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ARTICLE *in* NANOTOXICOLOGY · AUGUST 2014

Impact Factor: 6.41 · DOI: 10.3109/17435390.2014.948092

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ORIGINAL ARTICLE

Assessing orally bioavailable commercial silver nanoparticle product on human cytochrome P450 enzyme activity

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Abstract

Nanotechnology produces a wide range of medicinal compounds, including nanoparticulate silver, which are increasingly introduced in various forms for consumer use. As with all medicinal compounds, potential drug interactions are an important consideration for ingested silver nanoparticles. Nanoparticulate silver–drug interactions may be mediated through induced oxidative stress in liver tissue where the majority of systemically bioavailable silver nanoparticles is found. To investigate whether an orally ingested commercially available colloidal silver nanoproduct produces pharmacokinetic interference on select cytochrome P450 enzymes, a prospective, single-blind, controlled *in vivo* human study using simultaneous administration of standardized probes for P450 enzyme classes CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 was conducted. Oral ingestion of a commercial colloidal silver nanoproduct produces detectable silver in human serum after 14 days of dosing. This silver, however, elicits no demonstrable clinically significant changes in metabolic, hematologic, urinary, physical findings or cytochrome P450 enzyme inhibition or induction activity. Given their increasingly broad, diverse human exposures, future characterization of human cytochrome P450 enzyme activity for other systemically bioavailable nanotechnology products are warranted.

Keywords

Clinical study, *in vivo*, nanoparticles, pharmacokinetics, toxicology

History

Received 4 February 2014
Revised 15 July 2014
Accepted 18 July 2014
Published online 19 August 2014

Introduction

More than 1300 manufactured nanotechnology-enabled consumer products are available in the marketplace (The Project on Emerging Nanotechnologies, 2013). Engineered nanomaterials can be found in a variety of health, cosmetic, food and agricultural, recreational equipment and clothing products. This wide-ranging application of nanotechnology increases the likelihood of human exposure to nanomaterials through multiple routes of entry. Significantly, nanotechnology has also engendered tremendous enthusiasm for next-generation medicinal compounds (Villiers et al., 2009). Medicinal nanoproducts may be administered through various routes, including transdermal, ophthalmic, oral, inhaled and parenteral; however, bioavailability, biodistribution and possible accumulation of these nanoparticles have not been extensively reported. The United States Food and Drug Administration (FDA) acknowledges the promise and potential risk for nanoproducts, calling for studies of absorption, distribution, metabolism and excretion as related to drugs, from a staged approach to risk management (Hamburg, 2012). Despite this advocacy, the consequences of increasing human exposure to nanomaterials, both deliberate and inadvertent, and to larger, diverse populations are subject to debate, with little current consensus on the risks, toxicities, risk management and exposure (Becker et al., 2011; Grainger, 2009; Johnston et al., 2010; Oberdörster, 2010).

Nanoscale silver in a variety of forms comprises approximately a quarter of the inventory of current commercially available nanoproducts. Within this inventory, nanoparticulate silver has re-emerged notably as a nutraceutical medical modality (Atiyeh et al., 2007; Roy et al., 2007; Samberg et al., 2011). With centuries of silver therapeutic attributes, silver nanoproducts are claimed to provide unique physiochemical properties and biological activities, broadening its application as antibacterial, antiviral and anti-inflammatory therapy (Bhol et al., 2004; Carlson et al., 2008; Elechiguerra et al., 2005; Takenaka et al., 2001). Current *in vitro* evidence supports cellular toxicity across a diversity of organs in concentrations between 5–50 μg/mL. Specifically, the lung and liver appear to be important target tissues for sustained nanosilver exposure (Frőhlich, 2013; Hussain et al., 2005; Kawata et al., 2009; Lamb et al., 2010; Sung et al., 2008; Takenaka et al., 2001). Non-cytotoxic doses of nanosilver particles are shown to reduce cell mitochondrial function, cell proliferation and induce apoptosis in rat and human liver, and human mesenchymal cell lines *in vitro* (Arora et al., 2009; Lamb et al., 2010). We have previously shown in MDR1.C and Hep G2 cell lines exposed 24 h to a commercial orally formulated commercial 32-ppm silver nanoparticle solution at concentrations where cell viability was maintained (i.e. <1 μg silver/mL) that nanoscale colloidal silver exhibits the potential for drug–drug

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interactions via up-regulation of cytochrome P450 enzymes (Lamb et al., 2010). Commonly, drug–drug interactions are mediated through enzymes and transporters in liver cells and silver ion or nanoparticle-induced oxidative stress observed in rat-derived liver cell cultures supports this possibility (Hussain et al., 2005).

We have also previously reported that a controlled 14-day *in vivo* human oral exposure to the same commercial nanoscale silver nanoparticle solution does not prompt clinically important changes in human metabolic, hematologic, urine, physical findings or imaging morphology compared to placebo (Munger et al., 2013; Smock et al., 2014). Given this, *in vitro* and human *in vivo* background with the same silver nanocolloidal formulation, we sought to understand the potential for this nanoscale silver product to interfere with select cytochrome P450 activity through a prospective, single-blind, controlled *in vivo* human study using simultaneous administration of standardized probes for CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4.

Methods

Study population

This prospective, placebo-controlled, crossover design study was conducted at the University of Utah Lung Health Study Clinic and Center for Clinical and Translational Sciences at the University of Utah Hospital (UUCCTS). Each subject underwent a screening evaluation to assess enrollment eligibility. Male subjects, between 18–50 years of age, were enrolled. The following conditions were grounds for exclusion from the study: a history of any heavy metal allergy; asthma, chronic bronchitis or emphysema; renal impairment defined by a creatinine clearance $\leq$30 mL/min; or significant acute or chronic disease as determined by the investigators. Study subjects were prohibited from doing the following during the study: consumption of any medication or nutraceutical consumed within 20 days prior to each hospitalization for cytochrome P450 determination or within six times the elimination half-life, whichever is longer, except occasional use of acetaminophen; administration of enzyme-inducing drugs (e.g. rifampin), enzyme-inhibiting drugs (e.g. ketoconazole, clarithromyicn and ritonavir) within 3 months of baseline hospitalization; presence of alcohol abuse; excessive consumption of caffeine containing beverages (i.e. $\geq$4 cups or glasses daily) or unable to stop consumption 48 h prior to each UUCCTS stay; smoking >5 cigarettes or equivalent daily or not able to stop smoking 48 h prior to each UUCCTS stay; or ingestion of grapefruit or grapefruit-containing products within 48 h of each UUCCTS stay. Subjects unable to complete the study were excluded from analysis and replaced.

All study subjects were provided written informed consent before participating in any study procedures. The study was conducted in accordance with the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guidelines for Good Clinical Practice and the Declaration of Helsinki, and received approval from the University of Utah Institutional Review Board. This study is registered with ClinicalTrials.gov (Identifier: NCT01405794).

Study product

The silver nanocolloidal study product (manufactured by American Silver, LLC, Alpine, UT) is claimed to be a reduced elemental silver colloidal dispersion in water (refer USP 7,135,195 B2). The silver is in the form of zero-valent elemental silver nanoparticles with a silver oxide surface layer and without exogenous particle stabilizers. The reduced silver nanoparticle size averages 32.8 nm, with a size range of 25–40 nm (32 ppm silver, Lot #071511). Both silver particle and residual silver ion characterization in the product as received for human use is fully described recently (Munger et al., 2013). The average daily ingestion of elemental silver colloid formulation used in this study is estimated to be 480 mcg/day for the selected 32 ppm concentration (Munger et al., 2013).

Study protocol

At baseline (day 0) and end of the 14-day dosing periods, subjects underwent a medical and drug history, complete physical examination, comprehensive metabolic panel, blood count with differential and urinalysis to determine eligibility for the study (Figure 1). Laboratory tests were completed at Associated Regional and University Pathology, Inc., Salt Lake City, UT. In addition, blood and urine were collected for serum and urine silver concentrations at peak concentration ($\leq$2 h post-dose) on day-14 of each dosing period.

Each subject was admitted to the UUCCTS on three separate visits (3 days total) where Visit 1 was baseline measurements, followed at the same visit for measurement of drug inhibition (Visit 1: single-dose placebo and Visit 2: single-dose active nanoparticle solution) and Visit 3 (Day 14: single-dose active nanoparticle solution) for measurement of drug induction. A Day-14 UUCCTS admission for the placebo solution induction was considered unnecessary. At each visit, the subject was admitted at 1700 h for an overnight stay. After receiving a fixed-menu dinner, each subject received nothing-by-mouth through the last blood sample at 6 h after administration of the cytochrome P450 activity probes and either active silver nanocolloidal solution or placebo, depending on the study phase (dosed at 0700 h in the morning after admission). Subjects' heart rates and blood pressures were measured at time 0 (prior to cytochrome P450 probe and silver nanocolloidal solution or placebo administration), 15 and 30 min, 1, 2, 3 and 4 h after dosing. Subjects were kept at bed-rest for the initial 2 h post-dosing of the cytochrome P450 probes to avoid any unnecessary falls due to hypotension from the cytochrome P450 probes, namely, losartan and midazolam, respectively. Subjects were closely monitored for adverse effects, including signs or symptoms of lightheadedness, headache, severe fatigue, dizziness, gastrointestinal distress or sedation. Blood was drawn into a 10 mL heparin tube at time 0 (prior to probe and study drug administration) and 2, 4 and 6 h post-administration of probe agents and study medication. Urine was collected for 8 h after time 0. Eight hours post-study medication administration, subjects were discharged as long as they did not display untoward effects as noted above.

Each subject received the placebo (i.e. sterile water [no silver nanoparticles]) (placebo period) for 14 days, and then the active silver solution for 14 days (active period) to avoid any potential for residual silver carry-forward. A minimum 72-h washout period was required between the phases. Randomization was not performed due to the inability to predict residual silver nanoparticle retention in the human body and subsequent physiologic effects on the cytochrome P450 system. In each study phase, subjects received 15 mL of test solution (placebo in phase 1, then the silver solution in phase 2) daily from a pre-mixed oral syringe at baseline (day 0) in the UUCCTS and for 14 days thereafter at approximately 0900 each day. Each dose administration was observed by study personnel to ensure compliance. Subjects were blinded to the study product received.

Serum silver concentrations were determined by using Inductively Coupled Mass Spectrometry (ICP-MS, NMS Laboratories, Willow Grove, PA). Calibration samples in dilute

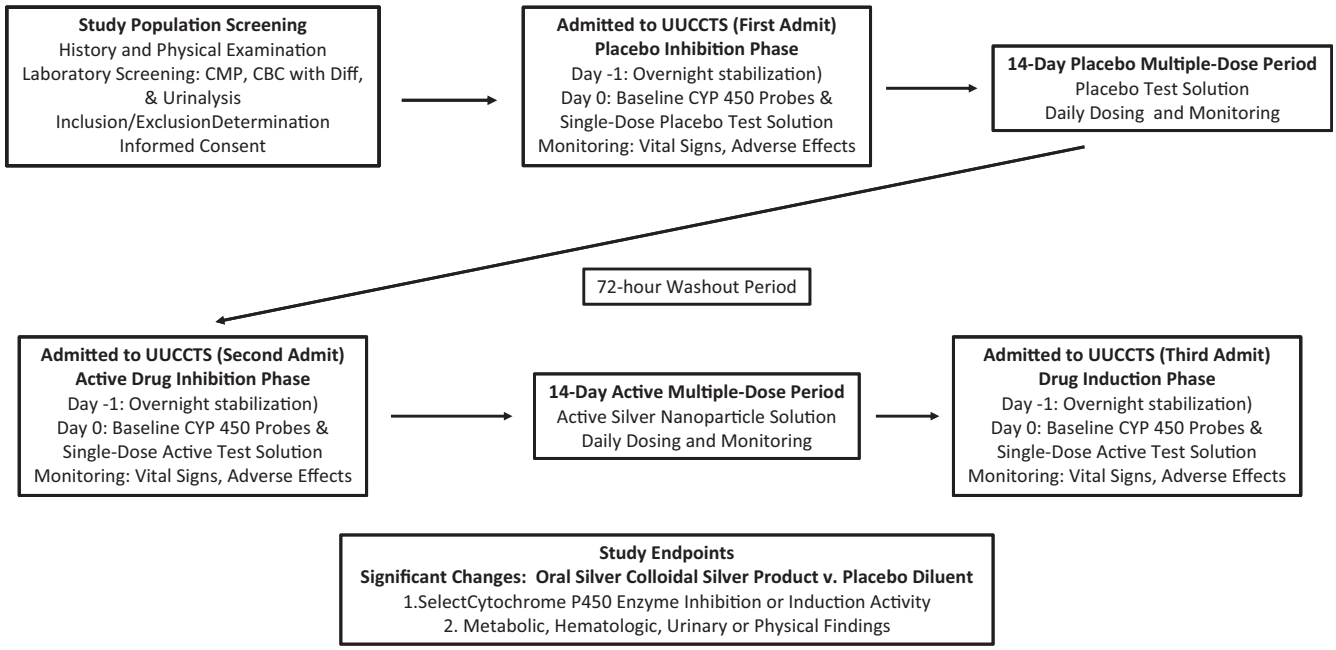

Figure 1. Study design and endpoints.

Table 1. Cytochrome P450 enzyme activity probe and drug analysis.

Cytochrome P450 enzyme	Probe	Clinical measurements: specific enzyme or enzyme/metabolite ratio	Laboratory method
1A2	Caffeine 100 mg p.o.	Plasma 2, 4, 6 h.	LC/MS/MS (Carlson et al., 2008)
2C9	Losartan 50 mg p.o.	Urine 8 h.	LC/MS/MS (Sung et al., 2008)
2C19	Omeprazole 20 mg p.o.	Plasma 2, 4, 6 h.	LC/MS/MS (Kawata et al., 2009)
2D6	Dextromethorphan 30 mg p.o.	Urine 8 h.	LC/MS/MS (Lamb et al., 2010)
3A4	Midazolam 2 mg p.o.	Plasma 2, 4, 6 h.	LC/MS/MS (Frőhlich, 2013)
2E1	Chlorzoxazone 250 mg p.o.	Plasma 2, 4, 6 h.	LC/MS/MS (Hussain et al., 2005)

p.o.: by mouth; LC/MS/MS: Liquid chromatography/tandem mass spectrometry.

nitric acid and controls in human serum were used in each ICP-MS assay cohort. The ICP-MS assay dynamic range of silver samples for this study was 0–40 mcg/L and assumed to represent ionic silver since this ICP-MS analytical method cannot distinguish nanoparticulate silver from silver ion in serum. Silver concentrations were determined at peak serum sampling, $\leq$2 h post-dose. Urine samples, once brought to the laboratory, were stored at 4 °C prior to analysis. Plasma samples were stored at −80 °C prior to analysis.

Cytochrome P450 probe testing

Cytochrome P450 enzyme activity was measured using standard published methods as outlined in Table 1 (Choi et al., 2008; Goh et al., 2010; Hukkanen et al., 2010; Klebanoff et al., 1999; Wang et al., 2004; Yu et al., 2004). Cytochrome P450 1A2 was measured as plasma paraxanthine and paraxanthine/caffeine ratio (Klebanoff et al., 1999), CYP2C9 as losartan and the losartan/E-3174 ratio (Choi et al., 2008), CYP2C19 as omeprazole and omeprazole/5-hydroxyomeprazole ratio (Wang et al., 2004), CYP2D6 activity as dextromethorphan and dextrorphan measured (Goh et al., 2010), CYP3A4 activity as midazolam (Yu et al., 2004) and CYP2E1 as chlorzoxazone and 6-hydroxychloroxazone (Hukkanen et al., 2010), respectively.

The analysis of losartan, losartan-COOH, dextromethorphan, and dextrorphan were adapted from published methods (Kolocouri et al., 2007; Li et al., 2007). Briefly 100 mcL of the sample, calibrator or control material was transferred to a 400 mcL automated liquid sampler (ALS) vial containing deuterium-labeled internal standard (losartan-D3 and dextrorphan-D3) and 200 mcL of water containing 10% acetonitrile. These preparations were injected onto a Quattro Premier XE LC-MS/MS system (Waters Corp., Milford, MA). Chromatographic separation was achieved using a Zorbax SB-C8 rapid resolution cartridge (2.1 mm × 30 mm; 3.5 micron) with gradient elution at 0.25 mL/min (0.1% formic acid in water, acetonitrile), ionized using positive ion electrospray, and detected using selected reaction monitoring of protonated molecular ions (Losartan: 423.1 > 207.1; losartan-COOH: 437.1 > 235.3; losartan-D3: 427.1 > 211.1; dextromethorphan: 272.1 > 171.2; dextrorphan: 258.3 > 157.1; dextrorphan-D3: 261.3 > 157.1). Concentrations were calculated using a multipoint calibration model which ranged from 5 to 5000 ng/mL using the system software (Quanlynx, Waters Corp., Milford, MA).

The analysis of caffeine and paraxanthine, midazolam, omeprazole and OH-omeprazole were measured by adaptation of published methods (Marier et al., 2004; Yu et al., 2004). A 250 mcL of the sample, calibrator or control material was fortified with deuterated internal standards (caffeine 13C3, midazolam-D4 and omperazole-D5), the pH was made basic with the addition of ammonium carbonate buffer (pH 9) and samples were extracted with methyl-*t*-butyl ether. The organic layer was collected and evaporated under a stream of air. Dried residues were reconstituted in 100 mcL of water containing 10% acetonitrile,

transferred to ALS vials, with a Quattro Premier XE LC-MS/MS system (Waters Corp., Milford, MA). Chromatographic separation was achieved using a Zorbax SB-C8 rapid resolution cartridge (2.1 mm × 30 mm; 3.5 micron) with non-linear gradient elution at 0.25 mL/min (0.1% formic acid in water/acetonitrile), ionized using positive ion electrospray, and detected using selected reaction monitoring of protonated molecular ions (caffeine: 195.2 > 138.3; paraxanthine: 181.2 > 124.3; caffeine-13c3: 198.2 > 140.3; midazolam: 326.2 > 291.3; midazolam-D3: 330.2 > 293.3; omeprazole: 346.1 > 198.2; OH-omeprazole: 362.1 > 214.1; omeprazole-D3: 349.1 > 198.1). Concentrations were calculated using a multipoint calibration model ranging from 1 to 5000 ng/mL using Quanlynx system software (Waters Corporation, Milford, MA).

Analysis of chlorzoxazone and HO-chlorzoxazone was performed by adapting the methods of Hukkanen et al. (2010). A 500 mcL of sample, calibrator, or control material was fortified with deuterated internal standard (chlorzoxazone-D3), Samples were deconjugated with β-glucuronidase, type IXA, (Sigma Chemical, St. Louis, MO) overnight at 37 °C. The sample proteins were precipitated with cold acetonitrile followed by centrifugation at 2000 × *g*. The supernatant was evaporated to dryness under a stream of air at 40 °C. Resulting dried residues were reconstituted in 100mcl of water with 10% acetonitrile, transferred to ALS vials and briefly centrifuged to pellet any insoluble material in the vial. The supernatants were analyzed with a Quattro Premier XE LC-MS/MS system (Waters Corp., Milford, MA). Chromatographic separation was achieved using a Zorbax SB-C8 rapid resolution cartridge (2.1 mm × 30 mm; 3.5 micron) with non-linear gradient elution at 0.25 mL/min (0.1% formic acid in water/acetonitrile), ionized using negative ion electrospray and detected using selected reaction monitoring of de-protonated molecular anions (chlorzoxazone: 168.1 > 132.1; HO-chlorzoxazone: 184.1 > 120.1; chlorzoxazone-D3: 171.1 > 134.1). Concentrations were calculated using a multipoint calibration model ranging from 1 to 5000 ng/mL using the system software (Quanlynx, Waters Corp., Milford, MA).

Table 2. Study participant changes in clinical findings.

Hemodynamic variable	32 ppm Mean change (active minus placebo period) [95% CI] (*p* value)
Weight (kg)	−0.4 [−0.1, 0.8] (0.13)
Body Mass Index (kg/m^2)	−0.1 [−0.04, 0.3] (0.14)
Systolic Blood Pressure (mmHg)	−1.3 [−3.0, 5.6] (0.54)
Diastolic Blood Pressure (mmHg)	−0.7 [−2.5, 3.8] (0.67)
Heart Rate (bpm)	−3.1 [−6.4, 0.3] (0.07)

CI, Confidence interval.

Statistical analysis

For clinical findings (Table 2) and metabolic panel analyses (Table 3), the active solution period value was compared to the placebo period value, using a mixed effects linear regression model, controlling for the baseline value. In these models, periods were nested with patients, providing a paired sample analysis. For the cytochrome P450 assay results (Table 4), where baseline values were not measured, the active solution period was compared to the placebo period using a paired sample *t*-test. All reported *p* values are from a two-sided comparison. The $n = 12$ subjects provided 80% power to detect a mean difference of 0.69 standard deviations (SD), using a two-sided alpha 0.05 paired sample comparison, assuming a correlation of $r = 0.70$ between the placebo and active phases.

The sample size was determined by using a two-sided alpha 0.05 comparison and assuming a correlation of $r = 0.70$ between the placebo and active phases. The $n = 12$ subjects with maximum

Table 3. Study participant baseline and end treatment in comprehensive metabolic panel and complete blood count with differential ($n = 12$).

Comprehensive metabolic panel and complete blood cell count with differential	Baseline mean ± SD	End of active treatment period mean ± SD	Total sample mean change (active minus placebo period) [95% CI: min, max] (*p* value)
Sodium [mmol/L]	141.8 ± 1.6	141.2 ± 1.9	0.02 [−0.7, 1.0] (0.71)
Potassium [mmol/L]	4.5 ± 0.4	4.4 ± 0.3	−0.03 [−0.2, 0.1] (0.74)
Chloride [mmol/L]	105.0 ± 2.4	105.5 ± 2.3	−0.04 [−1.1, 1.2] (0.94)
Carbon Dioxide [mmol/L]	26.5 ± 2.3	26.8 ± 2.2	−0.04 [−1.1, 1.0] (0.94)
BUN [mg/dL]	16.9 ± 4.0	16.7 ± 4.1	0.5 [−0.8, 1.8] (0.41)
Creatinine [mg/dL]	0.92 ± 0.16	0.88 ± 0.14	−0.02 [−0.04, 0.01] (0.21)
Glucose [mg/dL]	85.9 ± 10.7	83.5 ± 9.9	−0.7 [−4.2, 2.9] (0.71)
ALP [U/L]	89.0 ± 60.4	85.4 ± 48.5	2.0 [−1.0, 5.0] (0.18)
AST [U/L]	29.1 ± 7.9	30.9 ± 12.4	2.0 [−2.6, 6.5] (0.40)
ALT [U/L]	36.1 ± 11.7	34.7 ± 11.0	2.3 [−1.6, 6.3] (0.25)
Total Protein [g/dL]	7.1 ± 0.5	7.4 ± 0.5	0.2 [−0.001, 0.4] (0.051)
Total Bilirubin [mg/dL]	0.7 ± 0.3	0.8 ± 0.4	−0.03 [−0.11, 0.05] (0.49)
Albumin [g/dL]	4.3 ± 0.4	4.4 ± 0.4	0.11 [−0.01,0.23] (0.085)
Calcium [mg/dL]	9.4 ± 0.4	9.4 ± 0.5	0.1 [−0.003, 0.2] (0.057)
WBC Count [k/μL]	5.99 ± 1.24	5.92 ± 1.45	−0.09 [−0.68, 0.49] (0.75)
RBC Count [M/μL]	5.07 ± 0.50	5.07 ± 0.45	0.06 [−0.03, 0.14] (0.23)
Hemoglobin [gm/dL]	15.5 ± 1.4	15.5 ± 1.2	0.1 [−0.2, 0.4] (0.41)
Hematocrit [%]	45.0 ± 3.5	45.4 ± 3.5	0.8 [−0.1, 1.7] (0.095)
MCV [fL]	89.1 ± 3.8	89.6 ± 3.8	0.5 [−0.1, 1.2] (0.12)
MCH [pg]	30.7 ± 1.1	30.6 ± 1.3	−0.1 [−0.4, 0.2] (0.38)
MCHC [gm/dL]	34.5 ± 1.0	34.2 ± 0.9	−0.3 [−0.7, 0.1] (0.12)
Platelets [k/μL]	268 ± 59	253 ± 54	−4 [−16, 8] (0.53)
Granulocytes [%]	52.6 ± 6.9	53.4 ± 7.6	−0.7 [−4.0, 2.7] (0.70)
Lymphocytes [%]	36.7 ± 6.4	36.8 ± 6.9	1.3 [−1.5, 4.1] (0.36)
Monocytes [%]	6.4 ± 1.1	6.0 ± 1.3	−0.3 [−0.8, 0.3] (0.34)
Basophils [%]	0.8 ± 0.2	0.7 ± 0.3	0.001 [−0.1, 0.1] (0.99)
Eosinophils [%]	3.4 ± 1.5	3.1 ± 1.5	−0.2 [−0.7, 0.2] (0.36)

The last column shows the mean difference between the active period and placebo period, adjusting for baseline.

Table 4. Cytochrome P450 assay results on human serum exposed to orally dosed silver.

Ratio (Parent:Metabolite)	Treatment phase	Mean ± SE (min–max) $n = 12$	Difference from placebo [95% CI] (p value)*
Dextromethorphan:Dextrorphan	Placebo	4.16 ± 3.79 (0–45.78)	−0.90 [−11.76, 9.96] (0.86)
	Single dose	3.26 ± 2.83 (0–34.20)	
	Placebo	5.53 ± 5.04 (0–45.78)	−3.35 [−16.26, 9.55] (0.57)
	Multiple dose	2.18 ± 1.84 (0–16.86)	
Losartan:Losartan-COOH	Placebo	1.45 ± 0.32 (0.42–4.36)	0.11 [−0.17, 0.39] (0.41)
	Single dose	1.56 ± 0.29 (0.68–3.60)	
	Placebo	1.62 ± 0.42 (0.42–4.36)	0.14 [−0.22, 0.51] (0.38)
	Multiple dose	1.76 ± 0.45 (0.51–5.01)	
Caffeine:paraxanthine	Placebo	3.34 ± 0.28 (1.66–4.54)	0.004 [−0.68, 0.69] (0.99)
	Single dose	3.35 ± 0.37 (1.10–5.57)	
	Placebo	3.34 ± 0.28 (1.66–4.54)	0.34 [−0.87, 1.55] (0.55)
	Multiple dose	3.68 ± 0.64 (1.78–10.20)	
Omeprazole:OH-omeprazole	Placebo	2.18 ± 1.40 (0.56–7.77)	−0.61 [−3.14, 1.91] (0.54)
	Single dose	1.56 ± 0.58 (0.53–3.66)	
	Placebo	2.19 ± 1.14 (0.56–7.77)	−0.39 [−2.00, 1.22] (0.56)
	Multiple dose	1.81 ± 0.57 (0.57–4.49)	
Chlorozoxazone	Placebo	2311 ± 494 (809–5890)	20 (−1203, 1245) (0.97)
	Single dose	2332 ± 323 (812–4148)	
	Placebo	2311 ± 494 (809–5890)	−399 [−1419, 621] (0.41)
	Multiple dose	1912 ± 334 (696–4675)	
Midazolam	Placebo	9.62 ± 0.83 (5.6–13.0)	−0.52 [−2.05, 1.00] (0.46)
	Single dose	9.09 ± 0.96 (4.6–16.7)	
	Placebo	9.62 ± 0.83 (5.6–13.0)	−0.43 [−2.04, 1.17] (0.56)
	Multiple dose	9.18 ± 1.05 (4.5–16.5)	

*Paired t-test.

exposure, 32 ppm for 14 days, provided 80% power to detect a mean difference of 0.48 standard deviations (SD).

Results

Study sample

Thirteen healthy subjects were enrolled between June 2011 and November 2011. With the exception of one subject, each successfully completed both study phases. The single dropout was receiving placebo for 12 days, was hospitalized for a pulmonary embolism, and was subsequently discontinued from the study. The mean age of the subjects was 28.8 (range 20–50) years, all were male Caucasians, with an average body mass index of $29 \pm 6\,kg/m^2$ (range 21–43).

Safety studies

Changes in subject clinical findings are listed in Table 2. No statistically significant or clinically important changes in weight, body mass index, systolic or diastolic blood pressure or heart rate were noted. Physical examination did not reveal any clinically relevant findings in any subject (data not shown). The results of the comprehensive metabolic panel and complete blood cell count analyses are listed in Table 3. There were no significant or clinically important changes observed in metabolic, hematologic or urinary laboratory findings between active and placebo phases or with individual subjects. No adverse events were reported during the outpatient-administered multiple-dose phases of the study. The probe cocktail was relatively well-tolerated with two patients exhibiting moderate nausea for 2–3 h at the single- and multiple-dose phases. There was a well-tolerated decrease in supine blood pressure, as expected, during the first few hours post-dosing of the probe cocktail. No adverse events were considered clinically important.

Serum silver and urine findings

Mean serum silver concentration was detected in all subjects at 6.8 ± 4.5 mcg/L. No silver was detected in the urine, independent of dose or time period.

Cytochrome P450 enzyme activity

The parent-to-metabolite probe ratios for single-dose placebo versus single-dose active silver nanoparticle dispersion treatment did not demonstrate any statistically significant or clinically important inhibition of the studied cytochrome P450 enzyme activity (Table 4). After 14 days of multiple dose silver nanoparticle dispersion treatment, no statistically significant or clinically important induction of the select cytochrome P450 enzyme activity was demonstrated. There was a decrease in the parent-to-metabolite ratio of CYP2D6 probe dextromethophan. Although not statistically significant different from placebo, this change in the ratio is in the direction one would expect for enzyme induction activity, and may warrant further study.

Discussion

To assess the effects of oral exposure to a commercial nanoscale silver product on human cytochrome P450 enzyme activity, we conducted a 14-day prospective, controlled, crossover design study. Results indicate that ingestion of this colloidal silver dispersion produces detectable silver in human serum, likely in the form of ionic silver (i.e. not silver particulate) (Munger et al., 2013), but no clinically significant changes in metabolic, hematologic, urine or physical parameters, and no significant effects on cytochrome P450 enzyme inhibition or induction activity. To the best of our knowledge, this is the first *in vivo* human assessment of cytochrome P450 enzyme activity from a systemic human orally ingested nanoscale silver product.

Liver cell cultures are often used for *in vitro* studies of sustained nanosilver exposure, but based on the results of this study, may not be a target organ *in vivo* for orally administered nano-silver (Kulthong et al., 2012; Sung et al., 2008; Takenaka et al., 2001). Silver nanoparticles *in vitro* reduce cell mitochondrial functions, deplete glutathione and NADPH cytochrome c reductase activities, thereby increasing reactive oxygen species, leading to apoptosis in rat and human liver and mesenchymal cell lines (Arora et al., 2009; Hussain et al., 2005; Sung et al., 2008). These *in vitro* results suggest that drug metabolism may be affected by ingestion of silver nanoparticles if made bioavailable.

Huh7 cells treated with engineered silver-doped silica nanoparticles exhibited reduced induction of CYP1A and activity of ABC transporters (Christen & Fent, 2012). Kulthong et al. (2012). showed that exposing rat liver microsomal preparations to increasing concentrations of engineered silver nanoparticles resulted in strong inhibition of CYP2C and CYP2D, but not CYP1A, CYP2E1 or CYP3A activity. In contrast, when the same nanoparticles were orally administered to Sprague–Dawley rats at the same test concentrations, no significant effects on enzyme activity were observed. Furthermore, no silver nanoparticles were detected in the liver sections of the rats. Twenty-four hour exposure of MDR1.C and Hep G2 cell lines used in this study resulted in inhibition of human P450 enzyme proportional to the silver/microsomal protein ratio. In addition, CYP2C9, CYP2C19 and CYP3A4 enzymatic activities were most sensitive to inhibition caused by nanosilver exposure, CYP2D6 and CYP2E1 had intermediate inhibition, and CYP1A2, CYP2A6, and CYP2B6 had the least inhibition (Lamb et al., 2010). When the same colloidal silver nanoscale solution was administered to healthy humans in this study, however, no significant change or clinically important inhibition or induction of cytochrome P450 enzyme activity was observed.

Lack of correlation between *in vitro* and *in vivo* results for nanosilver toxicity is evident here and consistent with previous reports. One confounding issue is that oral administration of silver aqueous nanoparticles delivers both ionic silver and silver nanoparticles. Consistent with other silver nanoparticle preparations, we have shown that a substantial fraction of the silver nanoproduct used in this study is in the ionic form (Munger et al., 2013). Given the demonstrated very limited gastrointestinal absorption of metallic nanoparticles (Paek et al., 2013; Schleh et al., 2012), we presume that serum concentrations of silver measured in this report and a previous human oral ingestion study (Munger et al., 2013) represent ionic silver gut absorption, not particulate. Published research, however, shows that both silver ions and nanoparticles can exist inside cells following *in vitro* silver nanoparticle exposures (Wang et al., 2013). Nanoparticle effects even at low *in vitro* exposure concentrations have been suggested to be the predominant mediator of biological effects in erythroid cells (Wang et al., 2013). In addition, nanoparticulate surface site interactions may be important for cellular induced oxidative stress. In support of this proposed stress-inducing mechanism is a non-competitive best-fit inhibition mechanism for testosterone 6β-hydroxylation. With less than 10% inhibition at silver concentrations 2- to 10-fold greater than concentrations causing a 50% inhibition of the cytochrome P450 oxidation reactions, the electron flow through the flavoprotein is probably not the silver inhibition locus (Lamb et al., 2010). Size-dependent particle influences on cytochrome P450 enzymes may also be a possibility. Small (20–60 nm diameter) non-metallic, carboxy polystyrene particles inhibited the enzymatic activity of CYP3A4, CYP2D6 and CYP2C9 in insect cell preparations and normal liver microsomes (Frőhlich et al., 2010); however, the average nanosilver particle size here appears to exceed the largest cytochrome P450 substrate pocket dimension (Ekroos & Sjőgren, 2006; Munger et al., 2013; Ohkura et al., 2009; Otyepka et al., 2007), suggesting that the size of this silver nanoparticle preparation used in our study probably did not physically block probe metabolism to a statistically measurable or clinically meaningful level. Finally, a variety of other factors may explain observed differences between *in vitro* and *in vivo* responses to silver nanoparticles. Most importantly, the diverse methods of silver nanoparticle preparation that profoundly influence their resulting physiochemical properties and surface chemistry (e.g. stabilizers, solutions additives such as antioxidants and salts, PEGylation, ligand conjugation) (Choi et al., 2009; Johnston et al., 2010) also affect their consequent cell and enzyme interactions. Few of these methods control for report silver colloid shelf life or oxidative stability that produce silver ion coexisting by-products. Few studies to date can exclude co-existing silver ions as a confounding variable to silver nanoparticle mechanisms in biological experiments (Behra et al., 2013). Variations in liver physiology (e.g. blood flow, perfusion, fenestration, filtration efficiency), particle dosage and dosing frequency and patient characteristics (e.g. age, gender, body composition and other treatments) are also not controlled.

A series of *in vivo* studies were conducted to determine whether a health risk is posed to healthy humans through single- and multiple-dosing of a commercially available silver nanoscale particle solution product when administered as directed by the manufacturer; the same product studied here in this article. The first controlled single-blind, crossover study showed no significant or clinically important changes in human metabolic, hematologic, urine, physical findings or imaging morphology in 60 healthy subjects exposed for 3, 70 or 14 days at 10 or 32 ppm doses (Munger et al., 2013; Smock et al., 2014). There were also no detectable changes in reactive oxygen species or pro-inflammatory cytokine RNA changes from induced sputum samples. A second controlled study in healthy human volunteers showed no detectable *ex vivo* enhanced platelet aggregation effects in 18 healthy volunteers after 14-day exposure (Smock et al., 2014). The current study adds to this body of understanding by showing a lack of significant or clinically important changes in human selective cytochrome P450 enzyme inhibition or induction activity from the same silver colloidal nanoparticle solution. These studies provide the scientific, regulatory and healthcare industry a set of safety data on one single silver-engineered nanotechnology product. Furthermore, these studies were designed to offer a template for investigating other systemic nanoproducts into the future. This series of studies should be interpreted, however, with caution, as they do not provide a safety net for all systemically ingested nanomedicine products. These studies also do not offer information about dosing or the efficacy of this product.

A question should be raised why the toxicology studies described above do not result in human detectable toxicity from chronically ingested silver. The most common adverse effect of prolonged silver exposure is an irreversible bluish-gray or ash gray pigmentation of the skin called argyria, or in the eyes called argyrosis (Drake & Hazelwood, 2005; Gulbranson et al., 2000; Shelley et al., 1987). Argyria or argyrosis can occur through a variety of exposure routes, including oral ingestion, inhalation of dust or fumes, direct skin contact or through implantation or the use of silver-containing medical equipment (Barrie & Harding, 1947; Chung et al., 2010; Espinal et al., 1996; McKenna et al., 2003; Roseman et al., 1979; Suzuki et al., 1993). The highest rate of argyria or argyrosis occurs by exposure to soluble forms of silver as studied in this study (Drake & Hazelwood, 2005). The reasons for the lack of apparent toxicity in our studies may be multifactorial, including sub-toxic dosing, length of exposure, differences in clearance of silver ions and silver nanoparticles by humans as compared to animal models or some as yet unrecognized factors. Importantly, in animal models, the liver appears to be the target toxicity organ (Kim et al., 2010); however, the exact form of orally dosed silver (soluble and/or particulate) in many of these studies is not ascertained. In addition, handling of silver ions does not appear to be toxic in humans at least by examination of human cytochrome P450 enzyme activity or by serum transaminase levels (Munger et al., 2013). It should be noted that due to estrogen-related effects on cytochrome P450 enzymes, only men were studied in this study. Thereby, direct extension of these initial results to women should not be assumed. Despite the

findings of our work, caution should be exercised when ingesting high concentrations of silver ions or nanoparticles over prolonged periods in order to avoid silver toxicity in humans.

Conclusions

A commercially available colloidal silver nanoproduct dosed orally over 2 weeks to human volunteers produces detectable silver in human serum after 14 days of monitored dosing (Arora et al., 2009). No demonstrable clinically significant changes in metabolic, hematologic, urine, physical findings and cytochrome P450 enzyme inhibition or induction activity were observed, consistent with other clinical observations regarding the lack of coagulation perturbations reported for these same patients (Villiers et al., 2009). This is presumed to result from minimal systemic bioavailability of orally ingested nanoparticulate silver in these volunteers (Munger et al., 2013), and reduced oxidative stress induction and no observable liver toxicity for the bioavailable silver present in serum as silver ion in these human subjects (Klebanoff et al., 1999; Munger et al., 2013; Smock et al., 2014).

Acknowledgements

The authors acknowledge the nursing support of the University of Utah Center for Clinical and Translational Research towards the conduction of this protocol. Peer-reviewers for this journal are thanked for many high quality and insightful comments that improved this contribution greatly. The authors are grateful for many technical and editorial contributions and suggestions from Dr. D.W. Grainger (University of Utah, USA).

Declaration of interest

The authors disclose no financial, consulting, or personal relationships with other people or organizations that influenced this publication. The manuscript was not under any contractual relationship for review, comment or approval before publication. Research reported in this publication was supported by the National Center for Advancing Translational Sciences of the National Institutes of Health under Award Number 1ULTR001067. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. The authors declare no conflicts of interests. None of the funding bodies contributed to the study design, data interpretation, or preparation of the manuscript.

This study was presented, in part, at the 2012 Annual Meeting of the American Society for Clinical Pharmacology and Therapeutics, Washington, DC (March, 2012).

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