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BIODISTRIBUTION AND PHARMACOKINETICS OF COLON-SPECIFIC HPMA COPOLYMER – 9-AMINOCAMPTOTHECIN CONJUGATE IN MICE

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Abstract

A water soluble N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer - 9-aminocamptothecin (9-AC) conjugate was designed for oral colon-specific drug delivery for the treatment of colon cancer. Comparative studies between the polymer conjugate and free drug have been performed to assess their biodistribution and pharmacokinetics in mice. After oral administration of equal doses of the polymer conjugate or free 9-AC, the drug concentrations in major organs at fixed time points were determined using an HPLC-fluorescence assay. Only 2±1% of 9-AC released from the polymer conjugate was detected in small intestine (SI), and the mean peak concentration of free 9-AC was 45-fold higher than that from released drug. Colon-specific release of 9-AC produced high local concentrations. The mean peak concentration of released 9-AC in cecal contents, feces, cecal tissue, and colon tissue were, respectively, 3.2-fold, 3.5-fold, 2.2-fold and 1.6-fold higher than that using free 9-AC. In plasma, the high and sharp drug concentration profile from free drug was in contrast to the relatively low and flat pharmacokinetic profile obtained from drug released from the HPMA copolymer. There was no significant difference between released and free drug for the area under the concentration-time curve (AUC) and bioavailability values. As a consequence of the colon-specific release of unmodified 9-AC from the polymer conjugate, antitumor efficacy can be anticipated to be enhanced due to prolonged colon tumor exposure to higher and more localized drug concentrations.

Keywords

HPMA copolymer - 9-aminocamptothecin conjugate; aromatic azo bond cleavage; 1,6-elimination; oral administration; colon-specific drug release; biodistribution; pharmacokinetics

Introduction

The anticancer drug 9-AC is a semisynthetic camptothecin analogue [1–4], an inhibitor of topoisomerase I, a nuclear enzyme implicated in the cellular processes of replication, transcription, recombination, DNA repair, and chromosome segregation [5–8]. Inhibition of topoisomerase I by 9-AC via stabilizing the complex formed by the enzyme and DNA results

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in arrest in the G2 cell cycle phase and cell death [9]. 9-AC has demonstrated outstanding antitumor activity against a wide spectrum of solid tumors in animal models including colon, lung, breast, and malignant melanoma xenografts [10–12]. Thus far, however, clinical trials with 9-AC have been disappointing, and this has limited further development [13]. As an S phase-specific drug whose cytotoxicity operates during DNA synthesis, optimal therapeutic efficacy requires prolonged exposure to 9-AC concentrations exceeding a minimum threshold [14]. Due to greater sensitivity of humans (compared to animals) to its myelosuppressive effects, dose-dependent myelosuppression has precluded the use of the 9-AC levels required to achieve plasma concentrations necessary for optimal antitumor activity [15–17].

To improve its therapeutic effectiveness, drug delivery strategies that prolong tumor exposure to 9-AC are being actively pursued. Recently, we proposed a novel drug delivery system for colon-specific delivery of 9-AC for localized treatment of colon cancer. For this, 9-AC was attached to an HPMA polymer carrier via a spacer containing an aromatic azo bond and a 4-aminobenzylcarbamate group [18]. After oral administration, unmodified 9-AC can be colon-specifically released from the polymer by cleavage of the azo bond by the azoreductase activities of colonic microflora, followed by 1,6-elimination. This design may achieve local concentrations that are higher than the threshold concentration of 9-AC. In addition, due to the slow transit rate of the colon, tumors may be exposed to 9-AC for extended periods of time. Residence times in the colon may be up to ten times longer compared to the SI [19], which may enhance the relative antitumor activity in the colon with concomitant low systemic toxicity.

The purpose of the present study is to examine the biodistribution and pharmacokinetics of the HPMA copolymer–9-AC conjugate and of free 9-AC after oral administration to mice. 9-AC concentrations in major organs, target site—colon, and plasma were quantitatively analyzed by an HPLC-fluorescence assay. Pharmacokinetic parameters were calculated based on plasma 9-AC concentration-time profiles.

Materials and Methods

Abbreviations

9-AC—9-aminocamptothecin; AUC—area under the curve; C_{max}—maximal plasma concentration; CPT—camptothecin; DI—deionized; DMA—dimethyl acetamide; DMSO—dimethyl sulfoxide; F—absolute bioavailability; GI—gastrointestinal; HPMA—N-(2-hydroxypropyl)methacrylamide; IV—intravenous; LI—large intestine; MRT—mean residence time; PEG—polyethylene glycol; r.t.—room temperature; SI—small intestine; T_{max}—time to reach maximal plasma concentration.

Materials

9-AC was a generous gift from National Cancer Institute, NIH. HPMA copolymer - 9-AC conjugate was prepared as previously described [18,20]. Briefly, the polymer conjugate (Fig. 1) was prepared by radical copolymerization of HPMA (6.5 mmol) with 9-AC containing monomer, 9-N-{4-[4-(N-methacryloyl-N'-oxymethylcarbonyl-propyldiamino)-3-chlorophenyl-azo] benzylmethoxycarbonyl}aminocamptothecin (0.1 mmol), in the presence of 2,2'-azobisisobutyronitrile (0.43 mmol) as the initiator at 50°C for 24 h. The molecular weight (M_n = 24 kDa, M_w = 38 kDa, polydispersity = 1.6) was estimated by size exclusion chromatography (Pharmacia AKTA system). The content of 9-AC (2.2 wt%) was determined by UV spectroscopy at 360 nm using $\epsilon = 30200 \text{ M}^{-1} \text{ cm}^{-1}$. All other chemicals were from VWR (West Chester, PA). The solvents used were HPLC grade.

Animals

Female Swiss Webster mice, 24–26 g bodyweight, were purchased from Charles River (Wilmington, MA) and kept in a typical laboratory environment: four or five per cage with an air filter cover under light (12 h light/dark cycle) and temperature-control (22 ± 1 °C). The mice were fasted for 16 h prior to and during the experiments, while allowed free access to water. All animals received care in compliance with the “Principles of Laboratory Animal Care” and “Guide for the Care and Use of Laboratory Animals”. Experiments followed an approved protocol from the University of Utah Institutional Animal Care and Use Committee.

Drug administration and sample collection

The polymer conjugate and free drug solutions were prepared using different solvents. The HPMA copolymer – 9-AC conjugate was dissolved in DI water, whereas free 9-AC was dissolved in dimethyl acetamide (DMA), and then mixed with a diluent containing 51% polyethylene glycol (PEG) 400 and 49% 0.01 M phosphoric acid. The final solution consisted of 6% DMA, 48% PEG 400 and 46% 0.01 M phosphoric acid [21]. For oral administration, free drug or polymer conjugate were administered to the mice with a single bolus using feeding needles at doses of 1.25 mg/kg or 3.0 mg/kg of 9-AC or 9-AC equivalent. For intravenous (IV) injection, a single free drug dose of 1 mg/kg of 9-AC was administered by direct injection into a lateral tail vein, with a duration of infusion less than 1 min.

After oral administration of free drug or polymer conjugate, four mice per time point were anesthetized by halothane, bled by heart puncture, and sacrificed. Heparinized blood samples were immediately centrifuged at $5.5 \times g$ for 2 min on a tabletop centrifuge, and the plasma was separated and transferred to microcentrifuge tubes. Major organs were collected including lung, heart, liver, spleen, kidney, small intestine (SI), cecum, and colon. Cecal contents and feces were also collected and all samples were weighed. Only plasma was collected for the mice that received free drug intravenously (four mice per time point). All harvested samples were stored at -20°C and analyzed within one month. The tissue samples were stable at -20°C , and no significant differences were found between the samples stored at -20°C and -80°C .

Sample preparation

For concentration determination, all samples were acidified to convert 9-AC carboxylate to 9-AC lactone. The conversion of carboxylate to lactone form is reversible and pH-dependent; the active lactone form predominates at acidic pH, and the inactive open-ring carboxylate form is favored at neutral and alkaline pH [22]. Frozen plasma samples were thawed, mixed with 8.5% phosphoric acid and an internal standard—camptothecin (CPT) in DMSO, and kept at room temperature (r.t.) for 1 h. The mixture was loaded onto a preconditioned C_{18} solid-phase extraction cartridge (Waters, Milford, MA), washed with 2 ml DI water, and eluted with 0.3 ml methanol. The eluent was combined with 0.2 ml of 1% of phosphoric acid (to prevent lactone ring opening) and then filtered through a $0.45 \mu\text{m}$ nylon membrane filter before HPLC analysis.

Frozen organs, tissues, cecal contents, and feces were thawed, dispersed in 1% phosphoric acid, and homogenized with a mechanical blender (IKA, Wilmington, NC). The homogenates were then centrifuged at 15000 rpm for 15 min. The supernatant was mixed with CPT as an internal standard, extracted and filtered as described above, except washing the cartridge with 25% (v/v) methanol before elution.

HPLC assay

Although 9-AC has a relatively poor fluorescence compared to the parent compound CPT [23], acidification prior to analysis readily converts 9-AC to a fluorescent species. The 9-AC concentrations in all samples were determined using an HPLC-fluorescence assay based on a

method by Takimoto [23]. Briefly, an acidic chromatographic mobile phase was used to enhance 9-AC fluorescence resulting in increased sensitivity compared to post-column acidification methods. The sample was injected onto a C₁₈ silica gel column (250 mm x 4.6 mm, 5 µm mean particle size Ultrasphere ODS, Beckman Instruments, San Ramon, CA) at r.t. The samples were eluted with a mobile phase consisting of methanol/KH₂PO₄ aqueous solution (25 mM; pH 2.55; 4/6, v/v) at a flow rate of 1.0 ml/min. The excitation and emission wavelengths for fluorescence detection were 365 and 440 nm, respectively. The HPLC assay was accurate and reproducible with coefficients of variation ranging from 2% to 7%.

Pharmacokinetic analysis

Pharmacokinetic parameters were calculated using noncompartment model (WinNonlin Standard Edition Version 2.1; Pharsight, Cary, NC). The software was used to analyze 9-AC plasma concentration vs. time data after IV and oral administration of free drug or oral administration of polymer conjugates. The mean residence time (MRT) and absolute bioavailability (F) were also determined.

Statistical analysis

The results were expressed as mean ± standard deviation. Satterthwaite approximation was used to calculate the p-value for the two-sample t-test for independent samples from polymer-bound and free drug groups with unequal variances. The p-values less than 0.05 were considered significant.

Results

Distribution in small intestine

After 16 h of fasting, minimal contents were observed in the SI of mice. Whole small intestines (with fluids) were homogenized and the content of 9-AC was quantitatively determined using an HPLC assay. The distribution results of polymer-bound and free 9-AC in the SI after oral administration are displayed in Fig. 2. The results represent the total amount of 9-AC in the SI. Large differences have been observed in the distribution of polymer-bound and free drug. The mean peak 9-AC concentrations in SI were 1.3 nmol/g for HPMA copolymer – 9-AC conjugate, and 58 nmol/g for free 9-AC for an oral dose of 1.25 mg/kg of 9-AC or 9-AC equivalent. Only 2±1% of 9-AC was released from the polymer conjugate in SI, while the majority of free 9-AC remained in the SI for about 3 h following oral administration. The time dependence of the 9-AC concentration in the SI exhibited a sharp maximum approximately 2 h after administration. The sharp decrease in concentration at time intervals >2 h may be attributed to drug absorption through the SI and intestinal drug transit.

Distribution in cecum and colon

The biodistribution results after oral administration of HPMA copolymer – 9-AC conjugate or free 9-AC at a dose of 1.25 mg/kg of 9-AC or 9-AC equivalent for the cecum contents, feces, cecal tissue, and colon tissue are demonstrated in Figs. 3, 4, 5, and 6, respectively. In all cases, the 9-AC concentrations were considerably higher for the polymer-bound drug than for the free drug. The mean peak 9-AC concentrations in cecal contents, feces, cecal and colon tissues, respectively, were 275, 230, 11, and 5 nmol/g for 9-AC released from the HPMA copolymer conjugate, and 87, 66, 5, and 3.2 nmol/g for the administration of free 9-AC. Even 24 h after oral administration, 9-AC was detectable in cecum and colon. The mean 9-AC concentration in cecal contents, feces, cecal and colon tissues were 85, 106, 5.7 and 2.5 nmol/g for 9-AC released from the polymer, and 13, 11, 1.4 and 1.2 nmol/g for free drug after oral dosing, respectively. Since the aromatic azo bond in the HPMA copolymer conjugate was degraded by the microflora in cecum and colon [24], the curve 9-AC concentration vs. time exhibits a

maximum. The relatively sharp increase in 9-AC concentration (Figs. 3–6) is a consequence of colon-specific 9-AC release, and the subsequent decrease in concentration may be attributed to drug absorption via the cecum and colon and intestinal transit. Following the oral administration of free 9-AC, a flatter concentration profile was detected.

Pharmacokinetics

In order to examine drug absorption, plasma 9-AC concentrations were determined using an HPLC-fluorescence assay. For systemic availability, the mice received a single bolus intravenous injection of free drug at a dose of 1 mg/kg, and the plasma drug concentrations were measured at scheduled time points. The concentration vs. time profiles after oral administration of free drug or HPMA copolymer conjugate at a dose of 3 mg/kg is shown in Fig. 7. Two different patterns were displayed in the profiles. Following the oral administration of HPMA copolymer – 9-AC conjugate, 9-AC was detectable in the plasma after a 2 h time lag. The observed mean T_{max} from the polymer group (5.4 h) was longer than that of the free drug group (1 h). In contrast, the observed mean C_{max} from the polymer group (110 nM) was lower than that of the free drug group (350 nM). Though not detectable with free drug, the mean plasma concentration for released 9-AC 24 h after oral administration was 28 nM [25]. The plasma levels of 9-AC intact lactone and opened-ring carboxylate forms were similar in mice, and 9-AC lactone accounted for 62% of the total drug AUC [26]. Therefore, the concentration of active lactone form of 9-AC was more than 10 nM in plasma.

Pharmacokinetic parameters, calculated by a noncompartmental model, are provided in Table 1. The MRT (mean residence time) value of 9-AC from the polymer conjugate (10.2 h) was about two fold higher than that for free 9-AC (4.8 h). This indicated that the average residence time of 9-AC after oral dosing of polymer conjugate is longer than that of free drug. There was not a statistically significant difference ($p > 0.05$) in the AUC values between polymer-bound (1740 ± 330 nM·h) and free drug (1730 ± 340 nM·h). The plasma drug concentration vs. time profile (Fig. 8) after IV injection was used to estimate bioavailability. The results showed that the bioavailability values of 9-AC from the polymer conjugate ($35 \pm 4.1\%$) or free drug ($34 \pm 4.5\%$) were not significantly different ($p > 0.05$).

Distribution in other organs, and metabolites

The 9-AC concentrations in major organs (lung, heart, liver, spleen, and kidney), after oral administration of free drug or the polymer conjugate at a dose of 1.25 mg/kg of 9-AC or 9-AC equivalent, were low and undetectable by the HPLC assay. A few peaks were detected by mass spectroscopy in cecum and liver samples, suggesting metabolism of 9-AC. In the cecum, a peak at m/z 428.26 (calculated $M+Na$, 428.35) was identified as an acetylation product of the drug. In the liver, N-hydroxylation of the amine group [27] was identified as a peak at m/z 379.2 (calculated 379.35).

Discussion

A number of polymer conjugates containing aromatic azo bonds have been investigated to achieve colon-specific drug delivery [28–30]. Previously, an HPMA copolymer conjugate was designed for colon-specific delivery of 9-AC, which was bound via spacers containing peptide and aromatic azo bonds [31]. In vitro and in vivo [32] studies indicated that after entering the colon, the aromatic azo bond was cleaved first by azoreductase activities, followed by peptidase-catalyzed cleavage of the peptide bond in the drug derivative to generate free drug. However, subsequent cleavage of drug-containing fragment by peptidase was found to be slow, as only around 36.6% of 9-AC was released 24 h after incubation in rat cecal contents [31]. And around 1.43 nmol/g of 9-AC was detected in luminal content of the colon 12 h after oral administration [32]. In order to efficiently release 9-AC, a spacer containing 4-

aminobenzylcarbamate group was inserted between an aromatic azo bond and the drug. The cleavage of the aromatic azo bond unmasked the strong electron-donating amine group, thus initiating an electronic cascade, which allowed the immediate release of unmodified 9-AC from the conjugate by 1,6-elimination [18]. An in vitro degradation study demonstrated that around 85% of 9-AC was released from this conjugate within 12 h in rat cecal contents [18]. Here, we report the results of the in vivo study of this conjugate in mice.

In small intestine, only a tiny amount ($2\pm 1\%$) of 9-AC was released from the polymer conjugate. Although bacteria are distributed throughout the gastrointestinal tract, very few bacteria are present in the small intestine capable of degrading azo bonds. This result was consistent with our in vitro data, where we reported that less than 4% of 9-AC was released from the polymer 12 h after incubation in rat SI contents or mucosa [18]. In contrast, oral administration of free 9-AC resulted in a mean peak concentration 45-fold higher than that released from the polymer-bound drug in the SI. As a hydrophobic drug, free 9-AC was rapidly absorbed in SI owing to its large surface area, resulting in a peak concentration in plasma that was likely above its therapeutic window. The dose-limiting toxicities, including myelosuppression and diarrhea, have been observed at a peak concentration of 150 nM of 9-AC (lactone + carboxylate) in plasma in adult patients [33]. Therefore, an oral dose of 3 mg/kg of free 9-AC producing a peak plasma concentration of 360 nM is toxic (Fig. 7).

In the colon, abundant microflora and extended transit time were exploited for targeted drug release and prolonged drug exposure. The vast majority of bacteria are found in the distal gut and counts have been estimated to be 10^{11} per gram, compared to 10^4 per gram in the proximal small intestine [34–36]. Bacteria sensitivity of the HPMA copolymer 9-AC conjugate resulted in much higher concentrations of released 9-AC in the cecum contents, feces, cecal and colon tissues than that of oral free drug. Moreover, due to slow transit, some of released 9-AC still stayed in cecum and colon 24 h after oral administration of the polymer conjugate. This prolonged exposure of the drug may be of potential benefit to local therapy of colon cancer since 9-AC has a cell cycle dependent mechanism.

Drug absorption pattern for a colon-specific released drug is different from that of free drug [37]. A delayed absorption was observed for released 9-AC, since it took longer time to reach the cecum and colon than the SI after oral administration. T_{max} of 9-AC for the released 9-AC (5.4 ± 1.3 h) was longer than that for the free 9-AC group (1 ± 0.25 h). On the other hand, due to higher drug absorption rate in the SI than the LI, a higher C_{max} using oral free drug (350 ± 50 nM) was observed, compared to that from released drug (110 ± 23 nM).

Colon-specific absorption of released 9-AC affected its pharmacokinetic parameters. MRT for released drug (10.2 ± 0.61 h) was longer than that of free drug (4.8 ± 0.43 h). The longer residence time of released drug in colon resulted in sustained absorption [38,39]. The colon, like a homogeneous reservoir, elicited slow and constant drug input into systemic circulation similar to that observed with continuous infusion. Relatively flat plasma drug levels were measured following oral administration of the polymer conjugates. These steady plasma drug concentrations may provide not only a safety benefit by reducing the magnitude of peak plasma drug levels [40], but may also result in sustained drug exposure of tumor [41]. High absorption rates for oral free drug in the SI were compensated by the prolonged absorption times for released drug in the cecum and colon. AUC values of released drug were similar to that of free drug. Bioavailability (F) values were similar for released drug and free drug, and were close to those reported by Kirstein, with a median of 32% [42].

Metabolism of the drug was investigated at oral dose of 1.25 mg/kg, which was the maximal dose used in a schedule of daily $\times 5$ for 2 consecutive weeks [42]. A few metabolic peaks were detected in cecum and liver samples using mass spectrometry, indicating possible first-pass

elimination. There is limited information regarding the metabolism of 9-AC in the literature, however, it was reported that clearance of 9-AC was increased in patients receiving stable regimens of anticonvulsant drugs known to induce CYP450 enzymes [43].

The drug delivery system presented here permits the attachment of lectins [44] to achieve targeting to inflamed and/or cancerous tissue in the colon mediated by binding to colonic glycoproteins [45]. Attachment of peanut agglutinin, a lectin binding to the Thomsen-Friedenreich antigen, to HPMA copolymers resulted in increased binding to diseased colonic tissues [45]. Localized delivery to the diseased tissue may result in enhanced therapeutic efficacy [46].

Conclusions

The distribution of HPMA copolymer 9-AC conjugate was markedly different from that of free 9-AC after oral administration. In contrast to free drug dispersal along the whole GI tract, unmodified 9-AC was predominantly released from the polymer conjugate in cecum and colon. The absorption of drug from different regions of the GI tract was found to influence the pharmacokinetic profile and parameters. In conclusion, colon-specific delivery of 9-AC was achieved after oral administration of the HPMA copolymer 9-AC conjugate to the mice. High local drug concentration with prolonged exposure time provides a potential to enhance antitumor efficacy with low systemic toxicity for the treatment of colon cancer.

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$$x=1.25, y=160$$

Figure 1.
The structure of HPMA copolymer 9-AC conjugate.

Figure 2. 9-AC in SI after oral administration of free 9-AC or HPMA copolymer – 9-AC conjugate at a dose of 1.25 mg/kg of 9-AC or 9-AC equivalent in mice. Bars represent standard deviation (n=4 for each time point).

Figure 3. 9-AC in cecal contents after oral administration of free 9-AC or HPMA copolymer – 9-AC conjugate at a dose of 1.25 mg/kg of 9-AC or 9-AC equivalent in mice. Bars represent standard deviation. n=4

Figure 4. 9-AC in feces after oral administration of free 9-AC or HPMA copolymer – 9-AC conjugate at a dose of 1.25 mg/kg of 9-AC or 9-AC equivalent in mice. Bars represent standard deviation. n=4

Figure 5. 9-AC in cecum tissues after oral administration of free 9-AC or HPMA copolymer – 9-AC conjugate at a dose of 1.25 mg/kg of 9-AC or 9-AC equivalent in mice. Bars represent standard deviation. n=4

Figure 6. 9-AC in colon tissues after oral administration of free 9-AC or HPMA copolymer – 9-AC conjugate at a dose of 1.25 mg/kg of 9-AC or 9-AC equivalent in mice. Bars represent standard deviation. n=4

Figure 7. Plasma 9-AC concentration vs. time profile after oral administration of free 9-AC or HPMA copolymer – 9-AC conjugate at a dose of 3 mg/kg of 9-AC or 9-AC equivalent in mice. Bars represent standard deviation. n=4

Figure 8. Plasma drug concentration-time profile in mice after IV injection of free 9-AC at a dose of 1 mg/kg. Bars represent standard deviation. n=4

Table 1

9-AC pharmacokinetic parameters after oral administration of free drug or HPMA copolymer – 9-AC conjugate at a dose of 3 mg/kg of 9-AC or 9-AC equivalent in mice

	C_{max} (nM)	T_{max} (h)	AUC₀₋₂₄ (nM·h)	MRT (h)	F (%)
Free Drug	350±50	1±0.25	1730±340	4.8±0.43	34±4.5
Polymer Conjugate	110±23	5.4±1.3	1740±330	10.2±0.61	35±4.1

C_{max}: maximal plasma concentration

T_{max}: time to reach maximal plasma concentration

AUC: area under plasma concentration vs. time curve

MRT: mean residence time

F: Bioavailability