A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure

(chemoprotection/enzyme induction/isothiocyanates/sulforaphane/quinone reductase)

YUESHENG ZHANG*, PAUL TALALAY**, CHEON-GYU CHO†, AND GARY H. POSNER‡

*Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; and ‡Department of Chemistry, The Johns Hopkins University School of Arts and Sciences, Baltimore, MD 21218

Contributed by Paul Talalay, December 19, 1991

ABSTRACT Consumption of vegetables, especially cruciferous vegetables, reduces the risk of developing cancer. Although the mechanisms of this protection are unclear, feeding of vegetables induces enzymes of xenobiotic metabolism and thereby accelerates the metabolic disposal of xenobiotics. Induction of phase II detoxication enzymes, such as quinone reductase [NAD(P)H:quinone-acceptor] oxidoreductase, EC 1.6.99.2] and glutathione S-transferases (EC 2.5.1.18) in rodent tissues affords protection against carcinogens and other toxic electrophiles. To determine whether enzyme induction is responsible for the protective properties of vegetables in humans requires isolation of enzyme inducers from these sources. By monitoring quinone reductase induction in cultured murine hepatoma cells as the biological assay, we have isolated and identified (−)-1-isothiocyanato-(4R)-(methylsulfinyl)butane [CH₃-SO—(CH₂)₄—NCS, sulforaphane] as a major and very potent phase II enzyme inducer in SAGA broccoli (Brassica oleracea italica). Sulforaphane is a monofunctional inducer, like other anticarcinogenic isothiocyanates, and induces phase II enzymes selectively without the induction of aryl hydrocarbon receptor-dependent cytochromes P-450 (phase I enzymes). To elucidate the structural features responsible for the high inducer potency of sulforaphane, we synthesized racemic sulforaphane and analogues differing in the oxidation state of sulfur and the number of methylene groups: CH₃—SO—(CH₂)ₙ—NCS, where n = 0, 1, or 2 and n = 3, 4, or 5, and measured their inducer potencies in murine hepatoma cells. Sulforaphane is the most potent inducer, and the presence of oxygen on sulfur enhances potency. Sulforaphane and its sulfide and sulfone analogues induced both quinone reductase and glutathione transferase activities in several mouse tissues. The induction of detoxication enzymes by sulforaphane may be a significant component of the anticarcinogenic action of broccoli.

Individuals who consume large amounts of green and yellow vegetables have a lower risk of developing cancer (1–3). Feeding of such vegetables to rodents also protects against chemical carcinogenesis (4, 5), and it results in the induction in many tissues of phase II enzymes—e.g., quinone reductase (QR; NAD[P]H:quinone-acceptor) oxidoreductase, EC 1.6.99.2] and glutathione S-transferases (EC 2.5.1.18) (11–17). Although much evidence suggests that induction of these enzymes is a major mechanism responsible for this protection (18–20), the precise role of enzyme induction in protection of humans requires clarification. The preceding report (21) showed that measurement of QR activity in Hepa 1c1c7 murine hepatoma cells provides a rapid, reliable, and convenient index of phase II enzyme inducer activity in vegetables. Using this assay (21–24), we found that cruciferous vegetables (broccoli, cauliflower, mustard, cress, Brussels sprouts) were a rich source of inducer activity. We chose to investigate broccoli (Brassica oleracea italica) specifically because it is consumed in substantial quantities by Western societies and has been shown to contain abundant phase II enzyme inducer activity (21). In this paper we describe the isolation and identification of a potent major phase II enzyme inducer from broccoli.

MATERIALS AND METHODS

Source of Vegetables and Preparation of Extracts. SAGA broccoli was grown by Andrew Ayer (Maine Packers, Caribou, ME). SAGA is synonymous with Mariner broccoli (Petoseed, Arroyo Grande, CA) and was adapted for growing in Maine by Smith, Ayer, Goughan, and Arrow. The broccoli was harvested when ripe, frozen immediately, shipped to our laboratory in dry ice, and stored at −20°C until processed.

For preliminary survey of inducer activity in broccoli samples, florets were homogenized with 2 vol of water at 4°C, and the resultant soups were lyophilized to give powders, which were stored at −20°C. Portions (400 mg) of these powders were extracted for 6 hr with 14 ml of acetonitrile in glass-stoppered vessels on a wrist-action shaker at 4°C. The extracts were filtered through a sintered glass funnel and evaporated to dryness in a rotating evaporator (<40°C). The residues were dissolved or suspended in 100 μl of dimethyl formamide and assayed for inducer activity.

Assay of Inducer Activity. Inducer potency for QR was measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates (21, 24). The cells (10,000 per well) were grown for 24 hr and then exposed to inducer for 48 hr. Usually 20 μl of the solutions to be assayed (in acetonitrile or dimethyl formamide) was added to 10.0 ml of medium and 2-fold serial dilutions were used for the microtiter plates. The final organic solvent concentration was always less than 0.2% by volume. One unit of inducer activity is defined as the amount that when added to a single microtiter well (containing 150 μl of medium) doubles the QR specific activity. The inducer potency of compounds of known structure has been determined in the above system also, and it is expressed as

Abbreviations: QR, quinone reductase [NAD(P)H:quinone-acceptor] oxidoreductase, EC 1.6.99.2]; CD value, the concentration of a compound required to double the quinone reductase specific activity in Hepa 1c1c7 murine hepatoma cells.

*To whom reprint requests should be addressed.

†Enzymes of xenobiotic metabolism belong to two families (6): (i) phase I enzymes (e.g., cytochromes P-450), which functionize compounds, usually by oxidation or reduction; although their primary role is to detoxify xenobiotics, several cytochromes P-450 can activate procarcinogens to highly reactive ultimate carcinogens (7); and (ii) phase II enzymes, which conjugate functionalized products with endogenous ligands (e.g., glutathione, glucuronic acid, sulfate) and play primarily a detoxification role (8). QR is considered a phase II enzyme because it serves protective functions (9), is induced coordinately with other phase II enzymes, and is regulated by enhancer elements similar to those that regulate glutathione transferases (10).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. $1734 solely to indicate this fact.
the concentration required to double (CD value) the QR activity.

The inductions of QR and glutathione transferase activities in mouse organs were studied according to a standard protocol (25).

Synthesis of Compounds. (R,S)-Sulforaphane (CAS 4478-93-7) was prepared according to Schmid and Karrer (26) except that gaseous thiomethanol was replaced by sodium thiomethoxide. The sulfide analogues, CH3—S—(CH2)n—NCS, where n is 4 [erucin (CAS 4430-36-8)] or 5 [berteroin (CAS 4430-42-6)] were prepared as described (27), and the three-carbon analogue [iberin (CAS 505-79-3)] was prepared from phthalimidopropyl bromide (26). IR spectra of all three sulfide analogues showed strong absorptions near 2150 cm⁻¹, characteristic of isothiocyanates. ¹H NMR spectra of these compounds show sharp singlets at δ 2.10 ppm (CH3—S group). The sulfoxide analogues where n is 3 [ipherin (CAS 505-44-2)] or 5 [allysin (CAS 646-23-1)] were prepared by the same method as sulforaphane. IR spectra of these compounds showed strong absorptions near 2100 cm⁻¹, assigned to the —NCS group. ¹H NMR spectra also showed sharp singlets around δ 2.5 ppm, consistent with the presence of the CH3—SO₂ group. The sulfone analogues, CH3—SO₂—(CH2)n—NCS, where n is 3 [cherirolin (CAS 505-34-0)], 4 [erysolin (CAS 504-84-7)], or 5 (unreported) were prepared by known methods (28). ¹H NMR (δ ~ 2.9 ppm, for CH3—SO₂) and IR spectra of these compounds were consistent with the structures. Every analogue except 1-isothiocyanato-5-methylsulfonylpentane [CH3—SO₂—(CH2)₅—NCS] has been isolated from plants (29).

RESULTS

Isolation of Inducer Activity. We selected SAGA broccoli for study because acetonitrile extracts of lyophilized homogenates of this variety were especially rich in inducer activity (62,500 units per g) in comparison with other vegetables (21). Fractionation of acetonitrile extracts of SAGA broccoli by preparative reverse-phase HPLC (Fig. 1) with a water/methanol solvent gradient resulted in recovery of 70–90% of the applied inducer activity in the chromatographic fractions. Surprisingly, the majority (about 65–80%) in several chromatographies) of the recovered activity was associated with a single and relatively sharp peak [fractions 18–23; eluted at 64–71% (vol/vol) methanol]. This HPLC procedure was therefore adopted as the first step of the larger-scale isolation of inducer activity.

Lyophilized SAGA broccoli was extracted three times with acetonitrile (35 ml/g) for 6 hr each at 4°C. The pooled extracts were filtered and evaporated to dryness under reduced pressure on a rotating evaporator (<40°C). About 1 g of residue from 640 g of fresh broccoli (64 g of lyophilized powder) contained 3.6 x 10⁶ units of inducer activity. The residue was mixed thoroughly with 120 ml of methanol/water (25/75, vol/vol) and the insoluble fraction was discarded. Although not all of the residue obtained from the extraction was soluble in aqueous methanol, the solvent partition procedure resulted in substantial purification without significant loss of inducer activity. Portions of the extract were dried in a vacuum centrifuge and dissolved in small volumes of dimethyl formamide (0.75–1.0 ml per 50 mg of residue), and 50-mg portions were subjected to HPLC (nine runs) as described in the legend of Fig. 1. Fractions 18–23 from all runs were pooled, evaporated to dryness, applied in acetonitrile to five preparative silica TLC plates (100 x 200 x 0.25 mm), and developed with acetonitrile, which was run to the top of each plate three times. Four major fluorescence-quenching components were resolved, and nearly all (95%) of the inducer activity migrated at Rf 0.4. The active bands were eluted with acetonitrile, pooled, and fractionated by two runs on a second preparative reverse-phase HPLC in a water/

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Reverse-phase HPLC of acetonitrile extract of SAGA broccoli showing the distribution of absorbance at 280 nm, total inducer activity (units per fraction), and dry weight of each fraction. Lyophilized SAGA broccoli floret powder (16 g) was extracted three times (for 6 hr each) with 560-ml portions of acetonitrile on a shaker at 4°C. The extracts were filtered and evaporated to dryness on a rotating evaporator (<40°C). The residue (202 mg) was suspended in 3.0 ml of methanol and filtered successively through 0.45- and 0.22-μm porosity filters. The insoluble material was discarded. The filtrate was assayed for total inducer activity, and a 0.75-ml (50.5-ml aliquot of the methanol extract was subjected to HPLC on a reverse-phase preparative column (Whatman; Partisol 10 ODS-2; 90 x 1.0 cm) equilibrated with methanol/water (30/70, vol/vol), eluted at a rate of 2.0 ml/min, and 6.0-ml fractions were collected. Elution solvent: 30 ml of initial solvent, followed by 330 ml of a convex gradient (Waters Gradient program 5) to 100% methanol, and then by 90 ml of 100% methanol. The fractions were evaporated on a vacuum centrifuge (Savant Speed-Vac Concentrator), and the residues were weighed, redissolved in 0.1 ml of dimethyl formamide, and assayed for inducer activity. The activity applied (0.75 ml = 104,000 units) was recovered principally in fractions 18–23 (84,600 units, 81%), and minor amounts of activity were found in fractions 4, 49, 57, and 65. The total recovery of inducer activity in all fractions was 90% of that applied to the column.
acetonitrile gradient (Fig. 2). Ultraviolet absorption and inducer activity were eluted in a sharp coincident peak (at 66% acetonitrile) that contained all of the activity applied to the column. Evaporation (<40°C) of the active fractions gave 8.9 mg of a slightly yellow liquid, which contained 558,000 inducer units (overall yield 15%) and migrated as a single band on TLC.

**Identification of Inducer.** The identity of the inducer was established by spectroscopic methods and confirmed by chemical synthesis. It is (−)-1-isothiocyanato-(4R)-(methylsulfinyl)butane, known as sulforaphane or sulforaphane (CAS 4478-93-7):

\[
\text{H}_3\text{C} \quad \overset{\text{N}=\text{C}=\text{S}}{\smile}
\]

Sulforaphane has been synthesized (26) and isolated from leaves of hoary cress (30) and from other plants (31), and the absolute configuration has been assigned (32). The closely related olefin sulforaphane [4-isothiocyanato-(1R)-(methylsulfinyl)-1-(E)-butene (CAS 2404-46-8)] has been isolated from radish seeds and other plants (33, 34) and has also been synthesized (35, 36).

The following evidence establishes that (R)-sulforaphane is the inducer isolated from broccoli. UV spectrum (H_2O): \(\lambda_{\text{max}}\) 238 nm, \(\epsilon_{238}\) 910 M\(^{-1}\)cm\(^{-1}\); addition of NaOH (0.1 M) blue-shifted (\(\lambda_{\text{max}}\) 226 nm) and intensified (\(\epsilon_{226}\) 15,300 M\(^{-1}\)cm\(^{-1}\)) this absorption band, consistent with the behavior of isothiocyanates (37). IR (Fourier transform, neat): strong absorptions at 2179 and 2108 cm\(^{-1}\) and also at 1350 cm\(^{-1}\), characteristic of isothiocyanates (27). \(^1\)H NMR (400 MHz, C\(_2\)DCl\(_3\)): \(\delta\) 3.60 (t, 2H, \(J = 6.1\) Hz, \(-\text{CH}_2\)−NCS), 2.80−2.66 (m, 2H, \(-\text{CH}_2\)-SO−), 2.60 (s, 3H, \(-\text{CH}_3\)-SO−), and 1.99−1.86 ppm (m, 4H, \(-\text{CH}_2\)-CH\(_2\)). \(^{13}\)C NMR (400 MHz, C\(_2\)HCl\(_3\)): \(\delta\) 53.5, 44.6, 38.7, 29.0, and 20.1 ppm. Mass spectrometry (fast atom bombardment; thiglycerol matrix) gave prominent peaks at 178 (M + H\(^+\)) and 355 (M\(_2\) + H\(^+\)).

Electron impact mass spectrometry gave a small molecular ion (M\(^+\)) at 177, and chemical ionization mass spectrometry gave a small molecular ion (M + H\(^+\)) at 178 and prominent fragment ions with masses of 160, 114, and 72, consistent with the following fragmentation:

\[
\text{H} \quad \text{C} \quad \text{S} \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{N} \quad \text{C} \quad \text{S}
\]

Precise masses of molecular and fragment ions obtained by electron impact mass spectrometry were 177.0286 (calculated for C\(_4\)H\(_9\)NOS\(_2\)), 177.0283 (calculated for C\(_4\)H\(_9\)NOS\(_2\)), and 71.9909 (calculated for C\(_4\)H\(_9\)NOS\(_2\)). In addition, for the mass 160 fragment, the peaks at 161 (M + 1) and 162 (M + 2) were 8.43% (calculated, 8.44%) and 9.45% (calculated, 10.2%), respectively, of the parent ion. Similarly, for the mass 72 fragment, the peaks at 73 (M + 1) and 74 (M + 2) were 3.42% (calculated, 3.32%) and 5.23% (calculated, 4.44%), respectively, of the parent ion. Hence the isotope compositions corrected for the natural isotope abundance (of \(^{13}\)C, \(^{15}\)N, \(^{33}\)S, and \(^{43}\)S) were consistent with the relative intensities of the M + 1 and M + 2 ions of both fragments. The optical rotation of the isolated material was [\(\alpha\)\]\(_D\) = −63.6\(^\circ\) (c = 0.5, CH\(_2\)Cl\(_2\)), thus establishing that the product is largely, if not exclusively, the (−)-(R) enantiomer (literature [\(\alpha\)\]\(_D\) = −79\(^\circ\), −73.2\(^\circ\), −66\(^\circ\); refs. 26, 30, and 38, respectively). The spectroscopic properties of synthetic (R,S)-sulforaphane were identical to those of the isolated product.

**Relation of Structure to Inducer Activity Among Sulforaphane Analogues.** To define the structural features of sulforaphane (chirality, state of oxidation of sulfur of the thiomethyl group, number of methylene bridging groups) important for inducer activity, we synthesized (R,S)-sulforaphane and the following analogues and measured their inducer potencies: CH\(_3\)-S-(CH\(_2\))\(_n\)-N=C=S (\(n = 3, 4, 5\) or 5); CH\(_3\)-SO-(CH\(_2\))\(_n\)-N=C=S (\(n = 3, 4, 5\) or 5); and CH\(_3\)-SO\(_2\)-(CH\(_2\))\(_n\)-N=C=S (\(n = 3, 4, 5, 6\)).

**Induction of QR in Murine Hepatoma Cells.** The chirality of the sulfoxide does not affect inducer potency, since isolated (R)-sulforaphane and synthetic (R,S)-sulforaphane gave closely similar CD values of 0.4−0.8 \(\mu\)M. Sulforaphane is therefore the most potent monofunctional (see below) inducer that has been identified (19, 20). Both (R)- and (S)-sulforaphane were relatively nontoxic: the concentrations required to depress cell growth to one-half were 18 \(\mu\)M.

Sulforaphane and the corresponding sulfone (erysolin) were equipotent as inducers of QR, whereas the corresponding sulfide (erucin) was about one-third as active (Table 1). Oxidation of the side-chain sulfide to sulfone or sulfone enhanced inducer potency, and compounds with 4 or 5 methylene groups in the bridge linking CH\(_3\)S= and =N=C=S were more potent than those with 3 methylene groups (Table 1).

Mutants of Hepa 1c17 cells defective in the Ah (aryl hydrocarbon) receptor or expression of cytochrome P-450IA1 can distinguish monofunctional inducers (which induce phase II enzymes selectively) from bifunctional in-
When sulforaphane (defective in and foraphane activities of cytochromes P-450IA1), induction of QR was normal (data not shown). Sulforaphane is, therefore, like benzyl isothiocyanate, a monofunctional inducer (40) and is unlikely to elevate activities of cytochromes P-450 that could activate carcinogens.

Induction of QR and Glutathione Transferase Activities in Mice. When synthetic (R,S)-sulforaphane, erucin, and erucin were administered to female CD-1 mice by gavage (25), induction of QR and glutathione transferase activities was observed in the cytosols of several organs (Table 2). Sulforaphane and erucin (in daily doses of 15 μmol for 5 days) raised both enzyme activities 1.6- to 3.1-fold in liver, forestomach, glandular stomach, and mucosa of proximal small intestine, and to a lesser degree in lung. The sulfone (erucin) was more toxic, but even 5-μmol daily doses for 5 days elevated the specific activities of these enzymes in some tissues examined. We therefore conclude that sulforaphane and its analogues not only induce QR in Hepa 1c1c7 murine hepatoma cells but also induce both QR and glutathione transferase activities in a number of murine organs.

Discussion

Two considerations prompt the belief that sulforaphane is a major and probably the principal inducer of phase II enzymes present in extracts of SAGA broccoli. First, high yields of inducer activity were obtained at each step of the isolation, and even in the first HPLC (Fig. 1) more than 60% of the inducer activity was contained in a single chromatographic peak, the biological activity of which could not be fractionated. Second, when a totally independent method of isolation of inducer activity by high-vacuum sublimation of lyophilized broccoli (5 μm Hg pressure, 60–165°C, condenser at −15°C) was used, nearly all the isolated inducer activity was found in the methanol-soluble portion of the sublimate. Moreover, on HPLC (Fig. 2) this sublimed material gave rise to only a single isothiocyanate-containing fraction, which on TLC comigrated with authentic sulforaphane and after further purification by TLC provided a high yield of sulforaphane characterized unequivocally by NMR.

The finding that the majority of the inducer activity of SAGA broccoli probably resides in a single chemical entity, an isothiocyanate, is of considerable interest. Isothiocyanates (mustard oils) and their glucosinolate precursors are widely distributed in higher plants and are especially prevalent among cruciferous vegetables (29). Sulforaphane has been identified in species of Brassica, Erucera, and Iberis (29, 31).

Isothiocyanates have been shown to block chemical carcinogenesis. In rats, 1-naphthyl isothiocyanate suppressed hepatoma formation by 3-methylcholanthrene, 2-acetylamino-7-azafuorene, diethylthioleuamide, m-toluenediamine, and azo dyes (43–46). In mice, benzyl isothiocyanate blocked the neoplastic effects of diethylthioleuamide or benzaldehyde on lung and forestomach (47, 48), and a variety of phenylalkyl isothiocyanates reduced the pulmonary carcinogenicity of

| Table 1. Potency of induction of QR in Hepa 1c1c7 cells by sulforaphane and analogues |
|------------------------------------------|-----------------|
| Compounds | CD value, μM |
| CH$_3$S–(CH$_2$)$_n$N═C═S | n = 3 | n = 4 | n = 5 |
| (CH$_2$)$_4$NCS | 3.5 (Ibererin) | 2.3 (Erucin) | 1.7 (Berteroin) |
| O | 2.4 (Iberin) | 0.4–0.8 (Sulforaphane) | 0.95 (Alyssin) |
| CH$_3$S–(CH$_2$)$_n$N═C═S | 1.3 (Cheirolin) | 0.82 (Erysolin) | 0.98 |

Trivial names are given in parentheses. See Kjaer (29).

Table 2. Induction of QR and glutathione S-transferase (GST) in mouse tissues by sulforaphane and analogues

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Dose, μmol per mouse per day</th>
<th>Ratio of specific activities (treated/control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Forestomach</td>
<td>Glandular stomach</td>
</tr>
<tr>
<td>CH$_3$S–(CH$_2$)$_4$NCS</td>
<td>15</td>
<td>QR</td>
</tr>
<tr>
<td>Erucin</td>
<td>1.86 ± 0.08</td>
<td>2.51 ± 0.11</td>
</tr>
<tr>
<td>CH$_3$S–(CH$_2$)$_4$NCS</td>
<td>15</td>
<td>QR</td>
</tr>
<tr>
<td></td>
<td>GST</td>
<td>1.86 ± 0.08</td>
</tr>
<tr>
<td>Sulforaphane</td>
<td>1.62 ± 0.09</td>
<td>1.05 ± 0.21</td>
</tr>
<tr>
<td>CH$_3$S–(CH$_2$)$_4$NCS</td>
<td>5</td>
<td>QR</td>
</tr>
<tr>
<td>Erysolin</td>
<td>1.62 ± 0.09</td>
<td>1.05 ± 0.21</td>
</tr>
</tbody>
</table>

The compounds were administered to 6-week-old female CD-1 mice (4 or 5 mice per group) by gavage in indicated single daily doses in 0.1 ml of Emulphor EL 620P (GAF, Linden, NJ) for 5 days. Cytosols were prepared from the tissues 24 hr after the last treatment and assayed for enzyme activities (glutathione S-transferase was measured with 1-chloro-2,4-dinitrobenzene). The specific activities (nmol/min·mg⁻¹± SEM) of organs of vehicle-treated control mice were as follows. Liver: QR, 47 ± 0.70; GST, 1014 ± 69. Forestomach: QR, 1038 ± 155; GST, 1182 ± 74. Glandular stomach: QR, 3272 ± 85; GST, 1092 ± 81. Small intestine: QR, 664 ± 119; GST, 1372 ± 266. Lung: QR, 54 ± 5.8; GST, 439 ± 34. Data are presented as mean ± SEM. All ratios were significantly different from 1.0 with P < 0.01, except for *, P < 0.05, and †, P > 0.05.
the tobacco-derived carcinogenic nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone (49, 50). The antcarcinogenic effects of previously studied isothiocyanates may be related to their capacity to induce phase II enzymes, which are involved in the metabolism of carcinogens (51–57).

It will be important to establish whether the alterations of drug metabolism observed in humans and rodents after the ingestion of cruciferous vegetables (58, 59) can be ascribed to their content of sulforaphane. The finding that this isothiocyanate is a major monofunctional inducer of phase II enzymes in broccoli also provides the possibility of clarifying the relationship between enzyme induction and chemoprotection.

We thank Andrew Ayer for supplying generous quantities of SAGA broccoli. These studies were supported by grants from the National Cancer Institute (PO1 CA 44530) and from the American Institute for Cancer Research. All animal experiments were in compliance with National Institutes of Health guidelines (60) and were approved by the Committee on Animal Care of Johns Hopkins University.