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ORIGINAL ARTICLE

Comparison of pharmacokinetic and metabolic profiling among gossypol, apogossypol and apogossypol hexaacetate

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Abstract
Purpose To characterize the stability, pharmacokinetics and metabolism of analogs of gossypol, apogossypol and apogossypol hexaacetate to provide a basis for comparison.
Methods Gossypol, apogossypol and apogossypol hexaacetate were incubated in plasma or liver microsomes from various species, or administered to mice, respectively, from which the stability, metabolism and pharmacokinetic profiles of these analogs were quantitatively determined using a liquid chromatography-mass spectrometry (LC/MS/MS) method.
Results In various species of plasma, apogossypol and gossypol exhibited similar stability, while 20–40% of apogossypol hexaacetate was converted into apogossypol with concurrent formation of the corresponding di-, tri-, tetra-, and penta-acetates of apogossypol. (±)-Gossypol and (−)-gossypol showed comparable pharmacokinetic profile and oral bioavailability (12.2–17.6%) with some variations of clearance and V_{ss} following oral and intravenous administration to mice. At the same molar dose, apogossypol showed delayed T_{max}(1 h), a slower clearance rate and less distribution after administration to mice. Mono- and di-glucuronide conjugates of apogossypol were readily observed in mouse plasma following administration. Apogossypol formulated in sesame oil appeared to possess larger AUC and thus higher oral bioavailability than that formulated in cremophor EL:ethanol:saline. In contrast, intravenous apogossypol hexaacetate exhibited highest clearance rate partially due to its conversion into apogossypol. Concomitant with disappearance of apogossypol hexaacetate (i.v.), apogossypol converted from apogossypol hexaacetate was quantitatively detected, and accounted for ∼30% of total plasma apogossypol hexaacetate. Oral apogossypol hexaacetate showed no bioavailability with little apogossypol occurring in the plasma. In human and mouse liver microsomes, glucuronide conjugates of apogossypol and its acetates were readily identified with the exception of gossypol glucuronidation. Apogossypol appeared more stable in human and mouse liver microsomal preparations than gossypol and apogossypol hexaacetate.
Conclusions Apogossypol and gossypol show similar oral and intravenous pharmacokinetic profiles and in vitro stability although apogossypol appears to have a slower clearance rate, larger AUC, and better microsomal stability. Apogossypol hexaacetate converts to apogossypol in both in vitro and in vivo settings and lacks any quantifiable oral bioavailability.

Keywords Pharmacokinetics · Metabolism · Gossypol · Apogossypol · Apogossypol hexaacetate

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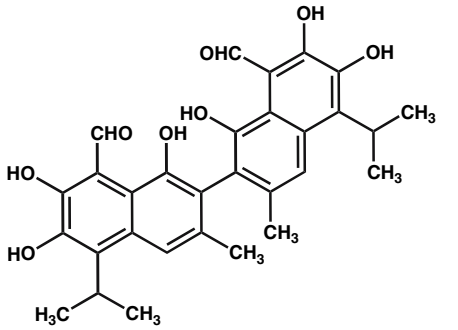
Introduction

Gossypol (Fig. 1), a biologically active constituent of cottonseed oil that was first extracted from the cotton plant

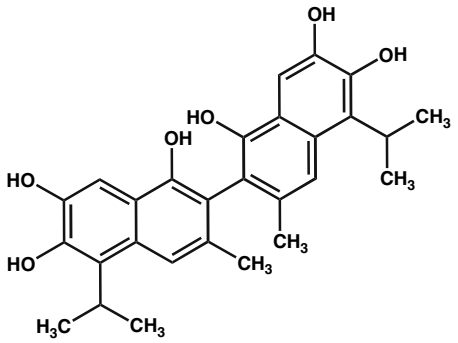
Gossypium hirsutum L about 90 years ago [1, 2], has been extensively studied in clinical trials conducted in China in the 1970s, and later in Austria and Brazil for its use as a male contraceptive agent [3] via metabolic inhibition of human sperm [4]. Extensive investigations of animal toxicity and recovery of fertility in men after discontinuation of gossypol treatment led the Special Program of Research, Development and Research Training in Human Reproduction at the World Health Organization to the decision that gossypol would not be acceptable as an antifertility drug due to its toxicity associated with hypokalemia and its effect of permanent infertility. During the period of the clinical trials, however, many efforts were made to alter the structure of gossypol in order to find analogs with improved efficacy and/or reduced toxicity. Some 70, highly purified, novel structural forms of gossypol were produced, and about 40 were tested for their antifertility activity. None were found to be more active than gossypol itself [3]. Large-scale resolution of racemic gossypol into its enantiomers, (−)- and (+)-gossypol, was another notable contribution of chemistry to gossypol research, which revealed that (−)-gossypol is approximately twice as active as racemic gossypol as an antifertility agent [5, 6]. The continuing legacy of gossypol research then focused on antiviral [7], anti-parasitic [8] and anti-inflammatory testing [9], and recently, strategically and thoughtfully turned to the life-threatening disease cancer [10, 11] following identification of gossypol as a cytotoxic agent against various carcinoma cell lines in both in vitro and in vivo settings [12–14].

Overexpression of antiapoptotic B-cell lymphocyte/leukemia-2 (Bcl-2) family proteins occurs in many human cancers; this occurrence has generated interest in these proteins as possible drug discovery targets 15. Soon after the finding that gossypol inhibits many enzymes including cationic lactate dehydrogenase [16], cell cycling by modulation of the regulatory proteins Rb and cyclin D1 [17, 18], as well as protein kinase C activity [19], Kitada et al. [20] showed that gossypol binds and antagonizes the antiapoptotic effect of Bcl-2 family proteins, and thus induces in vitro apoptosis in breast, colon, and prostate cancer cells that have high levels of Bcl-2 family proteins.

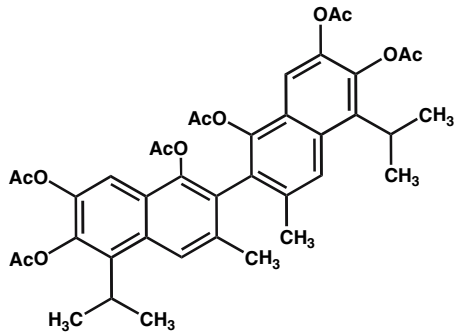
Recent structural modification of gossypol guided by a model of multidimensional nuclear magnetic resonance-based structural analysis of gossypol fitting into its target Bcl-2 family member revealed that apogossypol (Fig. 1), an analog of gossypol, displayed a proapoptotic activity comparable to gossypol demonstrated by fluorescence polarization assays and cell-based assays [21, 22]. Both gossypol and apogossypol occupied the BH3 domain of numerous Bcl-2 members and promoted apoptosis [21]. Fluorescence polarization assays, which monitors the binding of inhibitors to human Bcl-2-family proteins, further demonstrated that gossypol and apogossypol competed for BH3 binding



GOSSYPOL



APOGOSSYPOL



APOGOSSYPOL HEXAACETATE

Fig. 1 Chemical structures of gossypol, apogossypol and apogossypol hexaacetate

domain with $IC_{50} < 10$ μM for five of the Bcl-2 family proteins, Bcl-Xl, Bcl-2, Bcl-W, Mcl-1, and Bcl-B [23]. These findings formed the basis of our rationale that if apogossypol has improved pharmacokinetics and effectiveness in animal models over gossypol, it should be further developed. The present studies were therefore initiated to characterize the in vitro stability and metabolism of apogossypol and its pharmacokinetics in mice in comparison with gossypol to assess the suitability of apogossypol for further development as a pro-apoptotic agent. In the early stages of this project, we found that by protecting the six hydroxyl groups of apogossypol with the acetate groups produced a potential prodrug: apogossypol hexaacetate (Fig. 1), which seems to be physically more stable than apogossypol at room temperature. This created another objective of this

investigation to evaluate whether apogossypol hexaacetate can function as a viable prodrug. The present investigation was less focused on gossypol since a significant amount of data on gossypol is already available.

Methods and materials

Chemicals and materials

(±)-Gossypol CH_3COOH salt (M.W. 578.6; NSC19048), (−)-gossypol (M.W. 518.56; NSC726190), apogossypol (M.W. 462.5; NSC736630) and apogossypol hexaacetate (M.W. 714.75; NSC734247) were synthesized at RTI international (Research Triangle Park, NC, USA) under a National Cancer Institute contact to support the studies. Apogossypol was stored at −70°C, while gossypol and apogossypol hexaacetate were stored at −20°C. Apigenin (internal standard) was purchased from Indofine Chemical Co. (Hilsborough, NJ, USA). The NADPH regenerating system was purchased from BD Gentest (Woburn, MA, USA). HPLC grade acetonitrile and ammonium acetate were purchased from Fisher Scientific (Atlanta, GA, USA). Reagent grade ascorbic acid was purchased from Acros Organics (Pittsburg, PA, USA).

Stability of gossypol analogs in various species of plasma

Sample preparation and analytical methodology for determining gossypol, apogossypol, and apogossypol hexaacetate in various species of plasma was similar to that described previously [24]. To test the biostability of the analogs, mouse, rat, dog and human plasma were spiked with gossypol, apogossypol or apogossypol hexaacetate, at a final concentration of 5 μg/ml. The plasma mixtures were then incubated at 37°C for 1, 2, 4, 8 and 24 h. After incubation, acetonitrile (200 μl) was added to the plasma mixtures to extract the analytes for quantitative analysis using liquid chromatography-mass spectrometry (LC/MS/MS) method described below. The plasma samples containing apogossypol hexaacetate were analyzed for both apogossypol hexaacetate and apogossypol derived from apogossypol hexaacetate.

Analytical method for determining gossypol and its analogs in biomatrices

Analytical method for determining gossypol, apogossypol, and apogossypol hexaacetate in various biomatrices was similar to that described previously [24] with minor modifications for individual analogs. Briefly, separation of the compounds was achieved on a Perkin-Elmer (Norwalk, CT, USA) series 200 HPLC system consisting of an autosampler, and two micro-flow pumps with a Synergi Fusion RP, 100 × 2 mm, 4 μm column (Phenomenex; Torrance, CA, USA) at ambient temperature. The compounds and apigenin (internal standard) were eluted using a mobile phase composed of 5 mM ammonium acetate and methanol according to the following gradient program: 80% 5 mM ammonium acetate and 20% methanol was linearly increased to 5% 5 mM ammonium acetate and 95% methanol over a 4.5-min period after an initial 0.5-min hold, and remained constant for 2 min before returning to 80% 5 mM ammonium acetate and 20% methanol. The column was re-equilibrated to the initial conditions for 3 min. The flow rate was 400 μl/min. The injection volume for the method was set at 10 μl.

A PE Sciex API 3000 triple quadrupole mass spectrometer equipped with a Turbo Ion spray source and operating at 450°C in the positive ion mode was used for analysis of Gossypol and its analogs. The ion spray voltage, orifice and ring potentials were set at 5,000, 30 and 200 V, respectively. High purity nitrogen was used as the curtain and collision gas. The resulting pressure in collision cell was approximately 1.3×10^{-5} Torr. Quantitation was performed using multiple reaction monitoring for the following transitions: gossypol (m/z 519.3 → 501.3) apogossypol (m/z 463.2 → 231.4), apogossypol hexaacetate ammonium adduct (m/z 732.4 → 673.1), apigenin (m/z 271.1 → 152.9). A dwell time of 200 ms was used for each ion transition. Collision energies were optimized for each transition and ranged from 33 eV (Apogossypol) to 45 eV (apigenin). Mass calibration, data acquisition and quantitation of the analytes were performed using Applied Biosystem Analyst 1.4 software.

Stock and spiking solutions were prepared in acetonitrile. Calibration standards were prepared by spiking the corresponding plasma or microsomal preparation with known amounts of the analytes over a concentration range of 78-10,000 ng/ml (gossypol) 10 to 2,000 ng/ml (apogossypol), and 5 to 5,000 ng/ml (apogossypol hexaacetate) in the presence of apigenin (500 ng/ml). Peak area ratios of the gossypol analogs to the internal standard were used for the construction of calibration curves. The lower limit of quantification of gossypol, apogossypol and apogossypol hexaacetate in plasma was determined to be 78, 10 and 5 ng/ml, respectively, with corresponding correlation coefficients greater than 0.99.

Dose formulations

Intravenous and oral formulations of gossypol were prepared in PEG 400:cremophor EL:water (20:3:77, v/v/v) to contain 3 mg/ml of gossypol. For preparation, 30 mg of gossypol was dissolved in 2 ml of PEG 400 followed by addition of 0.3 ml of cremophor EL. After mixing, 7.7 ml

of water was added. For i.v. administration, 5% ethanol was added to the formulation.

Intravenous and oral formulations of apogossypol were prepared in cremophor EL:ethanol:saline (1:1:8, v/v/v) to contain 2.8 mg/ml of apogossypol. For preparation, a calculated amount of apogossypol was dissolved in ethanol and then mixed with an equivalent volume of cremophor EL. Saline (eight volumes) was then slowly added to the solution, with mixing. Additional oral formulation of apogossypol was prepared in sesame oil to contain 2.8 mg/ml of apogossypol. These excipients were determined to be the optimal vehicles, based on the data from our pilot experiments, for the i.v. and p.o. formulations of apogossypol.

Intravenous dose formulation of apogossypol hexaacetate was prepared in cremophor EL:DMSO:saline (1:1:8, v/v/v) to contain 1 mg/ml of apogossypol hexaacetate. This was the maximum concentration of apogossypol hexaacetate that was soluble in this formulation. For oral administration, the same excipients contained 3 mg/ml of apogossypol hexaacetate in a suspension form.

Drug administration and sampling

Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), or the Principles of Laboratory Animal Care (http://www.history.nih.gov/laws). To compare pharmacokinetic characteristics amongst the gossypol analogs, male CD_2F_1 mice (23–28 g) were administered the analogs via gavage or by tail vein injection at the same molar dose of 60 μmol/kg in a dose volume 10 ml/kg, with the exception that the intravenous apogossypol hexaacetate was administered at lower dose of 20 μmol/kg due to its limited solubility in excipients suitable for intravenous administration. For each compound and route of administration, four mice were anesthetized with CO_2/O_2 and then bled from retro-orbital sinus at 2, 5, 10 and 30 min, and 1, 2, 4, 8, 12 (at 16 h for gossypol) and 24 h after i.v. administration, or at 0.25, 0.5, 1, 2, 4, 8, 12 (at 16 h for gossypol), and 24 h after oral administration. For mice dosed with apogossypol or apogossypol hexaacetate, each blood sample was collected into a 0.6 ml tube containing EDTA and 0.06 ml of 10% ascorbate (yielding a final concentration of ascorbate 1%). For gossypol, blood samples were collected into Microtainer® tubes (0.6 ml) containing dried lithium heparin without ascorbate to provide plasma. Pharmacokinetic parameters were determined by non-compartmental model using WinNonlin® (Professional Version 4.1; Pharsight Corp; Mountain View, CA, USA). The area under the plasma concentration versus time curve from 0 to infinity ($AUC_{0\rightarrow\infty}$). was calculated by the logarithmic trapezoidal rule; C_{max} represented maximum observed plasma concentration following administration; for i.v. administration, C_{max} was extrapolated to the concentration at time 0 for all i.v. administration. V_{ss} indicated volume of distribution at steady state. $T_{1/2\beta}$ and $T_{1/2\gamma}$ were half-lives of the test compounds in the different elimination phases

Microsomal metabolism of gossypol and its analogs

The microsomal studies were similar to that described previously [25] using pooled CD1 mouse or human liver microsomes (Xenotech, LLC; Lexena, KS, USA). The Phase I in vitro reaction system was comprised of microsomal protein (1 mg/ml) and the NADPH regenerating system (consisted of 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, and 0.4 U/ml glucose-6-phosphate dehydrogenase), 120 μM sodium citrate buffer, 3.3 mM $MgCl_2$ in a 0.1 M potassium phosphate buffer at pH 7.4. A 5 μg/ml concentration of gossypol, apogossypol, or apogossypol hexaacetate was individually incubated with the reaction system at 37°C in a shaking water bath. The total volume of each reaction mixture was 0.5 ml. Triplicate samples, along with negative controls were quenched upon preparation and at 0.25, 1, and 2 h after incubation. Each sample was mixed with ice-cold acetonitrile containing an internal standard to stop the reaction and then centrifuged at 11,000 g for 5 min. Individual supernatants were transferred into autosampler vials for analysis by LC/MS/MS.

For the phase II (glucuronidation) reaction, microsomal proteins at 2.0 mg/ml were incubated with 2 mM of uridine 5′-diphospho-glucuronic acid cofactor solution A (BD Gentest, Woburn, MA) in H_2O and solution B containing 50 mM Tris-HCl, 8 mM $MgCl_2$ and 25 μg alamethicin in H_2O along with the NADPH generating system described above. The glucuronidation activity was thus measured after solubilisation of microsomal membrane. The reaction was initiated with the addition of gossypol, apogossypol, or apogossypol hexaacetate to a final concentration of 8 μg/ml. Each reaction mixture was incubated at 37°C for 1 h in a shaking water bath. Each reaction mixture was quenched using 200 μl of ice-cold acetonitrile and then centrifuged at 11,000 g for 5 min. The supernatant was transferred into autosampler vials for analysis.

To demonstrate the integrity and viability of the reaction systems, both negative and positive controls were used [25]. A total of three different negative control mixtures consisting of a no substrate control, a no microsome control, and a mixture incubated with microsomes previously quenched with acetonitrile were prepared. The corresponding positive control reactions with known phase I or phase II substrates included dealkylation of 7-ethoxy coumarin to 7-hydroxy coumarin (as the phase I mouse liver microsomes), hydroxylation of paclitaxel to 6α-hydroxypaclitaxel (as the phase I human liver microsomes), glucuronidation of acetaminophen (for both mouse and

human liver microsomes). These reactions were carried out in parallel with the reactions with gossypol, apogossypol, apogossypol hexaacetate using the same reagents and source of microsomes. The chromatograph eluent was monitored by electrospray ionization mass spectrometry using the Q1 positive mode scanning a mass range of 100–1,500 m/z. A list of potential phase 1 and phase II metabolites was generated using Metabolite ID version 1.3. The predicted metabolite masses were extracted from the Q1 scans of each reaction and the extracted ion chromatograms were compared to those generated from the negative control reactions.

Results

Plasma stability of gossypol, apogossypol and apogossypol hexaacetate

Incubation of gossypol at 37°C with mouse, rat, dog and human plasma for 1 h resulted in 76, 83, 92 and 94% degradation of gossypol, respectively At 4 h, only 10–15% of gossypol remained in mouse and rat plasma, and 1–4.7% remained in human and dog plasma (Fig. 2a). The stability of apogossypol in various species of plasma appeared to be similar to that of gossypol, with the exception of rat plasma. After a 1-h incubation in mouse, rat, dog or human plasma, 82, 46, 94 or 82% degradation of apogossypol occurred, respectively. Thus, in rat, apogossypol appeared to be more stable than gossypol (Fig. 2a, b). Ascorbic acid, when added into the plasma preparation at a final concentration of 1%, increased the stability of apogossypol in all the four species of plasma [24].

When apogossypol hexaacetate (5 μg/ml) was incubated with mouse, rat, dog, or human plasma at 37°C, more than a 95% loss of apogossypol hexaacetate was observed after 2 h in each species. Interestingly enough, concomitantly with the in vitro disappearance of apogossypol hexaacetate, apogossypol was formed quickly. Apogossypol derived from apogossypol hexaacetate reached a peak concentration in the plasma mixtures in less than 2 h (Fig. 2c–f).

Pharmacokinetic comparison among gossypol, apogossypol and apogossypol hexaacetate

Plasma concentration-time courses following single oral or i.v. administration of (±)-gossypol, (−)-gossypol, apogossypol, or apogossypol hexaacetate to mice are separately illustrated in panels of Fig. 3. Table 1 lists pharmacokinetic parameters in molar concentrations analyzed from mice administered at the same molar dose (60 μmol/kg) of (±)-gossypol, (−)-gossypol, and apogossypol with the exception that apogossypol hexaacetate was administered at 20 μmol/kg due to its limited solubility and administration volume. (Table 2)

Racemic gossypol and (−)-gossypol displayed a comparable pattern of the plasma concentration-time curves in mice as illustrated in Fig. 3a, b. The oral bioavailability of (±)-gossypol and (−)-gossypol was 12.2 and 17.6%, respectively (Table 1). However, there were deviations in the parameters such as clearance, V_{ss} and $AUC_{0\rightarrow\infty}$ between (±)-gossypol and (−)-gossypol. The peak time of

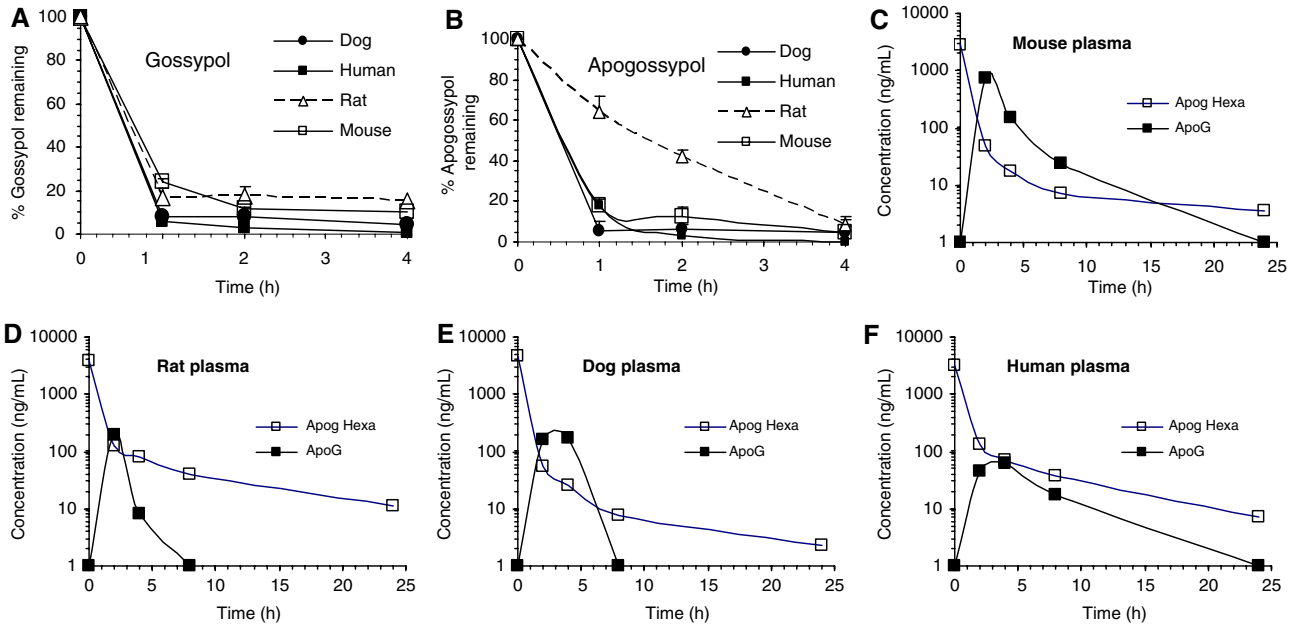


Fig. 2 Stability comparison of gossypol (**a**), apogossypol (**b**) and apogossypol hexaacetate (**c**–**f**) in plasma (5 μg/ml) of various species at 37°C. Note the spontaneous formation of apogossypol from apogossypol hexaacetate in the plasma

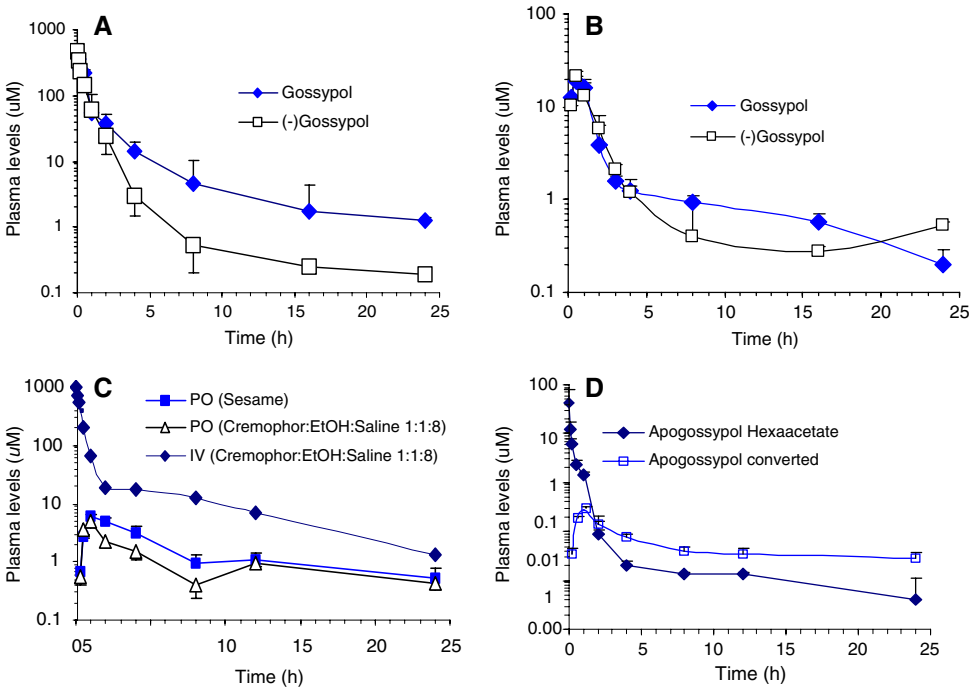


Fig. 3 Comparison of plasma concentration-time course patterns among gossypol analogs: **a** racemic- and (−)-gossypol (both 30 mg/kg, or ~60 μmol/kg) in PEG 400:cremophor EL:water (20:3:77) were administered to mice intravenously, or **b** orally. **c** apogossypol in different formulations was administered to mice i.v. and p.o. at 28 mg/kg (~60 μmol/kg). **d** apogossypol hexaacetate (10 mg/kg, ~20 μmol/kg) in DMSO:cremophor EL:saline (1:1:8) was administered i.v. to mice. The *panel D* also shows in vivo formation of apogossypol (*open square*) converted from intravenous apogossypol hexaacetate (*filled diamond*)

Table 1 Compartmental analysis of pharmacokinetic parameters of (−)-gossypol, (±)-gossypol, apogossypol, and apogossypol hexaacetate after administration to mice (n = 4–5/time point)

Compounds	(−)-Gossypol		(±)-Gossypol		Apogossypol			Apogossypol Hexaacetate
Routes	i.v.	p.o.	i.v.	p.o.	i.v.[a]	p.o.[a]	p.o.[b]	i.v.
C_{max} (μM)	572	21.5	663	21.1	27,644	10.7	13.4	155
T_{max} (h)		0.5		0.5		1	1	
$T_{1/2\beta}$ (h)	0.60	0.74	0.51	0.33	0.22	0.62	1.3	0.34
$T_{1/2\gamma}$ (h)	9.7	30.1	9.2	14.9	5.2	10.4	12.6	8.2
AUC $_{0\rightarrow\infty}$ (μmol•h/l)	237	42.3	422	52	737	75.4	99.4	5.3
Clearance (ml/h/kg)	243		137		82			2,646
V_{ss} (ml/kg)	351		672		219			2,712
F (%)		17.6		12.2		10.2	13.5	

Gossypol and apogossypol were administered at 60 μmol/kg, while apogossypol hexaacetate at 20 μmol/kg due to its limited solubility and administration volume

[a] Apogossypol was formulated in cremophor EL: ethanol: saline (1:1:8, v/v/v)

[b] Apogossypol was formulated in sesame oil

Table 2 Summary of biotransformation of gossypol, apogossypol and apogossypol hexaacetate by phase I and II enzyme reactions in mouse and human microsomes

Substrates	Metabolites observed			
	Phase I		Phase II (glucuronidation)	
	Mouse	Human	Mouse	Human
Apogossypol	NO[a]	NO[a]	Mono-,di- glucuronide	Mono-,di- glucuronide
Apogossypol hexaacetate	di-, tri-, tetra-, penta- acetate	di-, tri-, tetra-, penta- acetate	Di-acetate glucuronide	Di-, tetra-acetate glucuronide
Gossypol	NO[a]	NO[a]	NO[a]	Monoglucuronide

[a] NO annotates 'apparently not observed' during the experiment

(±)-gossypol and (−)-gossypol in plasma following oral administration was about 0.5 h, which appeared to be shorter than the T_{max} observed for its analog apogossypol. The oral C_{max} of (±)-gossypol was similar to that of (−)-gossypol, and moderately higher than apogossypol when the parameter was compared at the same molar dose (Table 1). Gossypol seemed to exhibit relatively higher clearance than apogossypol, resulting in rapid decline in its plasma concentration after the peak concentration.

Intravenous apogossypol disposition pattern showed the initial curvilinear portion of the data primarily representing the distribution of apogossypol into the tissues and the linear portion of the curve primarily representing renal elimination phase of apogossypol (Fig. 3c). Apogossypol showed a relatively long T_{max} and slow clearance; the latter may contribute to the relatively higher AUC of apogossypol in mice. For mice, administered oral doses of apogossypol, plasma concentrations of apogossypol were, in general, slightly higher for those mice given apogossypol in sesame oil than for those mice given apogossypol in cremophor EL:ethanol:saline. Peak plasma concentrations of apogossypol were observed at 1 h after administration of either oral formulation: apogossypol 10.71 ± 2.21 μM for mice given the cremophor EL:ethanol:saline formulation, and 13.40 ± 1.41 μM for mice given the sesame oil formulation. Apogossypol was still detectable in plasma at 24 h after oral administration and the mean plasma concentrations of apogossypol at this time were 0.93 ± 0.22 μM for mice given the cremophor EL:ethanol:saline formulation and 1.13 ± 0.32 μM for mice given the sesame oil formulation. From compartmental analysis of the data, the estimated half-lives of apogossypol were 10.4 h for mice given the cremophor EL:ethanol:saline formulation and 12.6 h for mice given the sesame oil formulation. Figure 4 illustrates oral pharmacokinetic patterns of apogossypol formulated in sesame oil or cremophor EL:ethanol:saline after administration to mice in comparison with the patterns of racemic gossypol and (−)-gossypol. Although oral apogossypol generally showed larger AUC than oral gossypol, which may be attributable to the slow clearance of apogossypol and its stability in liver metabolism circumstances (see below), the differences in pharmacokinetics between oral gossypol and oral apogossypol are moderate. It should be mentioned that, during the LC/MS/MS analysis of apogossypol administered to mice, several distinct peaks were observed in the total ion chromatograms. Based on their masses, these peaks appeared to represent mono- and diglucuronide conjugates of apogossypol, which were also observed in the in vitro liver microsomal phase II reaction mixtures (see below).

Following i.v. administration to mice, apogossypol hexaacetate was distributed to tissues and eliminated from plasma in three apparent phases with half-lives of 0.01, 0.34, and 8.2 h, respectively. Concurrent with the disappearance of apogossypol hexaacetate, plasma concentrations of apogossypol derived from apogossypol hexaacetate increased (Fig. 3d). A mean peak plasma concentration of the converted apogossypol was 0.18 μM at 1 h after i.v. administration of apogossypol hexaacetate. Plasma concentrations of apogossypol subsequently decreased with a terminal half-life of 26.2 h. The $AUC_{0\rightarrow\infty}$ value for the converted apogossypol was 1.52 μmol h/l (or 1.1 μg h/ml) and for the parent apogossypol hexaacetate was 5.3 μmol h/l, suggesting that ~30% of the in vivo apogossypol hexaacetate following intravenous administration had been converted to apogossypol. The magnitude and pattern of in vivo conversion of apogossypol hexaacetate to apogossypol in mouse plasma (Fig. 3d) was similar to what was observed in the in vitro mouse plasma stability study with apogossypol hexaacetate (Fig. 2c), with the exception that, in the in vivo setting, the formed apogossypol was presumably kinetically redistributed and eliminated, and therefore, its total plasma concentration was low. Following oral administration of 60 μmol/kg of apogossypol hexaacetate to mice, neither apogossypol hexaacetate nor apogossypol were detectable in plasma at most of the time points, suggesting poor oral bioavailability of apogossypol hexaacetate.

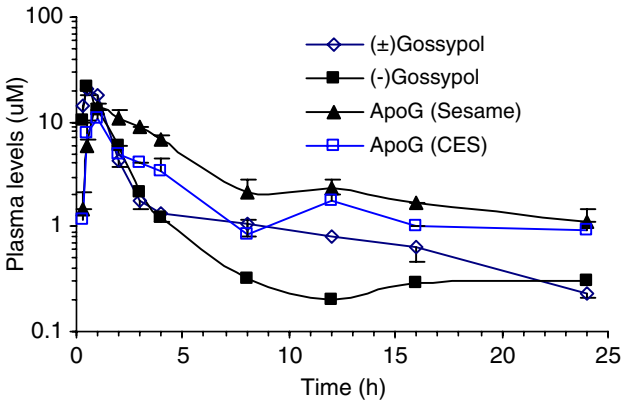


Fig. 4 Pharmacokinetic comparison of racemic gossypol, (−)-gossypol, apogossypol in sesame oil, and apogossypol in cremophor EL:ethanol:saline (CES; 1:1:8, v/v/v) after oral administration to mice at 60 μmol/kg

In vitro metabolism of gossypol, apogossypol and apogossypol hexaacetate in liver microsomes

For mouse and human phase I reactions of gossypol, apogossypol, and apogossypol hexaacetate, the predicted metabolism of these compounds included oxidation, hydrogenation, dehydrogenation, and gain of water. Upon analysis of the mouse and human microsomal reaction mixtures, no peaks were observed that were consistent with the predicted metabolites of either gossypol or apogossypol,

including gossypolone, gossypolonic acid and demethylated gossic acid. Apogossypol was not detected in the gossypol reaction mixtures. Additionally, there were no observed differences between the microsomal reaction mixtures and the negative controls reactions. Comparable losses of each parent molecule were observed between the plasma (Fig. 2) and microsomal reaction samples (Fig. 5). These data suggest that the loss of the parent compounds could be due to thermal instability rather than being enzymatically catalyzed.

For the reaction mixtures containing apogossypol hexaacetate, a decrease in apogossypol concentration was observed that appeared to be attributable to a loss of the acetate groups from the molecule. This was indicated by an increase in the di-, tri-, tetra-, and penta-acetates in the mixtures with a corresponding decrease in the concentration of apogossypol hexaacetate. Quantitative analysis of the three analogs in the microsomal preparations suggested that gossypol and apogossypol hexaacetate were rapidly degraded when incubated with mouse and human liver microsomes (Fig. 5). For example, after incubation at 37°C for 1 h, the remaining gossypol accounted for 19.2 and 23.9% of the total gossypol initially added to mouse and human liver microsomal reaction mixtures, respectively. Under the same condition, the remaining apogossypol hexaacetate accounted for 12.4 and 15.3% of the total apogossypol hexaacetate initially added to mouse and human liver microsomes, respectively. Apogossypol was relatively stable in the mouse and human liver microsomal preparations, with 84.7 and 79.1% remaining after 1 h in mouse and human liver microsomes, respectively.

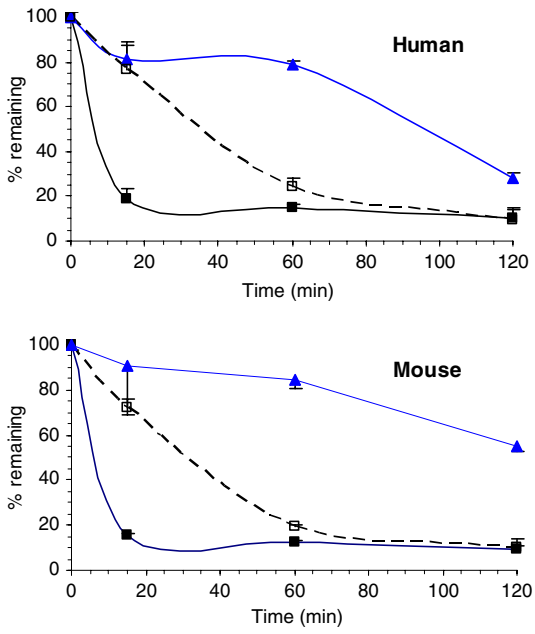


Fig. 5 Stability of gossypol (*open square*), apogossypol (*filled triangle*) and apogossypol hexaacetate (*filled square*) incubated in human (*up panel*) and mouse (*low panel*) liver microsomal preparations (5 μg/ml). Data represent the mean ± SD (n = 3)

For mouse and human glucuronidation reactions, the peaks in mass spectra were observed at masses corresponding to the mono and di-glucuronide conjugate transitions of apogossypol, which were also observed in mouse plasma following administration of apogossypol. The di-glucuronide transition contained the ammonium adduct, which gave an addition of 17 mass units. This adduct was also observed in the glucuronidation reaction mixtures prepared with apogossypol hexaacetate. In addition, the glucuronide of the di-acetate and tetra-acetate was observed in the apogossypol hexaacetate reaction mixtures. No glucuronidation was observed in the gossypol reactions but a loss of the parent compound was observed.

Discussion

Many pharmacokinetic and toxicological studies on gossypol have been completed using various species including human beings, dogs [26], dairy cows [27], rats and rabbits [28], Fischer-344 rats, Sprague-Dawley rats and mice [29, 30]. Table 3 briefly summarized these study results obtained from different laboratories using different methods. The half-lives of gossypol in the elimination phase following oral administration to human beings and animals appear to be relatively long, suggesting that gossypol may have high plasma and tissue protein binding that prevents it from being eliminated from the blood stream in the form of unbound free molecule. This property may be shared by its analog apogossypol that showed a similar $t_{1/2}$ value. The present report is the first to compare the pharmacokinetic profiles of gossypol, apogossypol and apogossypol hexaacetate in mice, and their relative stability in plasma and liver microsomes from various species. In general, racemic gossypol and its (-)enantiomer showed the similar pattern of the plasma concentration-time courses in mice. Racemic gossypol and (−)-gossypol had an oral bioavailability of 12.2 and 17.6%, respectively, after administration to mice. Interestingly, the result was consistent with the one reported 17 years ago [29] (Tables 1, 3). However, there were deviations in the parameters such as clearance, V_{ss} and $AUC_{0\rightarrow\infty}$ between racemic gossypol and (−)-gossypol. It was also noted that the oral elimination $t_{1/2}$ of (−)-gossypol appeared to be longer than that of racemic gossypol (Table 1).

The pharmacokinetic comparison among these analogs (Table 1) indicates that gossypol undergoes more extensive extravascular distribution and is cleared from the plasma compartment at a rate faster than apogossypol. Gossypol may react with basic amino acids such as lysine, glutamine

Table 3 Interspecies comparison of pharmacokinetic parameters of racemic gossypol in various species

Species	Humans [26]	Dogs [26]	Cows [27]	Rats [30] (SD)	Rats [29] (F-344)	Mice [29] (B_6C_3F)
Cl (ml/h/kg)		32 ± 12		160	1,840	1,230
Vd_{ss} (ml/kg)		1,190 ± 930		50	200	1,740
$t_{1/2}$ (h)	286 ± 179	74.5 ± 34	40–67	64.8	4.3	36
Bioavailability		30.9 ± 16.2		60	86	14.3

Clearance and Vd_{ss} were assessed via i.v. administration, while $t_{1/2}$ in the elimination phase was assessed via oral administration

and phenylalanine to form Schiff's bases [31, 32] to bind to target proteins [33, 34], resulting in its effectiveness. The oral bioavailability of gossypol and apogossypol appears comparable and ranges from 10 to 13.5%. The low oral bioavailability of gossypol and apogossypol may be explained by their relatively low trans-membrane permeability across intestinal epithelium, relative instability under the weakly basic condition of small intestine, and/or hepatic first-pass metabolism when administered orally. We observed that these analogs degraded faster at high pH (pH 11) as compared to low pH (pH 3). Various formulations for intravenous administration of gossypol and apogossypol have been tested, and the present formulations seem to be the best in terms of maximal solubility and chemical stability. Although formulation-related deviations in oral pharmacokinetics were observed, these deviations are not significant. Sesame oil appears to be a good formulation for oral apogossypol.

In view of the modest bioavailability of gossypol and apogossypol, it is somewhat surprising that apogossypol hexaacetate possesses essentially no bioavailability. This result suggests that it would not be a good candidate as a prodrug of apogossypol. Concomitant measurement of apogossypol in plasma following oral administration of apogossypol hexaacetate to mice revealed a low (<10 ng/ml) and intermittent presence of apogossypol in circulation. This suggests that the majority of apogossypol hexaacetate degraded in the gastro-intestinal tract and that a small portion was converted to apogossypol either before or after absorption.

Just like yellow–orange six-faced crystals of cobamamide that become deep red upon exposure to air, apogossypol powder gradually turns into black from gray when it is first synthesized. The black apogossypol powder seems stable at −70°C. We determined the stability of apogossypol at room temperature in two liquid formulations used in the present studies: (sesame oil ethanol):cremophor EL:saline (10:10:80) by a HPLC method. We found that after 72-h storage of apogossypol in the liquid formulations, there were 88 and 79% of the original apogossypol remaining in the (sesame oil ethanol):cremophor EL:saline (10:10:80), respectively, suggesting that apogossypol is generally quite stable in the liquids. Our in vitro NMR studies showed that apogossypol was stable in buffered solutions for many days [21]. However, when exposed to air at room temperature, it becomes much more labile. Under barometric pressure of 760 torr and at 25°C, water contains about 8.3 mg/l of saturated O_2, while under the same conditions; air contains 20.95% of O_2, which is about 24,000-fold higher than in water. The high density of O_2 in air may be contributable to the liability of apogossypol, causing it to be oxidized. Indeed, we found that in the presence of 1% ascorbic acid, a commonly known anti-oxidant, the stability of apogossypol under both liquid and air conditions was significantly increased [24].

Phase I reactions (or metabolism used synonymously) involve hydrolysis, reduction, and oxidation of drugs [35]. These reactions expose or introduce a functional group (-OH, -NH_2, -SH, or -COOH) to drugs and usually result in only a small increase in the hydrophilicity of drugs as we observed previously [25, 36]. Phenolic hydroxyl groups are present in both gossypol and apogossypol in addition to two aldehyde groups in gossypol only. Theoretically, one may expect that these groups would be modified. For example, the aldehyde groups might be oxidized to carboxylic acids with NAD+ as the cofactor. Heating gossypol up to 90°C in the presence of 40% NaOH can remove the two aldehyde groups without affecting phenolic hydroxyl groups [21]. However, our data from the in vitro microsomal metabolism study did not indicate the presence of metabolites such as gossypolone, gossypolonic acid, and demethylated gossic acid, suggesting that these gossypol analogs may not be vulnerable to enzymatic degradation. Functional groups exposed or introduced during phase I reactions are often sites for phase II glucuronidation [35]. Glucuronidation of apogossypol was observed in both in vitro mouse liver microsomal reaction and in vivo mouse plasma following oral administration of apogossypol. Based on this result, one may speculate glucuronidation of apogossypol occurs when it is administered to human subjects because the glucuronidation was demonstrated in human microsomal preparation (Table 2). For apogossypol hexaacetate, however, glucuronidation was observed only in the in vitro mouse liver microsomal reaction, not in mouse plasma obtained after oral administration of apogossypol hexaacetate. The disparity between in vitro presence and in vivo absence of apogossypol hexaacetate glucuronidation may be attributable to the fact that apogossypol hexaacetate displays

essentially no oral bioavailability, as evidenced by our present pharmacokinetic studies. Taken together, these observations further support the theory that oral drugs are subject to a 'first-pass effect' in liver when they are sufficiently absorbed from the gastrointestinal tract into the systemic circulation.

It was suggested about 80 years ago that the two aldehyde groups in gossypol may be important for some of its pharmacological effects [37]. This 80-year-old hypothesis needs to be reexamined today. From a series of gossypol derivatives studies, Sonenberg et al. [38] found that the most active compounds were gossypol and apogossypol in an in vitro mouse erythroleukemia cytocidal assay, and the latter was not active in the inhibition of erythrocyte anion transport or in a spermicidal assay, suggesting lower toxicity of apogossypol. Our most recent studies that compared the efficacy and toxicity of gossypol and apogossypol, following administration to Bcl-2 transgenic mice at the same molar doses for 3 weeks, indicated lower toxicity of apogossypol compared to gossypol (unpublished data). In vitro NMR studies showed that apogossypol was stable in buffered solutions for many days, while gossypol, was not stable under the same conditions [21]. The two molecules displayed similar affinities for five Bcl-2 proteins [23]. All the information prompts us to believe that it is worthy to pursue the further development of apogossypol as a proapoptotic agent for cancer.

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