Mitochondria act as a reservoir for the basic amine HIPDM in the lung

M. Miniati*, A. Paci**, F. Cocci**, G. Ciarimboli*, S. Monti*, M. Pistolesi*


ABSTRACT: The lungs are a site for the uptake, accumulation, and storage of exogenous basic amines. The compound N-N,N′-trimethyl-N′-(2-hydroxy-3-methyl-5-iodobenzyl)-1,3-propanediamine (HIPDM) is a basic amine that can be labelled with radioactive iodine and detected by external counting. Intravenously injected 125I-HIPDM is extracted by the human lung, where it is retained in a slowly effluxable pool.

In the present study, we measured HIPDM lung kinetics and subcellular distribution in rabbits given i.v. 125I-HIPDM. Rabbits were killed from 2 min to 5 h after injection, and the radioactivity retained in their lungs was measured. Subcellular lung fractions (nuclear, mitochondrial, lysosomal, microsomal, and postmicrosomal supernatant) were assayed for HIPDM radioactivity, protein content, and distribution of specific marker enzymes.

HIPDM lung clearance in rabbits was nearly identical to that of humans. Virtually all the HIPDM radioactivity in lungs (98±1%) was associated with subcellular membranous structures. The highest HIPDM specific radioactivity was found in the mitochondrial fraction, and the subcellular distribution profile closely resembled that of the mitochondrial marker enzyme succinate cytochrome c reductase. No redistribution of HIPDM among subcellular compartments was observed over a 5 h period after injection.

The data indicate that mitochondria act as reservoir for HIPDM in the lungs and contribute to the pulmonary persistence of this compound. HIPDM can be used to investigate the pulmonary uptake of basic amines in health and in lung disease.

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The lungs are a site for uptake, accumulation, and storage of a variety of exogenous chemicals, including drugs in clinical use. The available data on the pharmacokinetic function of the lung can be summarized as follows: 1) the compounds which concentrate most extensively in the lungs are amines with a pKa greater than 8, collectively grouped under the term of basic amines [1, 2]; 2) many of these molecules are amphiphilic in nature, inasmuch as they contain a large hydrophobic group and a side chain which is protonated at physiological pH [1, 2]; 3) in contrast to endogenous biogenic amines [3], a carrier-mediated, energy-dependent transport system is not involved in the pulmonary uptake of basic amines [4–6]; 4) no appreciable in vivo lung metabolism has been reported for most of the basic amines investigated so far [4, 6–12]; 5) basic amines with amphiphilic character, e.g. imipramine [8, 13], methadone [9, 13], chlorphenetermine [13], propranolol [10], verapamil [11], and amiodarone [14], are retained in the lungs in a slowly effluxable pool. At present, there is no obvious explanation for the observed persistence of these drugs in the lungs.

The radioiodinated compound N-N,N′-trimethyl-N′-(2-hydroxy-3-methyl-5-iodobenzyl)-1,3-propanediamine (HIPDM) is an amphiphilic basic amine similar to p empathilic drugs [15]. Although HIPDM was introduced as a radiopharmaceutical for brain perfusion imaging, preliminary biodistribution studies have indicated that the lungs, rather than the brain, are the main storage site for HIPDM [15]. As assessed in isolated perfused rat lungs, the pulmonary uptake of HIPDM is saturable, does not require an active transport system, and is competitively inhibited by other basic amines, such as imipramine, chlorpromazine and propranolol [16]. No significant in vivo metabolism of HIPDM has been detected in rat and rabbit lungs [17, 18].

Unlike other basic amines, the pulmonary uptake of HIPDM can be visualized by external detection [19, 20]. Intravenously injected 123I-HIPDM is quickly extracted by the human lung, where it is distributed according to regional blood flow [20]. The resulting lung scan is indistinguishable from that obtained by conventional perfusion radioisotopes. Furthermore, it has been observed that HIPDM is cleared from the human lung at a slow exponential rate [20]. The pulmonary persistence of HIPDM is increased in asymptomatic smokers and in patients with acute respiratory distress syndrome (ARDS) as compared to healthy nonsmokers [18, 20].
In the present study, HIPDM was used to investigate the mechanisms responsible for the pulmonary persistence of basic amines. The time course of HIPDM biodistribution was first evaluated in rabbits receiving i.v. bolus injection of labelled HIPDM. In additional experiments, the lung subcellular distribution profile of the compound was assessed by cell fractionation techniques.

Methods

Radiolabelling

The radiolabelling of HIPDM was carried out according to the method described by Kung et al. [15]. Briefly, 2 mCi of carrier-free Na\(^{125}\)I (Sorin Biomedica, Saluggia, Italy) were added to 0.25 mL of HIPDM solution (4 mg·mL\(^{-1}\) in 0.14 N HCl) in a sealed glass vial, and the reaction mixture was heated in a boiling water bath for 60 min. Radiochemical incorporation was evaluated by thin layer chromatography (Silica Gel 60-F; Merck, Darmstadt, Germany) using chloroform-ethanol-saturated ammonia (80:15:5 by volume) as solvent. The labelling yield was usually greater than 95% and the specific activity of the labelled compound was approximately 0.3 Ci·mmol\(^{-1}\). Free iodine was removed by eluting the reaction mixture through anion exchange resin (Biorex AG 1-X8; Bio-Rad, Richmond, CA, USA).

Biodistribution studies

Forty six adult New Zealand albino rabbits (Stefano Morini Laboratories, Reggio Emilia, Italy) weighing 2–3 kg were used throughout the study. They were fed with a standard rabbit chow. The protocol was approved by the Institutional Animal Care Committee. The animals were injected, through the marginal ear vein, with a 0.5 mL bolus of saline containing an average of 37±4 µCi (mean±SD) of labelled HIPDM. At preselected times, ranging from 2 min to 5 h after HIPDM injection, the animals were killed by an intravenous dose of sodium pentobarbital at various times (from 2 min to 5 h) after HIPDM administration. The lungs were excised, dissected free of major airways, minced, and homogenized in saline as described previously. The whole lung homogenate was assayed for HIPDM radioactivity. Counts in lung homogenate, lavage cells, and alveolar surfactant were expressed as percentage of the total HIPDM radioactivity recovered.

Lung cell fractionation studies

Twenty two adult New Zealand albino rabbits were used in these experiments. As for biodistribution studies, the rabbits received an i.v. bolus of labelled HIPDM and were killed with a lethal injection of sodium pentobarbital at various times (from 2 min to 5 h) after HIPDM administration. The lungs were excised, dissected free of major airways, weighed and minced. The lung mince was homogenized in 10 vol of ice-cold medium (0.25 M sucrose, 0.005 M Tris hydrochloride, 0.001 M MgCl\(_2\), pH 7.4 at 4°C) using a Polytron PT 10 homogenizer for 40 s at maximum speed. The resulting slurry was strained through two layers of gauze and subjected to differential centrifugation by a modification of the method described by de Duve et al. [23]. Prior to centrifugation, weighed aliquots of the homogenate and of the filtrate were set aside for further determinations as described below. All the subsequent manipulations were conducted at 4°C in tared tubes. Undisrupted cells and nuclei, henceforth referred to as nuclear fraction, were sedimented by an integrated force of 20,000 g-min. The supernatant, which contained the cytoplasmic extract, was further fractionated and three particulate fractions, mitochondrial, lysosomal and microsomal, were successively sedimented by integrated forces of 88,000, 200,000 and 4,440,000 g-min, respectively. All four sediments were washed once in cold medium (0.05 M Tris hydrochloride, 0.01 M MgCl\(_2\), 0.075 M NaCl, pH 7.4 at 4°C) by resuspension and recentrifugation. The washings were then discarded and the wet weight of each sediment was measured. Weighed samples of each sediment and of the final supernatant were taken for measuring the retained HIPDM radioactivity and the protein content. The latter was measured by the BCA Protein...
Assay (Pierce Chemical Company, Rockford, IL, USA) using bovine serum albumin as a standard. The same determinations were carried out on the samples of the homogenate and of the filtrate laid aside previously.

Additional aliquots of the subcellular fractions were assayed for the following enzymatic activities: succinate cytochrome c reductase (mitochondrial marker enzyme); arylsulphatase B (lysosomal marker enzyme); and NADPH-cytochrome c reductase (microsomal marker enzyme). Succinate cytochrome c reductase was measured as described by Sottocasa et al. [24]. Arylsulphatase B was assayed by the spectrophotometric method of Baum et al. [25], as modified by Hall et al. [26]. NADPH-cytochrome c reductase determination was carried out by a modification of the method of Siler Masters et al. [27].

Because the mitochondrial fraction of the mammalian lung is contaminated by lamellar bodies originating from alveolar type 2 pneumocytes [28], 10 additional lung cell fractionation experiments were carried out in which lamellar bodies were isolated from mitochondria in order to measure the partitioning of $^{125}$I-HIPDM between these two subcellular components. In these experiments, rabbits were killed by sodium pentobarbital overdose at 5 min and at 3 h after HIPDM i.v. injection. For the isolation of lamellar bodies, the methods described by Page-Roberts [29] and by Hassett et al. [30] were used, with some modification. Briefly, the crude mitochondrial sediment, obtained as described previously, was resuspended in 2 mL of 0.33 M sucrose in 0.01 M Tris hydrochloride, pH 7.4, at 4°C (0.33 M sucrose-Tris) and weighed. A weighed aliquot of the crude mitochondrial suspension was set aside for measuring HIPDM radioactivity. The residual suspension was layered on 5 mL of 0.80 M sucrose-Tris and centrifuged at 1,600,000 g·min in a tightly packed homogenate and of the filtrate laid aside previously.

**Results**

**Biodistribution studies**

Biodistribution data of HIPDM in rabbits are presented in Table 1. Two minutes after HIPDM i.v. administration, approximately 90% of the injected dose was recovered from the lungs. Radioactivity in the liver and in the total circulating blood volume accounted for 2 and 4% of the injected dose, respectively. Negligible amounts of radioactivity (<1% of the dose) were recovered from the other organs. As a function of time, the clearance of HIPDM from the lungs was biphasic, with an initial faster wash-out followed by a more gradual decline of radioactivity. In the liver and kidneys, the radioactivity increased progressively and, by 5 h after injection, averaged 12 and 5% of the injected dose, respectively. Throughout the study, the blood radioactivity declined to a constant value of about 1.5% of the injected dose; brain and spleen activity did not exceed 1% of the dose.

The residual suspension was layered on 5 mL of 0.80 M sucrose-Tris and centrifuged at 1,600,000 g·min to yield a tightly packed white pellet. Weighed aliquots of the purified mitochondrial and lamellar body sediments were assayed for HIPDM radioactivity.

Table 1. – Biodistribution of HIPDM in rabbits

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Lungs</th>
<th>Liver</th>
<th>Kidneys</th>
<th>Brain</th>
<th>Spleen</th>
<th>Blood</th>
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</thead>
<tbody>
<tr>
<td>2</td>
<td>90±7</td>
<td>2.1±0.4</td>
<td>0.8±0.5</td>
<td>0.5±0.1</td>
<td>0</td>
<td>3.9±2.6</td>
</tr>
<tr>
<td>15</td>
<td>50±12</td>
<td>5.2±1.2</td>
<td>4.0±0.8</td>
<td>0.9±0.3</td>
<td>0.1±0.0</td>
<td>2.7±0.0</td>
</tr>
<tr>
<td>30</td>
<td>52±11</td>
<td>6.2±1.2</td>
<td>4.2±1.2</td>
<td>0.7±0.3</td>
<td>0.3±0.2</td>
<td>1.8±0.5</td>
</tr>
<tr>
<td>45</td>
<td>39±11</td>
<td>8.3±3.5</td>
<td>4.5±1.6</td>
<td>0.7±0.3</td>
<td>0.3±0.2</td>
<td>1.8±0.4</td>
</tr>
<tr>
<td>60</td>
<td>31±5</td>
<td>7.1±0.8</td>
<td>5.3±0.9</td>
<td>0.8±0.1</td>
<td>0.3±0.2</td>
<td>1.6±0.0</td>
</tr>
<tr>
<td>90</td>
<td>22±2</td>
<td>11±2</td>
<td>5.6±0.5</td>
<td>1.0±0.3</td>
<td>0.5±0.1</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>120</td>
<td>23±7</td>
<td>13±3</td>
<td>6.9±1.3</td>
<td>1.0±0.1</td>
<td>0.4±0.2</td>
<td>1.8±0.4</td>
</tr>
<tr>
<td>150</td>
<td>21±6</td>
<td>13±3</td>
<td>6.1±0.6</td>
<td>1.0±0.1</td>
<td>0.5±0.1</td>
<td>1.4±0.2</td>
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<tr>
<td>180</td>
<td>21±7</td>
<td>13±2</td>
<td>4.9±1.1</td>
<td>1.0±0.2</td>
<td>0.5±0.3</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>240</td>
<td>17±5</td>
<td>15±4</td>
<td>4.5±1.1</td>
<td>0.8±0.1</td>
<td>0.3±0.1</td>
<td>1.5±0.4</td>
</tr>
<tr>
<td>300</td>
<td>13±4</td>
<td>12±1</td>
<td>4.9±2.2</td>
<td>0.7±0.2</td>
<td>0.4±0.1</td>
<td>1.4±0.3</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD of four different experiments. *: % of HIPDM injected dose in total circulating blood volume.

HIPDM: N-N-N′-trimethyl-N′-(2-hydroxy-3-methyl-5-iodobenzyl)-1,3 propanediamine.
lungs. Even at 5 h after injection, the radioactive concentration in the lungs was higher than that measured in the liver, brain, kidneys and spleen, by a factor of 10, 20, 8 and 6, respectively.

The time course of HIPDM clearance from the lungs was further characterized by applying nonlinear regression analysis to the experimental data. The efflux of HIPDM from the lungs was adequately described by a biexponential function (fig. 2). The half-times of the faster and of the slower exponential component were 18 min and 4.3 h, respectively (fig. 2). The mean times of the two exponential components, computed from the corresponding decay constants ($\lambda$), were 26 min and 6.2 h, respectively. The size of the slowly effluxable pool, estimated by backward extrapolation of the slower exponential component of HIPDM efflux, accounted for 30% of the injected dose.

In the three rabbits in which we did bronchoalveolar lavage (BAL), the radioactivity associated with lavage cells and alveolar surfactant did not exceed 3 and 0.2% of the total HIPDM activity recovered, respectively. Indeed, at any time after HIPDM i.v. injection, most of the radioactivity recovered (98±1%) was retained by the lung tissue after BAL.

### Lung cell fractionation studies

The subcellular distributions of HIPDM and of protein, obtained by averaging the data of 22 consecutive experiments, are presented in table 2. HIPDM and protein in each subcellular fraction are expressed as a percentage of their respective cumulative amounts recovered from all the fractions. In these experiments, the cumulative radioactivity recovered from the fractions averaged 98±3% of the radioactivity measured in the filtrate prior to centrifugation, thus indicating no significant loss of tracer in the fractionation procedure.

Virtually all the radioactivity retained in the lungs at any time was associated with subcellular membranous structures, the nuclear and mitochondrial fractions containing the bulk of the compound. Less than 2% of the total radioactivity in the lungs was recovered from the final supernatant. As a function of the time elapsed from the administration of the compound, the percentage of total radioactivity in each sediment did not appreciably change, suggesting no redistribution of HIPDM in subcellular compartments.

The relative specific radioactivity of HIPDM in the subcellular fractions was calculated by dividing the percentage of total radioactivity in each fraction by the corresponding percentage of total protein [23]. The mean values of HIPDM relative specific radioactivity in the fractions are given in table 2. A more illustrative analysis of the results is obtained by plotting the mean relative specific radioactivity of the fractions against their

### Table 2. HIPDM and protein in subcellular fractions of rabbit lung

<table>
<thead>
<tr>
<th>Fraction</th>
<th>HIPDM %</th>
<th>Protein %</th>
<th>HIPDM %/protein %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear</td>
<td>55±4</td>
<td>37±7</td>
<td>1.6±0.3</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>31±2</td>
<td>5±1</td>
<td>6.5±1.2</td>
</tr>
<tr>
<td>Lysosomal</td>
<td>7.1±1.8</td>
<td>1.8±0.5</td>
<td>4.1±1.1</td>
</tr>
<tr>
<td>Microsomal</td>
<td>4.8±1.6</td>
<td>3.7±0.9</td>
<td>1.4±0.6</td>
</tr>
<tr>
<td>Supernatant</td>
<td>1.9±0.9</td>
<td>53±8</td>
<td>0.04±0.01</td>
</tr>
</tbody>
</table>

HIPDM and protein in each subcellular fraction are expressed as the percentage of their respective cumulative amounts recovered from all fractions. HIPDM %/protein %: HIPDM relative specific radioactivity. Data are presented as mean±SD of 22 determinations carried out at preselected times from 2 to 300 min after HIPDM i.v. injection. For individual time points and further definitions see legend to table 1.
The above findings are consistent with the observation that rat and rabbit lungs are devoid of diamine oxidase activity [34]. Hence, the observed rate of clearance of radioactivity from the rabbit lung mostly reflects the behaviour of unmetabolized HIPDM. The pulmonary clearance of HIPDM in rabbits is similar to that observed in humans by external detection [20]. In fact, the mean time of the slower exponential component of HIPDM lung efflux in rabbits (6.2 h) approximates the value computed in normal nonsmokers (6.7±0.6 h) [20]. The amount of tracer cleared with the slower exponential component accounts for approximately 30% of the injected radioactive. Thus, in the rabbit lung, a sizeable fraction of HIPDM is retained in a slowly effluxable pool. The formation of a slowly effluxable pool has been described, in isolated-perfused lungs, for a variety of basic amines [8–11, 14]. In these experiments, the size of the slowly effluxable pool was similar to that observed for HIPDM, ranging 30–55% of the drug accumulated by the lungs [8, 9, 11, 14]. For imipramine, a pulmonary persistent pool with a mean residence time in excess of 5 h has also been demonstrated in vivo in rabbits receiving i.v. bolus injection of the labelled compound [13].

Because only those basic amines with amphiphilic character are retained by the lungs in a slowly effluxable pool, it has been speculated that such a persistent pool could result from the interaction of these molecules with lung phospholipids, namely with the alveolar surfactant [1, 2, 8, 13]. The present data do not support this hypothesis, inasmuch as it was found that very little HIPDM radioactivity was associated with alveolar surfactant [1, 2, 8, 13]. In the rabbit lung, most of the HIPDM radioactivity recovered from the lung mostly reflects the behaviour of unmetabolized HIPDM. The pulmonary clearance of HIPDM in rabbits is similar to that observed in humans by external detection [20]. In fact, the mean time of the slower exponential component of HIPDM lung efflux in rabbits (6.2 h) approximates the value computed in normal nonsmokers (6.7±0.6 h) [20]. The amount of tracer cleared with the slower exponential component accounts for approximately 30% of the injected radioactive. Thus, in the rabbit lung, a sizeable fraction of HIPDM is retained in a slowly effluxable pool. The formation of a slowly effluxable pool has been described, in isolated-perfused lungs, for a variety of basic amines [8–11, 14]. In these experiments, the size of the slowly effluxable pool was similar to that observed for HIPDM, ranging 30–55% of the drug accumulated by the lungs [8, 9, 11, 14]. For imipramine, a pulmonary persistent pool with a mean residence time in excess of 5 h has also been demonstrated in vivo in rabbits receiving i.v. bolus injection of the labelled compound [13].

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Discussion

The results of the present study indicate that the radioiodinated basic amine, HIPDM, is extracted by the rabbit lung from which it is cleared at a slow biexponential rate. As shown by previous experiments on the metabolism of HIPDM, more than 90% of the radioactivity recovered from the lungs of rats and rabbits is in the form of unmetabolized compound [17, 18]. By contrast, HIPDM is extensively metabolized in the liver [17, 18]. The above findings are consistent with the observation that rat and rabbit lungs are devoid of diamine oxidase activity [34]. Hence, the observed rate of clearance of radioactivity from the rabbit lung mostly reflects the behaviour of unmetabolized HIPDM. The pulmonary clearance of HIPDM in rabbits is similar to that observed in humans by external detection [20]. In fact, the mean time of the slower exponential component of HIPDM lung efflux in rabbits (6.2 h) approximates the value computed in normal nonsmokers (6.7±0.6 h) [20]. The amount of tracer cleared with the slower exponential component accounts for approximately 30% of the injected radioactive. Thus, in the rabbit lung, a sizeable fraction of HIPDM is retained in a slowly effluxable pool. The formation of a slowly effluxable pool has been described, in isolated-perfused lungs, for a variety of basic amines [8–11, 14]. In these experiments, the size of the slowly effluxable pool was similar to that observed for HIPDM, ranging 30–55% of the drug accumulated by the lungs [8, 9, 11, 14]. For imipramine, a pulmonary persistent pool with a mean residence time in excess of 5 h has also been demonstrated in vivo in rabbits receiving i.v. bolus injection of the labelled compound [13].

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radioactivity was still retained in the purified mitochondrial sediment. Thus, it appears that the mitochondria are important sites for the accumulation of HIPDM in the lungs.

The findings of the present study are in agreement with those of Yoshida et al. [35], who evaluated the subcellular distribution of the basic amines imipramine, quinine, and metoclopramide in the rat lung. The accumulation of these drugs was highest in the crude mitochondrial fraction and was correlated to the degree of lipid solubility, imipramine (the most lipophilic compound) exhibiting the highest relative accumulation in mitochondria [35]. The subcellular distribution of basic amines, described by Yoshida et al. [35], was measured in isolated rat lungs after 60 min of perfusion. In the present experiments, the subcellular distribution of HIPDM was examined in rabbit lungs at various times after in vivo i.v. injection of the labelled compound. It was observed that the preferential distribution of HIPDM in the mitochondrial fraction was constant over a period of time ranging from 2 min to 5 h after the i.v. administration of the compound. Since, during this time HIPDM is slowly cleared from the lungs at a biexponential rate (fig. 2), the above finding suggests that the persistence of HIPDM in the lungs is accounted for by the interaction of the compound with lung mitochondria.

The preferential distribution of basic amines in the mitochondria may result from binding to specific phospholipids, such as cardiolipin and phosphatidylethanolamine, known to be rich in mitochondrial membranes [36]. However, the mitochondria have a distinctive property over any other intracellular organelle, i.e. they express a significant membrane potential with a negative charge inside [37]. Indeed, a proton gradient is established across the inner mitochondrial membrane as a result of active extrusion of protons driven by electron transport chains [37, 38]. This gradient has a chemical component (pH gradient) and an electric component (membrane potential). The mitochondria of mammalian cells express this electrochemical gradient mostly as membrane potential of about -180 mV and, to a lesser extent, as pH gradient of about 1 unit [37]. Lipophilic compounds with a delocalized positive charge (e.g. HIPDM) may, therefore, penetrate the hydrophobic core of the inner mitochondrial membrane and attain an equilibrium across the membrane as predicted by the Nernst equation [37].

The in vivo results of this study support the concept that the mitochondria act as reservoir for the basic amine HIPDM in the lungs and, as such, contribute to the persistence of this compound in the lung tissue. The observation of an increased persistence of HIPDM in the lungs of smokers [20] and of patients with acute respiratory distress syndrome [18] may reflect some biochemical alteration of mitochondrial membranes that warrants further investigation. In this connection, it is worth considering that an unusual retention of the mitochondria-specific, fluorescent probe rhodamine 123 has been described in vitro in a variety of carcinoma cells [39], which express a significantly higher mitochondrial membrane potential as compared to normal epithelial cells. HIPDM or related compounds can be used to further investigate the pulmonary uptake of basic amines in health and in lung disease.

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