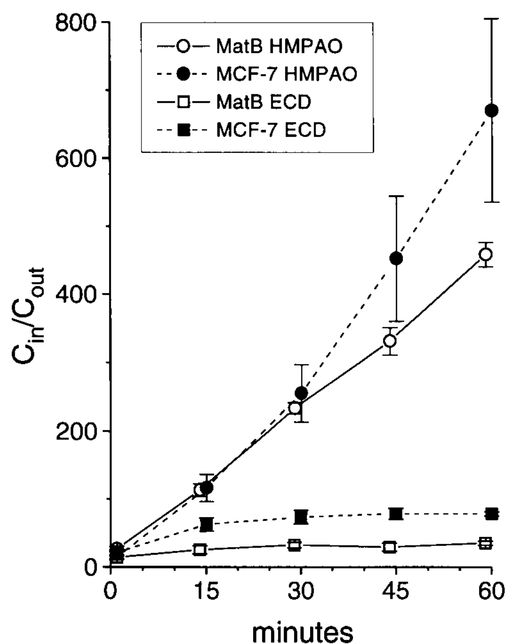

Fig. 3 Accumulation ratios (C_{in}/C_{out}) for HMPAO (circles) and ECD (squares) in MatB (open symbols) and MCF-7 (closed symbols) cells as a function of time. Data from Fig. 1 have been corrected for depletion and metabolism of tracer. Each point is mean ± s.d. for 4 experiments.

external medium. As seen in Fig. 1 (open circles), accumulation continued for 30–45 min, by which time a plateau was reached at a value of 240 kBq/10^6 cells (normalized to an initial extracellular tracer concentration of 1 MBq/m*l*). The extent of intracellular sequestration was sufficient that it led to depletion of tracer from the external medium by ~25% at 60 min (data not shown). In addition, metabolism of HMPAO was evident in the supernatant, to the degree that after 60 min only 35% of the parent drug remained (Fig. 2). When the curve in Fig. 1 was corrected for depletion of radioactivity from the external medium and metabolism of the parent drug, and converted to C_{in}/C_{out}, an almost linear relationship was obtained for HMPAO (Fig. 3).

The results with HMPAO in MCF-7 cells were very similar. The accumulation curve in Fig. 1 (closed circles) was almost identical and the extraction curve in Fig. 2 showed somewhat more extensive metabolism. When the curve in Fig. 1 was corrected for depletion and metabolism, again the relationship was almost linear (Fig. 3).

The general pattern observed with ECD in MatB cells was similar but not identical to that of HMPAO. Accumulation also reached a plateau (Fig. 1; open squares), but this was reached earlier (15–30 min) and both the initial accumulation and the plateau value were much lower than those observed with HMPAO. Because the accumulation was not as high, the depletion of tracer from the medium due to intracellular sequestration was also not as great as with HMPAO (to 10%; data not shown). In addition, the extent of metabolism was less (Fig. 2), with 65% of the parent drug remaining at 60 min. When the C_{in}/C_{out} values were plotted (i.e. corrected for the effects of depletion and metabolism), a plateau remained (Fig. 3).

The results with ECD in MCF-7 cells were slightly different. Accumulation was ~2-fold higher (Fig. 1; closed squares) and the extent of metabolism was greater (to 50% by 60 min, Fig. 2) than in MatB cells. The C_{in}/C_{out} values were also ~2-fold higher than those obtained in MatB cells, although the plateau remained (Fig. 3).

In studies in MatB cells performed at ECD concentrations which differed 100-fold (0.02 MBq/m*l* and 2 MBq/m*l*), the shapes of the curves were similar to those presented in Fig. 3. The relative accumulation of ECD (i.e. C_{in}/C_{out}, which takes into account the difference in added concentration) did not differ between concentrations; the plateau accumulation values were 23.0 ± 3.6 and 20.6 ± 5.7 for the low and high concentrations, respectively. The extent of metabolism observed in the supernatant medium was 9.3 and 9.0% for the low and high concentrations over the course of 60 min.

DISCUSSION

These experiments show that both HMPAO and ECD accumulate in tumor cells but there are differences between the two agents and between rodent and human tumors, particularly for ECD. Although the accumulation curves (Fig. 1) show the same general shape, the explanation for the shape is quite different for the two tracers. The plateau with HMPAO occurs due to depletion of the tracer from the supernatant (intracellular sequestration) and metabolism of the tracer (less parent drug available; Fig. 2). When the accumulation curve is corrected for these two effects (i.e. converted to C_{in}/C_{out}), a linear relationship is seen (Fig. 3). The slight upward curvature and large standard deviations in the data for HMPAO in MCF-7 cells may be artifacts of slight over-correction for these effects. These observations are in agreement with the results of Suess et al. who showed linear accumulation of HMPAO in rat cerebellar cells.[18] Furthermore, there is little difference between the rodent and human cell lines in the accumulation of HMPAO. The reported GSH contents of MatB and MCF-7 cells growing in culture are similar.[14,19]

The plateau with ECD is in part due to depletion and metabolism of the tracer. However, both of these effects are seen to a lesser extent than with HMPAO and when correction is made for these effects, a plateau remains. Both accumulation in and metabolism of ECD by the human cell line is greater than that observed with the rodent cell line, consistent with the species differences observed in cerebral accumulation of the tracer.[6] However, the major determinant of accumulation may be passive diffusion with metabolic trapping being a minor component. The observation of similar relative accumulation at tracer concentrations which differed by a factor

of 100 supports this interpretation. Further, the C_{in}/C_{out} values attained by ECD are similar to those we have observed for passive partitioning into aerobic cells of BMS181321, a tracer with a similar partition coefficient to ECD.[20]

The differences between HMPAO and ECD accumulation and retention in tumor cells may be related to their mechanisms of trapping, although the higher partition coefficient of HMPAO may also play a role. The theory that HMPAO reacts with intracellular GSH[3,4,10,18] has been questioned by some authors, who did not find a relationship between biodistribution of HMPAO and tissue GSH content in animals partially depleted of GSH.[21] However, as postulated recently by Sasaki et al.[22] the high specific activity of HMPAO and its rapid rate of reaction with GSH may allow even low levels of GSH to trap HMPAO quantitatively; if this is correct, accumulation of HMPAO may be independent of cell type and species, as seen in the present results. Other authors have recently suggested that HMPAO retention is dependent upon the balance between intracellular and extracellular redox state.[5] Unlike HMPAO, trapping of ECD requires an active process[6,8] and thus can only occur in viable cells; moreover, those cells must contain the appropriate esterase enzyme. It has recently been shown that ECD esterase activity can vary among cell lines[7] and tissues,[8] and that the intracellular distribution of esterase activity (membrane versus cytoplasm) can affect ECD accumulation.[7]

In summary, these experiments have shown differences between HMPAO and ECD in their accumulation and retention in breast cancer cells *in vitro* but underscore the need for further evaluation of the clinical role for these agents, but particularly HMPAO, in tumor characterization.

ACKNOWLEDGMENT

We thank Patricia Firby and Tassawwar Muzzammil for assistance in maintaining the breast cancer cell lines. MatB was provided by Dr. G. Batist, McGill University. Neurolite® kits were donated by DuPont Pharma and Amersham Canada Ltd. has supported our work by donation of Ceretec™. Ms. H.A. Hua was supported in part by the Medical Research Council of Canada and Ms. J. Duncan by a Pharmaceutical Manufacturers Association of Canada scholarship.

REFERENCES

1. Neirinckx RD, Canning LR, Piper IM, Nowotnik DP, Pickett RD, Holmes RA, et al. Technetium-99m d,l-HM-PAO: A new radiopharmaceutical for SPECT imaging of regional cerebral blood perfusion. *J Nucl Med* 28: 191–202, 1987.
2. Vallabhajosula S, Zimmerman RE, Picard M, Stritzke P, Mena I, Hellman RS, et al. Technetium-99m ECD: A new brain imaging agent: *In vivo* kinetics and biodistribution studies in normal human subjects. *J Nucl Med* 30: 599–604, 1989.
3. Neirinckx RD, Burke JF, Harrison RC, Forster AM, Andersen AR, Lassen NA. The retention mechanism of technetium-99m-HM-PAO: Intracellular reaction with glutathione. *J Cereb Blood Flow Metab* 8: S4–S12, 1988.
4. Ballinger JR, Reid RH, Gulenchyn KY. Technetium-99m HM-PAO stereoisomers: Differences in interaction with glutathione. *J Nucl Med* 29: 1998–2000, 1988.
5. Jacquier-Sarlin MR, Polla BS, Slosman DO. Oxide-reductive state: The major determinant for cellular retention of technetium-99m-HMPAO. *J Nucl Med* 37: 1413–1416, 1996.
6. Walovitch RC, Cheesman EH, Maheu LJ, Hall KM. Studies of the retention mechanism of the brain perfusion imaging agent ^{99m}Tc-bicisate (^{99m}Tc-ECD). *J Cereb Blood Flow Metab* 14: S4–S11, 1994.
7. Jacquier-Sarlin MR, Polla BS, Slosman DO. Cellular basis of ECD brain retention. *J Nucl Med* 37: 1694–1697, 1996.
8. Inoue Y, Momose T, Watanabe T, Ohtake T, Nishikawa J, Sasaki Y. Properties of enzyme to catalyze Tc-99m ECD (abstr). *J Nucl Med* 37: 189P, 1996.
9. Grunwald F, Menzel C, Pietsch T, Pavics L, Hufnagel A, Biersack H-J. Increased technetium-99m-HMPAO uptake in Grade II astrocytoma. *J Nucl Med* 26: 804–806, 1995.
10. Suess E, Malessa S, Ungersbock K, Kitz P, Podreka I, Heimberger K, et al. Technetium-99m-d,l-hexamethyl-propyleneamine oxime (HMPAO) uptake and glutathione content in brain tumors. *J Nucl Med* 32: 1675–1681, 1991.
11. Shih WJ, Coupal J, Magoun S. The biodistribution of technetium-99m-hexamethylpropylene amine oxime. *Semin Nucl Med* 24: 180–183, 1994.
12. Tanada S, Murase K, Inoue T, Sugawara Y, Miki H, Okumura A, et al. Comparison of kinetics in brain tumors between Tc-99m ECD and Tc-99m HMPAO with dynamic brain SPECT and compartmental model (abstr). *J Nucl Med* 33: 969–970, 1992.
13. Hirano T, Suzuki H, Higuchi T, Watanabe N, Oriuchi N, Shibazaki T, et al. Grade II astrocytoma visualized by technetium-99m-ECD SPECT. *J Nucl Med* 37: 273–275, 1996.
14. Schecter RL, Woo A, Duong M, Batist G. *In vivo* and *in vitro* mechanisms of drug resistance in a rat mammary carcinoma model. *Cancer Res* 51: 1434–1442, 1991.
15. Ballinger JR, Cowan DSM, Boxen I, Zhang ZM, Rauth AM. Effect of hypoxia on the accumulation of technetium-99m-glucarate and technetium-99m-gluconate by Chinese hamster ovary cells *in vitro*. *J Nucl Med* 34: 242–245, 1993.
16. Ballinger JR, Sheldon KM, Boxen I, Erlichman C, Ling V. Differences between accumulation of ^{99m}Tc-sestamibi and ^{201}Tl-thallous chloride in tumour cells: Role of P-glycoprotein. *Quart J Nucl Med* 39: 122–128, 1995.
17. Ballinger JR, Hua HA, Berry BW, Firby P, Boxen I. ^{99m}Tc-Sestamibi as an agent for imaging P-glycoprotein-mediated multidrug resistance: *In vitro* and *in vivo* studies in a rat breast tumour cell line and its doxorubicin-resistant variant. *Nucl Med Commun* 16: 253–257, 1995.
18. Suess E, Huck S, Reither H, Hortnagl H, Angelberger P. Uptake mechanism of technetium-99m-d,l-HMPAO in cell cultures of the dissociated postnatal rat cerebellum. *J Nucl*

Med 33: 108–114, 1992.

19. Batist G, Tulpule A, Sinha BK, Katki AG, Myers CE, Cowan KH. Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. *J Biol Chem* 261: 15544–15549, 1986.
20. Ballinger JR, Wan Min Kee J, Rauth AM. *In vitro* and *in vivo* evaluation of a technetium-99m-labeled 2-nitroimidazole (BMS181321) as a marker of tumor hypoxia. *J Nucl Med* 37: 1023–1031, 1996.
21. El-Shirbiny AM, Sadek S, Owunwanne A, Yacoub T, Suresh L, Abdel-Dayem HM. Is $^{99}Tc^{m}$ hexamethylpropyleneamine oxime uptake in the tissues related to glutathione cellular content? *Nucl Med Commun* 10: 905–911, 1989.
22. Sasaki T, Toyama H, Oda K, Ogihara-Umeda I, Nishigori H, Senda M. Assessment of antioxidative ability in brain: Technetium-99m-*meso*-HMPAO as an imaging agent for glutathione localization. *J Nucl Med* 37: 1698–1701, 1996.