Synchrotron X-Ray Fluorescence Microscopy of Gallium in Bladder Tissue following Gallium Maltolate Administration during Urinary Tract Infection

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A mouse model of cystitis caused by uropathogenic Escherichia coli was used to study the distribution of gallium in bladder tissue following oral administration of gallium maltolate during urinary tract infection. The median concentration of gallium in homogenized bladder tissue from infected mice was 1.93 µg/g after daily administration of gallium maltolate for 5 days. Synchrotron X-ray fluorescence imaging and X-ray absorption spectroscopy of bladder sections confirmed that gallium arrived at the transitional epithelium, a potential site of uropathogenic E. coli infection. Gallium and iron were similarly but not identically distributed in the tissues, suggesting that at least some distribution mechanisms are not common between the two elements. The results of this study indicate that gallium maltolate may be a suitable candidate for further development as a novel antimicrobial therapy for urinary tract infections caused by uropathogenic E. coli.

Urinary tract infections (UTIs) are common in humans and dogs, and most of these infections are caused by uropathogenic strains of Escherichia coli (UPEC) (1, 2). In addition to patient discomfort and economic burdens from treatment costs and lost work time, failure to cure these infections can lead to serious complications, including pyelonephritis and septicemia (3). Antimicrobial resistance in UPEC complicates therapy and is of concern for both canine and human patients because these pathogens may be zoonotic (4–6). An increasing prevalence of antimicrobial resistance among UPEC from human and canine patients has created a need for a new approach to treatment (2, 7–9).

Uropathogenic strains of E. coli are distinguished by their ability to invade and persist in the urinary tract (10). By invading the transitional epithelial cells, UPEC are able to evade many host defenses and can persist in the urinary tract despite the presence of bactericidal concentrations of antimicrobial drugs in urine (11, 12). The virulence of UPEC is associated with genes coding for siderophores and siderophore receptors, suggesting that siderophore iron uptake pathways are essential for UPEC survival in the urinary tract (10, 13). Compounds which arrive at the transitional epithelium and interfere with bacterial iron metabolism may therefore be suitable alternatives to traditional antimicrobial therapy.

The element gallium, as gallium(III), is similar in size and behavior to iron(III) (14). Unlike iron(III), gallium(III) is not known to undergo reduction under physiological conditions (14). Gallium binds readily to transferrin, and its transport in mammalian tissues is thought to depend on transferrin receptor pathways (15). The similarities between gallium and iron also affect bacterial systems. In vitro, gallium binds to E. coli siderophores and exerts antimicrobial activity against Gram-negative and intracellular pathogens by disrupting iron metabolism (16–18). The salts gallium citrate and gallium nitrate are used in human medicine for medical imaging and treating hypercalcemia of malignancy, respectively, but they must be administered intravenously due to negligible oral bioavailability. Gallium maltolate, a complex of gallium(III) with three maltol groups, is of particular interest as a potential UTI therapy as it can be administered orally (19).

While gallium distribution has been described in a limited number of tissues, its distribution in the urinary bladder is unknown (15, 20). Confirmation of gallium distribution to the transitional epithelium following oral administration of gallium maltolate will support further investigation of gallium maltolate as a new antimicrobial therapy for UTIs caused by UPEC.

Metal detection by analytical methods such as inductively coupled plasma mass spectrometry require tissue homogenization, possibly with centrifugal separation or fine dissection to achieve a sample suitable for introducing into the instrument. Hard X-ray microprobe analysis is a synchrotron-based analytical technique which includes synchrotron X-ray fluorescence (XRF) imaging and X-ray absorption spectroscopy (XAS). Synchrotrons are electron accelerators which generate intense, focused X-rays that can be used to probe the electron structure of atoms within a variety of sample types, yielding valuable information about the distribution and chemical characteristics of elements within the sample. Elements of interest in intact samples can be localized on a micron scale using XRF imaging, and XAS can be used to determine the species of the elements of interest in regions of interest in the sample (21, 22). Hard X-ray microprobe analysis has been used to evaluate the distribution of elements in a variety of biological samples, including cardiac muscle, macrophages, and neurons (21, 23,

Received 27 March 2013 Returned for modification 4 May 2013 Accepted 14 July 2013 Published ahead of print 22 July 2013 Address correspondence to Katherine R. Ball, katherine.ball@usask.ca. Copyright © 2013, American Society for Microbiology. All Rights Reserved.
As tissue homogenization is not required for analysis, these methods are well suited for investigating the distribution of metal-based drugs within tissues.

The objectives of this study were to use a well-characterized mouse model of UPEC cystitis, XRF imaging, and XAS to confirm the arrival of gallium in the bladder mucosa after oral administration of gallium maltolate. The relationship of gallium and iron distribution was also investigated to gain insight into potential mechanisms for gallium distribution in the bladder.

**MATERIALS AND METHODS**

**Mice.** All procedures involving mice were approved by the University of Saskatchewan Committee for Animal Care (protocol 20080060). Six 8- to 10-week-old female C56BL/6 mice were anesthetized with isoflurane and infected transurethrally with 10⁸ CFU of a canine clinical uropathogenic *Escherichia coli* isolate (M2B, isolated at the Western College of Veterinary Medicine Bacteriology Laboratory) in a volume of 50 µl as previously described. Treatments were started 48 h after infection. Three mice received gallium maltolate (provided by Lawrence Bernstein) in distilled water at 200 mg/kg of body weight by gavage once daily for 5 days (treatment group). The remaining three mice each received distilled water by gavage once daily for 5 days (control group). Two mice in the treatment group died suddenly during handling approximately 24 h following the last treatment, shortly before the remaining mice were euthanized for sampling. Gross necropsy findings included enteritis in one mouse and mild icterus in the other. Euthanasia of the remaining mice was accomplished by isoflurane exposure, and tissues were harvested aseptically from all mice. One-third of each bladder from the treated mice was submitted to the Prairie Diagnostic Services Toxicology Laboratory to quantify gallium concentrations in tissue homogenate by inductively coupled plasma mass spectrometry.

**Confirmation of infection.** Tissues were stored at −20°C prior to microbiological culture, which was performed within 24 h of sample collection. Samples from kidneys and bladders were plated on MacConkey II agar (BD Canada, Mississauga, ON, Canada) and on Trypticase soy agar with 5% sheep blood (BD Canada) and incubated at 37°C for 18 to 24 h. Identification was based on colony type and morphology, Gram-staining characteristics, and growth on MacConkey II agar.

**Synchrotron X-ray fluorescence imaging and X-ray absorption spectroscopy.** X-ray fluorescence maps and X-ray absorption spectra were collected using the Pacific Northwest Consortium/X-ray Science Division (PNC/XSD 20 ID) beamline at the Advanced Photon Source located at Argonne National Laboratory. Formalin-fixed, paraffin-embedded tissue from each mouse was sectioned at 20 µm and mounted on high-purity quartz slides (SPI Supplies, West Chester PA). Adjacent sections cut at 5 to 7 µm and stained with hematoxylin and eosin were used for light microscopy to confirm map locations. For XRF and XAS, incident energy was selected with a double-crystal Si(111) monochromator, with the second crystal detuned by 10% for harmonic rejection. Gallium metal foil was used for energy calibration prior to and during data collection. Kirkpatrick-Baez mirrors were used to focus the beam to 5 µm by 5 µm. X-ray fluorescence data were collected at 11 keV with a 10-s integration time and 5-µm horizontal and vertical steps. A minimum area of 20,000 µm² was mapped for each sample. Gallium, iron, and zinc Kα fluorescence signals were monitored using a 7-element germanium detector (Canberra Industries, Inc., Meriden, CT) positioned at 45° to the sample and at 90° to the incident beam. X-ray absorption spectra at the Ga K-edge were collected from each sample at regions with the strongest Ga signal using 0.5-eV steps and an integration time of 5 s. At least two spectra were collected at each region.

**Data reduction and statistical analysis.** Gallium and iron XRF data were processed using ImageJ software. Due to the proximity of the gallium Kα and zinc Kα emission energy ranges, the gallium data were corrected for the influence of the zinc signal prior to correlation analysis. Counts for gallium Kα emission were collected between 9.04 keV and 9.28 keV, and counts for zinc Kα emission were collected between 8.36 keV and 8.92 keV. The detector resolution was approximately 200 eV over the range of these emission ranges. Assuming that the emission energy had a Gaussian distribution about the tabulated energy for the emission line, counts from the zinc signal falling in the gallium Kα count window were estimated and then subtracted from the counts recorded in the gallium Kα window. The iron signals and zinc-corrected gallium signals were normalized to the incident beam intensity (I₀) prior to plotting element distribution maps. Spearman’s correlation was used to evaluate the relationship between I₀-normalized corrected counts in the gallium Kα emission range and the I₀-normalized counts in the iron Kα emission range at each pixel from XRF maps. This analysis was conducted with a commercial statistical software package (Stata/IC 10.1 for Windows; StataCorp, College Station TX).

**RESULTS**

**Microbiology.** Bacterial growth consistent with *E. coli* was observed from the bladders of all control mice. No bacterial growth was observed from the bladders from mice treated with gallium maltolate. There was no bacterial growth from the kidneys of any mice, indicating that the infection model was limited to cystitis.

**Localization of gallium in bladder.** The median gallium concentration in homogenized tissue samples was 1.93 µg/g (Table 1). X-ray fluorescence at the gallium K-edge was detected in tissue samples from all treated mice. X-ray absorption spectra were collected at the gallium K-edge from the regions with the strongest gallium Kα signal from each mouse (Fig. 1). The sharp increase in X-ray fluorescence around 10.37 keV is characteristic for gallium and confirmed the presence of this element in the mapped regions from the treated mice. The infection point identified on the first derivative plot of the spectra was located at 10.374 keV for all treated mice. The XRF maps revealed a nonhomogeneous distribution of gallium within the mucosa (Fig. 2a and b). Regions of strong fluorescence in the Ga Kα window were not observed in tissues from the control mice. In treated mice, the gallium distribution was similar but not identical to the iron distribution (Table 1 and Fig. 2c and d).

**DISCUSSION**

These results are consistent with previous studies which demonstrated absorption of gallium after oral administration of gallium maltolate. The mean gallium concentration in homogenized bladder samples from treated mice was approximately five times the serum concentrations reported after oral administration.

**TABLE 1** Gallium concentrations in homogenized bladder and urine samples with Spearman’s rho for gallium and iron counts in XRF maps obtained from mice with urinary tract infections following gallium maltolate administration by gavage at 200 mg/kg/day for 5 days.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Gallium concn (µg/g)</th>
<th>Spearman’s rho (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.90</td>
<td>0.60 (&lt;0.0001)</td>
</tr>
<tr>
<td>B</td>
<td>2.30</td>
<td>0.86 (&lt;0.0001)</td>
</tr>
<tr>
<td>C</td>
<td>1.93</td>
<td>0.74 (&lt;0.0001)</td>
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interactions with other atoms and molecules (14). In the pres-
states, and these similarities contribute to similarities in their
chemical characteristics, such as ionic radius and valence
iron and gallium signals from treated mice suggest that, in the
ent study, the Spearman’s rho values of less than 0.9 between
centrations required to inhibit growth of
mediated pathways (14). Gallium and iron share numerous
site of infection.
- axis scale to facilitate observation of the small edge jump at the gallium
K-edge in this sample.
of 50 mg/kg (one-quarter of the dose used in the present study)
(29). The mean tissue concentration in this study exceeds the con-
centrations required to inhibit growth of Pseudomonas aeruginosa
and Mycobacterium tuberculosis in vitro, suggesting that oral ad-
ministration of gallium maltolate can produce potentially clini-
cally relevant gallium concentrations in homogenized bladder tis-
sue (17, 18). Further characterization of the antimicrobial activity
of gallium maltolate against UPEC, including determination of
MICS, will be important to better predict its potential clinical util-
ity for treating urinary tract infections caused by UPEC.
Gallium concentrations in homogenized tissue samples are
useful to rule out gallium compounds as potential therapeutic
agents, since if no gallium is detectable in the sample, it is unlikely
that it achieves relevant concentrations in any part of the tissue. As
homogenized samples contain all tissue layers, researchers cannot
distinguish gallium within the target region of the sample from
gallium in other regions of the sample. The hard X-ray micro-
probe results confirm that gallium reaches the bladder mucosa
after gallium maltolate administration and thus meets one of the
basic requirements of an antimicrobial compound: it arrives at the
site of infection.
Gallium distribution has previously been ascribed in large
part to iron transport mechanisms, particularly transferrin-
mediated pathways (14). Gallium and iron share numerous
chemical characteristics, such as ionic radius and valence
states, and these similarities contribute to similarities in their
interactions with other atoms and molecules (14). In the pres-
ent study, the Spearman’s rho values of less than 0.9 between
iron and gallium signals from treated mice suggest that, in the
bladder, gallium is partially distributed via mechanisms that do
not involve iron. Observations in bone marrow, liver, spleen,
and tumor tissues have similarly indicated that gallium and
iron are not identically distributed in tissues (15). The positive
values obtained for Spearman’s rho suggest that inhibition of
one metal’s distribution by the other is not a large component
of the relationship between gallium and iron in the bladder.
This is consistent with a common high-capacity transport sys-
tem coupled with additional specific pathways to account for
the differences in distribution. The common distribution path-
way may include transferrin and transferrin receptors, as pre-
viously described, while the greater aqueous solubility of gal-
lium than of iron at physiological pH may explain differences
in the distribution of non-protein-bound gallium and iron
(15).
Iron transport in mammalian tissues is heavily dependent
on transferrin and its receptors and on transporters from solute
 carrier family 11, notably SLC11A2 (divalent metal transport-
er-1) and SLC11A3 (ferroportin) (30, 31). Iron uptake via
transferrin receptors involves endocytosis, release from trans-
ferrin, reduction, and subsequent transport of ferrous iron out
of the vesicles by SLC11A2 (32). While SLC11A2 will accept a
variety of divalent cations as substrates, ferric iron must be
reduced to its ferrous form before it can be transported (31). It
is unlikely that gallium, present as a trivalent cation unable to
be reduced under physiological conditions, would be effi-
ciently transported by SLC11A2 or SLC11A3. Characterization
of the transport mechanisms for gallium will be essential for
predicting gallium distribution within other tissue types and
for identifying potential drug-drug or drug-nutrient interac-
tions that could lead to clinically significant alterations in phar-
macokinetics. Additional hard X-ray microprobe studies may
provide critical insight into gallium transport mechanisms by
revealing intracellular distribution and chemistry.
To our knowledge, this is the first application of synchrotron-
based techniques to the identification of antimicrobial distribu-
tion within tissues. The simultaneous mapping of multiple ele-
ments of interest by synchrotron XRF imaging provides informa-
tion not only about the distribution of a single element
but also about the distributional relationships between elements.
This capability is useful at the level of tissues, as was accomplished
in this experiment, and improvements in spatial resolution should
make synchrotron XRF imaging an even more useful method for
tracking elemental distribution within biological samples. In this
study, we used a spatial resolution of 5 μm, which is suitable for
mapping elements in tissues and allows the analysis of larger re-
gions of the sample in the limited available instrument access time.
However, spatial resolutions of less than 100 nm have been re-
ported, with speculation that 30-nm resolution should soon be
possible (33). Accompanied by method refinements to ensure cor-
correct identification of cellular elements, this spatial resolution
should allow the characterization of intracellular elemental distri-
bution without the need to disrupt tissue architecture.
The utility of XAS in hard X-ray microprobe analysis of tissues
extends beyond confirming the presence of a specific element. The
spectra collected in this study, particularly those from mice B and
C, are of sufficient quality to suggest that the determination of
gallium species is possible in these samples. The edge energy in-
flexion point, approximately 7 eV above the tabulated electron
binding energy for the gallium K-edge, is similar to previously

FIG 1  X-ray absorption spectra from regions of strong gallium fluorescence in
bladder tissue from three mice treated with gallium maltolate. Letters beside
the spectra indicate individual mice. The inset for mouse A uses an expanded
y-axis scale to facilitate observation of the small edge jump at the gallium
K-edge in this sample.

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reported observations for Ga_2O_3 and for Ga(NO_3)_3 (34, 35). While these spectroscopy results are preliminary, they demonstrate that near-edge X-ray absorption spectroscopy is possible and that extended X-ray absorption fine-structure spectroscopy (EXAFS) may be possible at the gallium K-edge in tissue samples despite the low gallium concentrations observed during treatment. Additional XAS studies of gallium at the site of infection will allow the refinement of in vitro antimicrobial susceptibility testing strategies by providing the necessary information to more closely reproduce the chemistry of the site of infection.

Along with the negative bladder cultures from the control mice, positive bladder cultures from all of the control mice suggest that gallium maltolate administration leads to antimicrobial activity in the bladder tissues. While only a small number of mice were included in this experiment, these results are consistent with findings by other research groups that gallium maltolate administration reduces bacterial loads in tissues (29, 36). Along with in vitro pharmacodynamic investigations, further study with larger groups of animals is required to more accurately characterize the antimicrobial activity of gallium maltolate in tissues. In addition to this, the unexpected death of two gallium maltolate-treated mice in the present study should prompt toxicological investigations of this compound. While enteritis and icterus are not classical signs of gallium toxicity, we cannot exclude with certainty the potential of toxicity (37). This is particularly true where few toxicology data are available for comparison and where very small groups of animals were involved. Toxicological characterization of gallium maltolate will be essential for determining whether this compound should be considered further as a candidate for clinical use as an antimicrobial therapy.

The distribution characteristics of gallium in the urinary bladder following oral administration of gallium maltolate suggest that this compound may be a suitable candidate for further development as an antimicrobial therapy for UTIs caused by UPEC. This is further supported by its apparent antimicrobial efficacy in a small group of mice, but further efficacy studies using larger groups will be necessary to confirm this observation. Toxicology investigations are indicated to establish whether gallium maltolate may exert unacceptable toxic effects at potentially therapeutic doses. Elucidation of the major uptake and transport mechanisms for gallium will be essential for predicting potential drug-nutrient and drug-drug interactions, as the behavior of iron does not accurately reflect the behavior of gallium. As XRF imaging and in situ XAS techniques evolve to provide spatial resolution in the 30-nm range, studies of the intracellular distribution and chemistry of gallium should aid in directing investigations of the mechanisms of gallium transport and uptake.
ACKNOWLEDGMENTS

We thank J. Y. Ko and Robert Gordon for their assistance with data collection at the Advanced Photon Source and Lawrence Bernstein for providing gallium maltolate.

This research was supported by a Collaborative Health Research Projects grant from NSERC and CIHR. K.R.B. and F.S. are CIHR Fellows in Health Research Using Synchrotron Techniques. K.R.B. is supported by an Interprovincial Graduate Student fellowship. The PNC/XSD facilities at the Advanced Photon Source and research at these facilities are supported by the U.S. Department of Energy—Basic Energy Sciences, a Major Resources Support grant from NSERC, the University of Washington, Simon Fraser University, and the Advanced Photon Source. Use of the Advanced Photon Source, an Office of Science User Facility operated for the U.S. Department of Energy (DOE) Office of Science by Argonne National Laboratory, was supported by the U.S. DOE under contract no. DE-AC02-06CH11357.

Experimental work was conducted at the Western College of Veterinary Medicine (University of Saskatchewan, Saskatoon, Saskatchewan, Canada) and at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL, USA).

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