Short-Term Dose–Response Characteristics of 2-Iminobiotin Immediately Postinsult in the Neonatal Piglet After Hypoxia-Ischemia

S. Tracey Bjorkman, PhD; Zoe Ireland, PhD; Xiyong Fan, MD, PhD; Willem M. van der Wal, PhD; Kit C.B. Roes, PhD; Paul B. Colditz, MBBS, MBiomedEng, DPhil (Oxford); Cacha M.P.C.D. Peeters-Scholte, MD, PhD

Background and Purpose—To determine the optimal dose of 2-iminobiotin (2-IB) for the treatment of moderate to severe asphyxia in a neonatal piglet model of hypoxia-ischemia.

Methods—Newborn piglets were subjected to a 30-minute hypoxia-ischemia insult and randomly treated with vehicle or 2-IB (0.1 mg/kg, 0.2 mg/kg, or 1.0 mg/kg). aEEG background and seizure activity were scored after hypoxia-ischemia every 4 h until 24 h and at 48 h and neurobehavioral scores were obtained. Brain tissue was collected and processed for analysis of caspase-3 activity, histology, and tyrosine nitration.

Results—A dose range of 0.1 to 1.0 mg/kg/dose of 2-IB improved short-term outcome as demonstrated by an increased survival with a normal aEEG and decreased nitrotyrosine staining in the 2-IB–treated animals, indicating decreased cellular damage. Neurobehavior, caspase-3 activity in thalamus, and histology scores were not significantly different.

Conclusions—Based on survival with a normal aEEG, 0.2 mg/kg 2-IB is likely to be the most appropriate dose for use in future clinical trials in neonates with perinatal hypoxia-ischemia. (Stroke. 2013;44:809–811.)

Key Words: hypoxia-ischemia, brain ■ 2-iminobiotin ■ neuroprotection ■ neonatal nursing ■ nitric oxide synthase

Perinatal hypoxia-ischemia (HI) is a significant cause of neonatal brain injury. Neonatal animal models of HI show that excessive production of nitric oxide (NO), mediated by nitric oxide synthases (NOS), play an important role in the pathogenesis of neuronal injury after HI in the neonate. Three isoforms of NOS exist: the constitutively expressed neuronal NOS, endothelial NOS, and the inducible NOS. In vitro studies have shown that selective inhibition of neuronal NOS and inducible NOS can be achieved by the NOS inhibitor 2-iminobiotin (2-IB).

To transition treatment to the human term neonate, it is important to know the dose–response effect of 2-IB to identify the optimal dose to be given after perinatal HI. The aim of this study was to determine the dose–response characteristics of 2-IB in a preclinical animal model of perinatal HI and to establish the most effective dose (range) for future clinical trials. This study was performed in our piglet model of inhalational HI, which is clinically, electrophysiologically, and neuropathologically comparable with the term born human neonate.

Methods

Please see the online-only Data Supplement for expanded Methods. Experiments were performed in accordance with National Health and Medical Research Council guidelines (Australia) and approved by the University of Queensland Animal Ethics Committee.

The HI insult was performed in term neonatal piglets (n=47) as previously described; 6 animals served as sham-operated controls. HI piglets were randomly assigned to blinded treatment with vehicle or 2-IB at 0.1, 0.2, or 1 mg/kg/dose i.v. immediately post-HI and dosing repeated every 4 h until 20 h (6 doses in total). aEEG background pattern, presence of epileptic activity, and neurobehavior were scored.

At 48 h postinsult animals were euthanized and tissue analyzed for caspase-3 activity, tyrosine nitration, and histology (see the online-only Data Supplement).

Results

In total, 47 piglets were subjected to HI; 16 piglets were only mildly affected (continuous normal voltage at 30 min post-HI; see the online-only Data Supplement for examples) and excluded from further analysis. Of the remaining 31 piglets, 10 were vehicle-treated, 7 received 0.1 mg/kg/dose, 9 received 0.2 mg/kg/dose, and 5 received 1.0 mg/kg/dose. There was no difference in birth weight, postnatal age, pH, arterial BE, P O2, P O2, duration of hypotension, heart rate, or temperature between treatment groups (see the online-only Data Supplement).
There was a significant overall effect of 2-IB on survival with a normal aEEG at 48 h ($P=0.0047$; Table 1). Treatment was effective in all 3 dosing groups, with the 0.2 mg/kg/dose group showing the highest proportion of surviving animals with a normal aEEG at 48 h. 2-IB treated piglets also demonstrated significantly less tyrosine nitration in thalamus, parietal, and temporal cortex at all doses versus vehicle-treated piglets (Figure). No nitrotyrosine-modified substrates were observed in sham-operated piglets.

Caspase-3 activity in thalamus was not significantly different between groups ($P=0.096$; Table 2). aEEG-patterns over time are shown in the online-only Data Supplement. Electrographic seizure activity was detected in all HI-injured piglets from about 4 h onwards until 48 h but was abolished in the 0.2 and 1.0 mg/kg dose groups between 24 and 48 h. Neurobehavioral and histology scores did not show significant differences (see the online-only Data Supplement).

### Discussion

The aim of the present study was to determine the short-term dose–response characteristics of 2-IB for treatment of moderate to severe perinatal HI. Animals treated with 2-IB demonstrated greater survival with a normal aEEG at 48 h and reduced tyrosine nitration. Decreased nitrotyrosine staining supports the NO pathway as a potential mechanism of neonatal HI injury. The aEEG background pattern is known to be an early predictor of brain injury in term infants with hypoxic-ischemic encephalopathy. Because piglet HI brain injury closely mimics that of human HI brain injury, the effect of 2-IB on aEEG background pattern can be considered a good biomarker of clinical outcome. In our piglet model, 2-IB treatment promoted recovery of aEEG background pattern and reduced epileptic activity.

In human term-equivalent rats, 2-IB neuroprotection has been demonstrated at 10 mg/kg/dose when administered s.c. three times in 24 h. Previous studies in newborn piglets examined only 0.2 mg/kg/dose and reported neuroprotection at 24 h with 6 doses (i.v.) every 4h. Our current study supports that the full dose range of 0.1 to 1.0 mg/kg 2-IB is safe; the 0.2 mg/kg/dose shows the most promising short-term outcome data in our piglet model of perinatal HI. Limitations of this study include the treatment with 2-IB immediately postinsult, and the potential bias caused by the exclusion of mildly affected piglets. Because hypothermia is currently an established treatment for perinatal HI in high-income countries, preclinical studies of combination therapy of 2-IB with delayed hypothermia would be essential before a clinical trial can be considered. For translation into human clinical studies (perinatal HI without hypothermia), we believe that a starting dose

#### Table 1. Proportion of Animals With Normal aEEG at 48 h

<table>
<thead>
<tr>
<th>Drug Dose</th>
<th>Number Survived With Normal aEEG at 48 h After HI</th>
<th>Number Not Survived or Without Normal aEEG at 48 h After HI</th>
<th>Point Estimate for Probability of Survival With Normal aEEG at 48 h</th>
<th>Difference (Treated – Vehicle) With 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>10</td>
<td>0.00 (0.00, 0.31)</td>
<td>Reference</td>
</tr>
<tr>
<td>2-IB (0.1)</td>
<td>3</td>
<td>4</td>
<td>0.43 (0.14, 0.77)</td>
<td>0.43 (0.07, 0.76)</td>
</tr>
<tr>
<td>2-IB (0.2)</td>
<td>6</td>
<td>3</td>
<td>0.67 (0.33, 0.89)</td>
<td>0.67 (0.30, 0.88)</td>
</tr>
<tr>
<td>2-IB (1.0)</td>
<td>1</td>
<td>4</td>
<td>0.20 (0.03, 0.69)</td>
<td>0.20 (−0.13, 0.64)</td>
</tr>
<tr>
<td>Sham</td>
<td>6</td>
<td>0</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Point estimates for each arm as well as estimated differences between 2-IB-treated groups and vehicle, both with 95% confidence interval.

### Figure

Tyrosine nitration at 48 h after hypoxia-ischemia. The amount of nitrated tyrosine (expressed as amount of pixels; mean±SEM) in parietal cortex, temporal cortex, and thalamus was found to differ significantly vs the vehicle-treated group. *$P<0.05$ vs vehicle group; **$P<0.001$ vs vehicle group.
comparable with the 0.2 mg/kg dose in the piglet will be optimal and safe.

Acknowledgments
We thank Stephanie Miller and Dr Ir. Mira Wenker.

Sources of Funding
Funding support was provided from Neurophyxia B.V., National Health and Medical Research Council (Australia), and UQ Center for Clinical Research.

Disclosures
None.

Table 2. Mean Caspase-3 Activity in the Thalamus

<table>
<thead>
<tr>
<th>Drug Dose</th>
<th>Log-Scale</th>
<th>Original Scale</th>
<th>Original Scale</th>
<th>Ratio of Mean Caspase-3 Activity in the Thalamus at 48 h (pmol AMC/min/mg protein)</th>
<th>With 95% CI</th>
<th>With 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>2.60 (1.52, 3.68)</td>
<td>13.48 (4.59, 39.63)</td>
<td>Reference</td>
<td>Mean Caspase-3 Activity in the Thalamus at 48 h (pmol AMC/min/mg protein)</td>
<td>Ratio of Mean Caspase-3 Activity in the Thalamus (Treated/Vehicle)</td>
<td>With 95% CI</td>
</tr>
<tr>
<td>2-IB (0.1)</td>
<td>1.77 (0.81, 2.74)</td>
<td>5.88 (2.24, 15.41)</td>
<td>0.44 (0.10, 1.85)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-IB (0.2)</td>
<td>0.99 (0.11, 1.87)</td>
<td>2.68 (1.11, 6.47)</td>
<td>0.20 (0.05, 0.80)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-IB (1.0)</td>
<td>1.73 (0.76, 2.69)</td>
<td>5.63 (2.15, 14.76)</td>
<td>0.42 (0.17, 1.77)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Point estimates for each arm (on the log and original scale) as well as estimated ratio of geometric means between 2-IB–treated groups and vehicle, all with 95% confidence interval.

References
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Supplemental Methods

Animal Model
Large white newborn piglets (n=53) were obtained from the University of Queensland Gatton Piggery. Average (±SEM) postnatal age and weight was 13.62 h (±0.93) and 1.61 kg (±0.03) respectively.

The HI insult was performed as previously described. In brief, piglets were anaesthetised, ventilated and an umbilical arterial catheter inserted for monitoring blood pressure and arterial blood gases. Hypoxia was induced (n=47) by decreasing inspired oxygen (O_2) to 4% for 30 min and decreased to 2% if low amplitude EEG (laEEG; <5 µV) was not reached within the first 4 min; O_2 was manipulated as necessary to maintain mean arterial blood pressure (MABP) >70% baseline, heart rate >130 bpm and laEEG <5 µV. Hypotension was induced for the final 10 min of the HI insult by decreasing O_2 if necessary until MABP was <70% of baseline. Six animals served as sham-operated controls undergoing all procedures except the HI insult. Animals were housed in pairs following recovery from anaesthesia until euthanasia at 48 h post-HI.

Post-insult monitoring of aEEG and seizures
The aEEG and seizure monitoring protocol can be found in detail elsewhere. aEEG (BRM2; Natus, San Carlos USA) was recorded for 30 min every 4 h during the first 24 h after HI, and at 48 h post-insult. Blinded analysis of the aEEG was performed off-line using Analyze software (BrainZ Instruments). aEEG background pattern was scored as continuous normal voltage (CNV), discontinuous normal voltage (DNV), burst suppression (BS), continuous low voltage (CLV) or flat trace (FT) and, presence of epileptic activity scored as no seizures (NS), single seizure (SS), repetitive seizures (RS) or status epilepticus (SE). Animals were observed during all aEEG recordings and during feed times for presence of clinical seizures. Clinical seizures were treated with phenobarbitalone (20 mg/kg i.v., Sigma, Croydon, VIC, Australia) and midazolam (0.2 mg/kg i.v., Sandoz, Pyrmont, NSW, Australia). If seizures continued, piglets were euthanased with an overdose of pentobarbitalone (Sodium Pentobarbitalone, Virbac, NSW, Australia, 325mg/ml, 2ml/kg).

Neurobehavioural scoring
Animals were assessed for neurobehaviour at 4, 8, 12, 16, 20, 24 and 48 h as previously described. Animals were assessed on nine measures such as level of consciousness, respiration, ability to stand and walk, the righting reflex and presence of clinical seizures. Each measure was assigned a score of 2=normal, 1=moderately abnormal or 0=pathologic. Measures were totalled to achieve a maximal score of 18=normal.

Pharmacokinetic analysis
Blood samples for pharmacokinetic (PK) analysis were taken at the following time points: 15, 30, and 60 min after infusion of the first dose, just before infusion of the second dose, and 15 min, 30 min, 1 h, and 4 h after infusion of the 6th dose. CSF samples were taken 15 minutes and 4 h after the 6th dose, and at 48 h. Concentrations of 2-IB in plasma and CSF were determined using HPLC (Waters Corporation, Milford, MA, USA). For each dose, all PK parameters were calculated from curves constructed from each animal. Non-compartmental analysis was applied using the constant infusion model and the validated WinNonlin® 5.2 program (Pharsight Corporation, Mountain View, CA, USA). C_{max} (maximum plasma concentration), AUC_{last} (area under the plasma concentration-time curve from time of
administration until the last measurable plasma concentration) and AUC∞ (area under the curve after a single dose from time of administration until infinity) were determined. The lower limit of quantification (LLOQ) of 2-IB in plasma and CSF was 5 ng/mL. Values below LLOQ after Cmax were excluded from the PK evaluation.

**Tissue collection**
At 48 h piglets were anaesthetised (1-2% isoflurane using a facemask), intraperitoneally injected with an overdose of pentobarbitone (Sodium Pentobarbitone, Virbac, NSW, Australia, 325mg/ml, 2ml/kg) and perfused intracardially with saline to remove blood from the brain. Brains were removed and sliced coronally (3-4 mm). Sections from the right hemisphere were immersion fixed in 4% paraformaldehyde overnight while sections from the left hemisphere were dissected into frontal, parietal, temporal, occipital cortex, striatum, hippocampus and thalamus, snap frozen and stored at -80°C.

**Assay of caspase-3 activity**
Tissue pieces were homogenized in 10 volumes of ice-cold 50 mmol/L Tris-HCl/ 5 mmol/L EDTA (pH 7.3). Protein concentrations were determined by BCA protein assay (Pierce BCA Kit, Thermo Scientific, Rockford, IL, USA). Activated caspase-3 activity was determined by cleavage of DEVD-AMC at 25°C (RT) with the Multimode Analysis Software and Paradigm detection platform (Bechman Coulter Australia Pty Ltd, Gladesville, NSW, Australia) and expressed as pico moles AMC released/milligram protein/minute.5,6

**Histology**
Paraffin-embedded tissue sections (4µm) were stained with haematoxylin and eosin (HE) to assess neuronal injury. Blinded examination of thalamus, hippocampus, striatum, frontal, parietal, temporal and occipital cortex was undertaken and injury graded 0–9 with zero representing no injury and nine representing severe injury.1 For each region, two sections with an interval of 40 µm were scored and averaged. Sections were scored according to degree of morphological changes as follows (see Table S1): 0=no injury; 1-3 neuronal necrosis (damage to individual neurons); 4-6 laminar necrosis (damage to a group or layer of neurons); 7-9 confluent infarct (damage to all cells within a defined area). Total histological injury score was the sum of all brain region scores (maximum possible score=63).

**Immunohistochemistry**
Sections were incubated with rabbit anti-nitrotyrosine polyclonal antibody (1:200, Millipore Australia Pty. Ltd, North Ryde, NSW, Australia) followed by incubation with goat-anti-rabbit secondary antibody (Vector-Labs, Burlingame, CA) and revealed using diaminobenzamidine (DAB - Sigma Chemical Co.-Aldrich). Full section images (resolution 600 dpi) were scanned, made binary and degree of nitrotyrosine staining measured in parietal and temporal cortex, striatum and thalamus with ImageJ 1.42q software as described previously (two sections with an interval of 40 µm were scored and averaged).7

**Statistical analysis**
Piglets with moderate to severe brain injury whose aEEG pattern did not recover to CNV within 30 minutes after the HI event were included for analysis. Primary outcomes were survival to 48 h with a normal aEEG (CNV) and activated caspase-3 activity in thalamus at 48 h after HI. The primary outcomes were analysed by logistic regression (survival with normal aEEG at 48 h) or analysis of variance (on log transformed caspase-3 activity), including treatment arm (excluding sham animals) as factor. An overall test of differences between treatment arms was performed at a two-sided significance level of 5%.
Subsequently, point estimates and 95% confidence intervals (CI) comparing 2-IB dose arms versus vehicle were determined. In addition, within group point estimates with 95% CI for each treatment arm were determined. A difference on log scale directly translates into a ratio (with CI) on the original scale. Hence, differences between groups for caspase-3 activity are also presented as ratios with 95% confidence intervals.
Supplemental Figures

Figure S1: Example of a) mild HI and recovery of the aEEG within 30 min after the insult and b) moderate to severely asphyxiated piglet. Mild piglets were excluded while moderate to severely asphyxiated piglets were included in the analysis.

Figure S2: Mean aEEG patterns over time. Score 1=continuous normal voltage, score 2=discontinuous normal voltage, score 3=burst suppression, score 4=continuous low voltage, score 5=flat trace.
Figure S3: Neurobehavioural scores. Animals were scored at 4 hourly intervals during the first 24 h and at 48 h. Scores are expressed as mean±SEM. *p<0.05 Sham c.f. 0.1mg/kg, 0.2mg/kg and vehicle group. **p<0.01 Vehicle c.f. 0.2mg/kg group; sham c.f. 0.2mg/kg, 1.0mg/kg and vehicle group. ***p<0.001 Sham c.f. 1.0mg/kg and vehicle group.
### Supplemental Tables

#### Table S1: Histology scoring system

<table>
<thead>
<tr>
<th>Neuropathological score</th>
<th>Percentage of area affected</th>
<th>Morphological changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>No injury</td>
</tr>
<tr>
<td>1</td>
<td>&lt; 20</td>
<td>Neuronal necrosis</td>
</tr>
<tr>
<td>2</td>
<td>20-50</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&gt; 50</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>&lt; 20</td>
<td>Laminar necrosis</td>
</tr>
<tr>
<td>5</td>
<td>20-50</td>
<td>Confluent infarct</td>
</tr>
<tr>
<td>6</td>
<td>&gt; 50</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>&lt; 20</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>20-50</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>&gt; 50</td>
<td></td>
</tr>
</tbody>
</table>

#### Table S2: Physiological variables (mean ± SEM) at the end of the HI insult.

<table>
<thead>
<tr>
<th>Drug dose</th>
<th>pH</th>
<th>Actual BE</th>
<th>pO2</th>
<th>pCO2</th>
<th>MABP &lt; 30 mm Hg</th>
<th>HR</th>
<th>temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>6.97±0.04</td>
<td>-19.38±0.83</td>
<td>14.1±1.5</td>
<td>47.7±5.0</td>
<td>9.28±1.35</td>
<td>160.3±8.5</td>
<td>38.4±0.1</td>
</tr>
<tr>
<td>2-IB (0.1)</td>
<td>7.01±0.03</td>
<td>-19.0±1.1</td>
<td>16.5±1.4</td>
<td>46.6±3.5</td>
<td>8.74±1.94</td>
<td>153.5±6.7</td>
<td>38.3±0.1</td>
</tr>
<tr>
<td>2-IB (0.2)</td>
<td>7.00±0.03</td>
<td>-18.6±1.2</td>
<td>15.3±1.4</td>
<td>47.5±3.0</td>
<td>7.36±1.73</td>
<td>140.1±9.6</td>
<td>38.4±0.1</td>
</tr>
<tr>
<td>2-IB (1.0)</td>
<td>7.05±0.03</td>
<td>-18.25±1.33</td>
<td>19.0±2.2</td>
<td>45.9±4.0</td>
<td>8.87±2.23</td>
<td>160.3±10.9</td>
<td>38.5±0.1</td>
</tr>
<tr>
<td>Sham</td>
<td>7.48±0.02</td>
<td>+5.91±1.0</td>
<td>102.5±7.5</td>
<td>40.4±0.9</td>
<td>0</td>
<td>152.5±5.8</td>
<td>38.7±0.1</td>
</tr>
</tbody>
</table>

BE=base excess, HR=heart rate, MABP<30 mmHg=time (min) mean arterial blood pressure <30 mmHg during the HI insult.

#### Table S3: PK analysis for 2-iminobiotin.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>CSF</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose level First infusion</td>
<td>mg/kg</td>
<td>0.10</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>ng/mL</td>
<td>n/a</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>ng/mL</td>
<td>n/a</td>
</tr>
<tr>
<td>$AUC_{\text{last}}$</td>
<td>hr*ng/mL</td>
<td>n/a</td>
</tr>
<tr>
<td>$AUC_{\text{last}}$</td>
<td>hr*ng/mL</td>
<td>n/a</td>
</tr>
<tr>
<td>Last infusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>ng/mL</td>
<td>n/a</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>ng/mL</td>
<td>n/a</td>
</tr>
<tr>
<td>$AUC_{\text{last}}$</td>
<td>hr*ng/mL</td>
<td>n/a</td>
</tr>
<tr>
<td>$AUC_{\text{last}}$</td>
<td>hr*ng/mL</td>
<td>n/a</td>
</tr>
</tbody>
</table>
Table S4: Histology score (mean ± SEM) at 48 h after HI. Treatment with any dose of 2-IB did not significantly alter histological outcomes c.f. vehicle treated piglets

<table>
<thead>
<tr>
<th>Drug dose</th>
<th>Frontal cortex</th>
<th>Parietal cortex</th>
<th>Temporal cortex</th>
<th>Occipital cortex</th>
<th>Basal ganglia</th>
<th>Thalamus</th>
<th>Hippo-campus</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>5.4±1.1</td>
<td>6.4±1.3</td>
<td>4.7±1.7</td>
<td>6.5±1.0</td>
<td>4.9±1.7</td>
<td>3.0±1.3</td>
<td>5.4±1.9</td>
</tr>
<tr>
<td>2-IB 0.1</td>
<td>4.4±1.4</td>
<td>5.0±1.5</td>
<td>4.1±1.4</td>
<td>4.9±1.5</td>
<td>3.4±1.7</td>
<td>1.8±0.1</td>
<td>5.8±1.4</td>
</tr>
<tr>
<td>2-IB 0.2</td>
<td>3.7±1.0</td>
<td>5.2±0.8</td>
<td>3.0±0.9</td>
<td>4.6±0.4</td>
<td>4.5±1.0</td>
<td>1.4±0.2</td>
<td>4.9±1.3</td>
</tr>
<tr>
<td>2-IB 1.0</td>
<td>5.4±1.3</td>
<td>5.7±1.3</td>
<td>3.7±1.4</td>
<td>5.9±1.1</td>
<td>5.1±1.7</td>
<td>2.3±0.4</td>
<td>5.7±1.1</td>
</tr>
</tbody>
</table>

Sham animals received a score of 0 (data not shown).
Supplemental References


