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Antitumor Efficacy of Colon-Specific HPMA Copolymer/9-Aminocamptothecin Conjugates in Mice Bearing Human-Colon Carcinoma Xenografts

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Abstract

The antitumor activity of a colon-specific *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer – 9-aminocamptothecin (9-AC) conjugate (P-9-AC) was assessed in orthotopic and subcutaneous animal (HT29 xenograft) tumor models. P-9-AC treatment of mice bearing orthotopic colon tumors, with a dose of 3 mg/kg of 9-AC equivalent every other day for 6 weeks, resulted in regression of tumors in 9 of 10 mice. A lower dose of P-9-AC (1.25 mg/kg of 9-AC equivalent) every other day for 8 weeks inhibited subcutaneous tumor growth in all mice. No liver metastases were observed. Colon-specific release of 9-AC from polymer conjugates enhanced antitumor activity and minimized the systemic toxicity.

Graphical abstract

Keywords

Introduction

Colorectal cancer is the third-most-common cancer in both men and women in the United States. The American Cancer Society estimates that 153 760 cases of colorectal cancer were diagnosed, and 52 180 people died from this disease in 2007.^[1] Colorectal cancer is also the third-leading cause of cancer-related deaths in the United States, accounting for 10 and 11% of cancer deaths in men and women, respectively. The lifetime risk of developing colorectal cancer is 1 in 17 for both men and women with 90% of cases occurring after the age of 50 years. Two types of active drugs are currently used for the treatment of colorectal cancer: thymidylate synthase inhibitors, such as 5-fluorouracil (5-FU), and topoisomerase-I inhibitors, such as irinotecan (CPT-11). It appears that topoisomerase-I inhibitors are more efficient than thymidylate synthase inhibitors in colorectal-cancer management. In advanced colorectal cancer, 5-FU monotherapy produced a response rate of only 10–15%,^[2] compared to a 10–35% response rate with CPT-11 in 5-FU refractory colon-cancer patients.^[3–5] The patients receiving CPT-11 also showed a survival advantage in comparison with the 5-FU group with one-year survival rates of 45% and 32%, respectively.^[6] Recently, the US Food and Drug Administration (FDA) approved two new target therapies, bevacizumab and cetuximab, to treat metastatic colorectal cancer.^[1] Bevacizumab blocks vascular endothelial growth factor (VEGF), thus cutting off the blood supply to tumor cells resulting in cell death. Cetuximab blocks the epidermal growth-factor receptors (EGFR) that promote tumor-cell growth. AEE788 and ZD6474 are dual-inhibitors of VEGF and EGFR.^[7,8] A more-potent antitumor response can be achieved through concomitant inhibition of both the EGFR and VEGF receptors using AEE788 or ZD6474, compared to using monoinhibitors. Some of these new, targeted therapies, such as AEE788 and ZD6474, are in clinical trials.

9-AC and other camptothecin analogues primarily target topoisomerase I, a nuclear enzyme that embeds in the double-helix structure of DNA. Consequently, these molecules engage in almost all biological transactions of DNA such as replication, transcription, recombination and repair.^[9–12] Topoisomerase I relaxes torsionally strained DNA through the formation of transitory single-strand breaks.^[13] The broken strand is rapidly religated with concomitant

release of active tyrosine from the end of the DNA.^[14] As a result of the transient relaxation and unwinding of supercoiled DNA by topoisomerase I, the replication fork proceeds down the DNA strand and is used as a template for the synthesis of a new DNA strand.^[15] Camptothecins convert topoisomerase I into a cellular poison by inhibiting the religation step via stabilizing the transient, cleavable DNA/topoisomerase I complex and forming an enzyme/drug/DNA ternary cleavable complex. Following the collision of the replication fork with this cleaved strand of DNA, the cell cycle arrests in the G2 phase, thus leading to cell death.^[16–19] 9-AC demonstrated not only much-stronger topoisomerase-I inhibitory activity than irinotecan in studies *in vitro*, but also antitumor activities against a variety of human-tumor xenografts including colon cancer in preclinical studies. However, in spite of its impressive preclinical antitumor activities, clinical development of 9-AC has been disappointing because of its dose-limiting toxicity, such as neutropenia and thrombocytopenia, which were noted after systemic drug administration via intravenous infusion.^[14]

To overcome the disadvantage of systemic treatment, water-soluble *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers have been exploited as carriers for colon-regional drug delivery. Kopecková et al.^[20–22] evaluated bioadhesive HPMA-copolymer/5-aminosalicylic acid (5-ASA) conjugates with side-chains terminating in acylated fucosylamine, a targeting moiety to the lectin-like structures present in the colon. These polymers have a two-fold specificity: bioadhesion and drug release in the colon. 5-ASA was released from the polymer *in vitro* by the azoreductase activities in the cecal content of guinea pigs, rats and rabbits, as well as in human feces. A higher adherence to guinea-pig colon was observed for the polymer containing fucosylamine moieties, compared to that without fucosylamine moieties. Lu et al.^[23] synthesized HPMA-copolymer/cyclosporine conjugates containing lectins as the targeting moieties for the treatment of inflammatory bowel diseases. The cyclosporine derivative was released *in vitro* by the incubation of the polymer conjugate with the rat cecal contents. Sakuma et al.^[24] designed and synthesized HPMA-copolymer/9-AC conjugates where 9-AC was bound via spacers containing amino acid residues and aromatic azo bonds. *In vitro* evaluation indicated that the azo bond was reduced first, followed by the release by peptidases of unmodified 9-AC from the 9-AC-containing fragment. However, the release of unmodified 9-AC was slow. Less than 37% of 9-AC was released 24 h after incubation with the rat cecal contents. An *in vivo* study demonstrated that the plasma concentration of 9-AC increased gradually and reached approximately 30×10^{-9} M at 24 h after oral administration of the polymer, and the bioavailability was only 1.7% in rats.^[25] To ensure a highly efficient release of unmodified 9-AC, a new polymer conjugate containing a novel spacer between 9-AC and the polymer carrier was prepared. Rapid release of 9-AC in the colon was achieved by the cleavage of this spacer through aromatic azo bond degradation followed by 1,6-elimination. Within 12 h after incubation of the polymer with the rat cecal contents, nearly 85% of the 9-AC was released.^[26] Biodistribution and pharmacokinetic studies indicated that, as a consequence of the rapid release, the maximal plasma concentration of 9-AC (110×10^{-9} M) was reached at approximately 5.4 h after oral administration of the polymer conjugate, with a bioavailability of 35% in mice.^[27] In this study, we investigated the antitumor activities of

the HPMA-copolymer/9-AC conjugate, containing a novel spacer, against human-colon carcinoma xenografts in nude mice.

Experimental Part

Chemicals

9-AC was a generous gift from the National Cancer Institute, NIH. All of the other chemicals were from VWR (West Chester, PA).

HPMA-Copolymer/9-AC Conjugate

P-9-AC (P is the HPMA copolymer backbone) was prepared as previously described.^[26] Briefly, the HPMA-copolymer conjugate, whose structure is shown in Figure 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}amino-camptothecin (0.1 mmol), in the presence of 2,2'-azoisobutyronitrile (AIBN, 0.43 mmol) as the initiator at 50 °C for 24 h. The molecular weight ($M_w = 38$ kDa; $M_n = 24$ kDa; polydispersity index = 1.6) was estimated by size-exclusion chromatography using Superose 6 HR 10/30 column (AKTA/FPLC system, GE Healthcare), phosphate-buffered saline (PBS) buffer +30% acetonitrile. PolyHPMA standards of narrow polydispersity (1.1–1.2) were used for calibration. The content of 9-AC (2.2 wt.-%) was determined by UV spectroscopy at 360 nm using $\epsilon = 30\,200\text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

Animals

Female nu/nu nude mice (25–28 g) were purchased from the National Cancer Institute and used for tumor transplantation. The animals were maintained under constant temperature (23 ± 2 °C), in a humid atmosphere ($50 \pm 5\%$), and under a 12 h light/dark cycle with food and water available *ad libitum*. All of the animals were acclimated for at least one week before tumor implantation. The animal experiments were performed according to “Principles of Laboratory Animal Care” and “Guide for the Care and Use of Laboratory Animals”. The experimental protocols were approved by the University of Utah Institutional Animal Care and Use Committee.

Tumor Models

Human-colon tumor cells, HT29, were obtained from ATCC (American Type Culture Collection) (Manassas, VA) and maintained as a monolayer in a McCoy medium supplemented with 10% fetal bovine serum (FBS). Cells in the logarithmic growth phase were used for tumor implantation. To produce *subcutaneous tumors*, 1×10^6 HT29 cells per mouse were subcutaneously (s.c.) injected into the left flank of nude mice. The treatment started seven days after s.c. inoculation, when the tumor volume was $36 \pm 12\text{ mm}^3$. To induce *orthotopic colon tumors*, nude mice were anesthetized by intraperitoneal (i.p.) injection of a mixture of xylazene ($10\text{ mg} \cdot \text{kg}^{-1}$) and ketamine ($50\text{ mg} \cdot \text{kg}^{-1}$). A small incision was made on the lower left of the midline and the cecum was gently exteriorized. Then, 1×10^6 HT29 cells in 50 μL of Matrigel tumor suspension were injected subserously at the junction of the mesocolon and the cecum. This part of the cecum wall is coated with

fold visceral peritoneum and is continuous with the mesocolon; there is more soft tissue in the subserosa of this part. Consequently, a small volume of a 50 μL tumor suspension can be carefully injected subserously. After injection, the Matrigel will become solid at body temperature, and localize between the layer of the mucosa and the serosa of the cecum wall. The cecum was then returned to the peritoneal cavity, and the abdominal wall and skin were closed.

Dose Formulation and Administration

For oral administration, P-9-AC was dissolved in deionized water at 10 mg of conjugate per mL, equivalent to 0.22 mg $\cdot$ mL⁻¹ of 9-AC. The free drug was dissolved in dimethylacetamide (DMA) first, then diluted with a solution comprising 51% poly(ethylene glycol) (PEG) 400 and 49% 0.01 M phosphoric acid. The final solution comprised 6% DMA, 48% PEG 400 and 46% 0.01 M phosphoric acid; the concentration of 9-AC was 0.27 mg $\cdot$ mL⁻¹. P-9-AC and 9-AC were administered by oral gavage every other day during the treatment period. Two doses were used, namely a higher dose of 3 mg $\cdot$ kg⁻¹ and a lower dose of 1.25 mg $\cdot$ kg⁻¹ of 9-AC or 9-AC equivalent.

Antitumor Activity

The antitumor efficacy was assessed by tumor-volume (TV) change and tumor-volume inhibition (TVI). The tumor growth was measured twice a week with calipers. The tumor volume was calculated by the formula: length $\times$ (width)²/2, where the length was the longest diameter and the width was the shortest diameter perpendicular to the length. The tumor volume inhibition was expressed according to the following formula: TVI (%) = 100 – [(mean TV in treated group) / (mean TV in control group)] $\times$ 100.

Toxicity

The toxicity of the drug was assessed by relative bodyweight (RBW) and survival rate. The relative bodyweight was calculated by the formula: RBW% = 100 – [(bodyweight change at day y) / (bodyweight at day zero)] $\times$ 100. The mice were weighed twice a week during the experimental time. The survival rate was calculated by the formula: survival% = [(number of surviving animals at day y)/(number of animals at day zero)] $\times$ 100. The mice were examined for overt signs of any adverse drug-related side effects, and inspected daily for mortality.

Histological Assessment

Conventional procedures were used for histological assessment. After sacrifice of the mice, the cecal tissues were immediately removed, fixed in PBS (pH = 7.3) buffered 10% formalin solution, embedded in paraffin and cut into sections 6 μm thick by the histological laboratory at the University of Utah Hospital. The histological slides were stained with hematoxylin and eosin (H&E), and examined under using optical microscopy.

Carcinoembryonic-Antigen (CEA) Concentration Measurement

A solid-phase enzyme-linked immunosorbent assay (ELISA) was used to determine the CEA concentrations. A commercially available kit (Ref 5201, Diagnostic Automation, Inc. Calabasas, CA) was used according to the manufacturer's instructions.

Statistical Analysis

The results were presented as the mean $\pm$ the standard deviation (SD). The statistical significance of the results was evaluated by one-way analysis of variance (ANOVA) for more than two groups, or by the Student's *t*-test for two groups. The difference was considered statistically significant when the *p* value was less than 0.05. Statistical analysis was performed using the GraphPad Prism program (Version 4.02, San Diego, CA).

Results

Antitumor Activity Against Orthotopic Tumors

Nude mice bearing orthotopic, human-colorectal HT29 carcinoma xenografts were used to examine the antitumor activity of P-9-AC. Seven days after tumor initiation (by cell injection), the tumor-bearing mice were divided into three groups: i) treatment with P-9-AC; ii) treatment with 9-AC; and iii) no treatment. The HPMA-copolymer conjugate, P-9-AC, and the free drug, 9-AC, were orally administered to the mice using a feeding needle at a dose of $3 \text{ mg} \cdot \text{kg}^{-1}$ of 9-AC (or 9-AC equivalent) every other day. Treatment started 7 days after inoculation. The drugs were administered every other day for 6 weeks. The P-9-AC treatment resulted in complete regression of the orthotopic tumors in 8 out of 9 mice. In the polymer-treatment group, no mice showed any manifestation of disease for 8 weeks. At the end of the experiments, the mice were euthanized. At necropsy, eight out of nine mice presented a macroscopically normal colon, no liver metastases and disseminated tumors were observed. In contrast, large tumors were found in the cecums of the nontreated (control) mice, four weeks after tumor implantation. All of the mice treated with $3 \text{ mg} \cdot \text{kg}^{-1}$ of free 9-AC died within two weeks due to the toxicity of the free drug.

To demonstrate the efficacy of colon-wall implantation to create localized and invasive orthotopic tumors, necropsy and histology were performed. The tumors were identified using H&E staining, and demonstrated an invasive pattern. After two weeks, the tumors grew beyond the limits of *muscularis propria* and spread toward the mucosa of the cecum (Figure 2A). Three weeks after implantation, the tumor invaded the mucosa and kept growing as a large mass in the lumen (Figure 2B–C).

Antitumor Activity against Subcutaneous Tumors

Mice bearing subcutaneous tumors were treated every other day by oral administration of P-9-AC and 9-AC for 8 weeks. Two doses were used, namely $1.25 \text{ mg} \cdot \text{kg}^{-1}$ and $3.0 \text{ mg} \cdot \text{kg}^{-1}$ of 9-AC or 9-AC equivalent. At the lower-dose level ($1.25 \text{ mg} \cdot \text{kg}^{-1}$), P-9-AC delayed tumor growth with TVI = $96 \pm 1\%$ eight weeks after treatment (Figure 3A). The activity of the free 9-AC at $1.25 \text{ mg} \cdot \text{kg}^{-1}$ was similar to that of P-9-AC (Figure 3A). At the higher dose ($3 \text{ mg} \cdot \text{kg}^{-1}$), P-9-AC produced tumor regression with no recurrence, observed through the completion of the study with TVI = $99 \pm 1\%$ eight weeks after treatment (Figure 3B). At

necropsy (day 56), no mice presented any liver metastases or disseminated tumors. In contrast, all of the mice treated with $3 \text{ mg} \cdot \text{kg}^{-1}$ (free) 9-AC died within two weeks due to the non-specific 9-AC toxicity. Mice in the nontreated group had to be sacrificed before the end of the experiment due to excessive tumor growth. Typical large differences in subcutaneous tumor sizes between nontreated mice and mice treated with P-9-AC ($3 \text{ mg} \cdot \text{kg}^{-1}$ 9-AC equivalent) are demonstrated in Figure 3C.

Toxicity of Free and HPMA-Copolymer-bound 9-AC

The non-specific toxicity of P-9-AC and 9-AC was estimated by body-weight determination in the colon-tumor models. In the P-9-AC treatment groups, no significant weight loss was observed at the lower ($1.25 \text{ mg} \cdot \text{kg}^{-1}$) dose level (data not shown); the group of mice treated with $3 \text{ mg} \cdot \text{kg}^{-1}$ of 9-AC equivalent exhibited an average 12% weight loss (Figure 3D). The weight loss in the higher-dose group was transient and not accompanied by any other concomitant overt signs of toxicity. On the contrary, mice treated with $3 \text{ mg} \cdot \text{kg}^{-1}$ of (free) 9-AC lost bodyweight gradually (Figure 3D), and eventually died within two weeks (Figure 4). The lower dose of the free drug, as well as the administration of the solvent mixture only (control) did not produce significant bodyweight loss (data not shown).

CEA Concentration in Serum

The serum CEA levels were correlated with the tumor size for the tumor models used (Figure 5). It is important to note that due to small tumor sizes, the method was less sensitive.

Discussion

In clinical trials, 9-AC was initially formulated in DMA/PEG and then later as a colloidal dispersion (CD). The solubility of 9-AC is greatly enhanced in a DMA vehicle. A lyophilized colloidal dispersion of 9-AC revealed not only enhanced water solubility, but also a slightly improved toxicity profile with regard to neutropenia, compared with the original DMA formulation.^[28] At first, 9-AC was administered in DMA solvent at a maximum tolerated dose (MTD) of $0.85 \text{ mg} \cdot \text{m}^{-2}$ per day (total dose $2.55 \text{ mg} \cdot \text{m}^{-2}$) as a continuous intravenous infusion on days 1–3 and repeated every 2 weeks. In 1997, the formulation was changed to a colloidal dispersion based on preliminary phase I data. The dose of 9-AC/CD was $1.1 \text{ mg} \cdot \text{m}^{-2}$ per day (total dose $3.3 \text{ mg} \cdot \text{m}^{-2}$) as a continuous intravenous infusion on days 1–3 and repeated every 2 weeks. The dose of 9-AC/CD is nearly 30% higher than that for 9-AC/DMA with a similar toxicity profile.^[29] Unfortunately, 9-AC in both formulations failed to demonstrate antitumor activity in clinical trials because of dose-limited toxicity. In Phase I trials of a 72 h infusion of 9-AC, dose-dependent myelosuppression was the major toxicity.^[30–32] In subsequent Phase II trials, despite showing modest activity in ovarian cancer^[33] and malignant lymphoma,^[34] 9-AC didn't demonstrate antitumor activities against lung cancer^[35] or colon cancer^[36] on both 72 h infusion and 120 h infusion schedules. Thus, further drug development has been hampered in clinical trials.

To reduce the systemic toxicity of 9-AC, a drug-delivery-based approach was developed in our study. By colon-targeted delivery of 9-AC, the HPMACopolymer/9-AC conjugates are exploited to improve therapeutic efficacy and biocompatibility. The targeting of 9-AC specifically to the colon has the potential to overcome the disadvantages of systemic treatment such as continuous intravenous infusions for colon cancer. This local therapy ensures direct treatment at the disease site with a reduction in associated systemic adverse effects. Apparently, therapeutic effects depend on local concentrations of 9-AC around colon tumors whereas systemic 9-AC exposure might cause side effects. Previously, HPMACopolymer/9-AC conjugates were designed and synthesized for oral colon-targeted drug delivery in our laboratory.^[26] In addition, *in vitro* drug release from the polymer conjugates, and biodistribution and pharmacokinetics after oral administration of the polymer conjugates in mice were evaluated.^[27] In this study, the antitumor activities of the HPMACopolymer/9-AC conjugates against human-colon carcinoma xenografts in nude mice were investigated.

Colon-specific release of unmodified 9-AC from P-9-AC provided potent antitumor activity against orthotopic colon tumors. Local-regional drug delivery by the HPMACopolymer and prolonged drug exposure in the colon played important roles in the induction of cytotoxic effect towards orthotopic tumors. This drug-delivery system allowed direct delivery of 9-AC into the tumor site over a short time due to fast drug release in the colon. Using a pharmacokinetic model, it was predicted that, in rats, 9-AC was gradually released from P-9-AC in the cecum and reached a peak point (about 60% of total oral dosing released) at approximately 6 h after oral administration. At 24 h after oral administration of P-9-AC, 7% of the total dose remained in the cecum.^[37] In mice, the mean peak concentrations of released 9-AC in the 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 those obtained using free 9-AC.^[27] Therefore, it was expected that colon-targeted drug delivery will maximize the antitumor effect against local-regional tumors by producing high levels of 9-AC in the colon for a prolonged time. Colon-specific release of unmodified 9-AC from P-9-AC may result also in systemic effects against colon tumors. The drug-delivery system improved the pharmacokinetic properties of 9-AC by sustained absorption of the released drug. As a lipophilic drug, 9-AC rapidly diffused into systemic circulation across lipid membranes, following its release from the polymer in the colon. Due to slow colonic transit, a large amount of 9-AC might be absorbed during the transit time, thus creating sustained levels of 9-AC in the blood. It has been reported that for an oral dose of $3 \text{ mg} \cdot \text{kg}^{-1}$ of 9-AC or 9-AC equivalent (P-9-AC) to mice, the mean residence time (MRT) of 9-AC was $10.2 \pm 0.61 \text{ h}$ for the drug released from the polymer conjugate; this was longer than the MRT of free 9-AC ($4.8 \pm 0.43 \text{ h}$).^[27] Even 24 h after oral administration of P-9-AC, the mean plasma concentration of 9-AC was 28 nM ; this was above the threshold drug concentration.^[38,39] Lethal damage to tumor cells can be enhanced by prolonging the circulation time of 9-AC at concentrations above the threshold drug concentration. The duration and concentration of 9-AC in systemic circulation illustrated the efficiency of the polymer conjugates for the treatment of subcutaneous tumors.

As a consequence of drug absorption only through the colon, reduced systemic toxicity was observed for the P-9-AC conjugates compared with the free drug. The colon has a smaller

surface area compared to the small intestine; thereby, the drug absorption rate in the colon is lower than that in the small intestine. A lower peak concentration of the 9-AC in plasma was obtained for the colon-specific released drug. It has been demonstrated that the peak concentration of free 9-AC in plasma after oral administration is about three-fold higher than that from the colon-specific released drug, because free 9-AC is rapidly absorbed in the small intestine.^[27] Reducing the magnitude of the peak plasma drug levels by colon-specific release provides a safety benefit. It was found that a peak concentration above 150×10^{-9} M of 9-AC in plasma is toxic for adult patients.^[40] The reduction in toxicity using the polymer conjugates is clearly a critical factor in enabling an improvement in the therapeutic index over the free drug.

DNA topoisomerase I (topo I) is the principle target for 9-AC and its analogues in colon tumor cells. Topo I activity was relatively high in colon tumors, in comparison to lung and breast tumors. In addition, topo I concentrations are significantly increased in malignant colonic tissues from advanced human-colon adenocarcinoma or from xenografts of colon cancer in immunodeficient mice.^[2,41,42] The differences in activity between the tumor groups suggest that the potential efficacy of inhibitors of topo I in colon tumors may be greater than in lung and breast tumors.^[41,43,44] It has been reported that, on the basis of topo I inhibition, camptothecins potently induce apoptosis in human-colon tumor cells, such as HT29. As a result of topo I inhibition, the nuclei of the HT29 cells fragment, thus leading to the release of nuclear-envelope proteins into the cytosol.^[45] The specific effects of camptothecins on the proteome of HT29 cells are predominantly characterized by increased levels of nuclear-envelope proteins, such as lamin A, lamin A/C transcript variants, and lamin A mutant forms (progerins).^[46] Therefore, as a representative of human colon cancer, HT29 tumor was exploited to evaluate the antitumor efficacy of P-9-AC conjugates.

CEA, a 180 kDa oncofetal glycoprotein, is one of the first widely investigated, soluble tumor markers, and has been used to monitor responses of therapy and disease progression.^[47] The function of CEA is known as a potentiator of metastasis by modulating immune responses, by facilitation of intercellular adhesion, and by cellular migration.^[48] Thus, increased expression of CEA could contribute to metastatic potential, as well as the ability of local tumor expansion.^[49] As observed in humans, the colon is the main site of CEA production, and circulating CEA can be detected in most patients with CEA-positive tumors. Though increased CEA levels have been found under both benign and malignant conditions, it has been reported that over 95% of patients with benign conditions have a CEA level below $5 \text{ ng} \cdot \text{mL}^{-1}$, whereas a CEA level above $5 \text{ ng} \cdot \text{mL}^{-1}$ has usually been found in cases of colorectal carcinomas. Colorectal-carcinoma patients with a high level of serum CEA on the first diagnosis have a poor prognosis or distant metastases,^[50] and a preoperative CEA level greater than or equal to $5 \text{ ng} \cdot \text{mL}^{-1}$ predicts poor survival chance for colorectal-carcinoma patients. In addition, increasing CEA level is associated with tumor recurrence after surgery. In animal experiments, since the tumor was the source of elevated serum CEA, the serum CEA was used as a marker of tumor growth.^[51] However, despite benefits in prognosis and follow-up, the CEA exhibited a low sensitivity (30–40%) particularly for early small colon tumors.^[52] In our experiments, CEA secreted by the HT29 human-colon tumor cells was detected in the serum. The tumor sizes correlated with the CEA levels in the serum: the CEA levels increased with increasing tumor sizes. When the

tumor size was small (less than 150 mm³), the CEA concentration in the serum was not detectable.

Conclusion

A preliminary study of the antitumor activity of HPMA-copolymer/9-AC conjugates towards orthotopic and subcutaneous tumor models of human-colon carcinoma was performed; free 9-AC served as a control. Oral administration of the HPMA-copolymer/9-AC conjugate resulted in remarkable antitumor activity in HT29 xenografts in nude mice with concomitant reduction in systemic toxicity when compared with free-drug treatment. It is evident that more experiments are needed to fully evaluate the potential of P-9-AC, including comparison with free 9-AC at equitoxic doses. The main conclusion, however, which warrants further studies, is the fact that higher doses of P-9-AC (9-AC equivalent) could be administered without side effects. This is encouraging since one of the main problems in clinical trials of 9-AC is the dose-limiting toxicity of the free drug. Consequently, it appears that the HPMA-copolymer/9-AC conjugates have potential in colon-cancer treatment.

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References

1. 2007 <http://www.cancer.org/downloads/STT/CAFF2007PWSecured.pdf>.
2. Boyer J, McLean EG, Aroori S, Wilson P, McCulla A, Carey PD, Longley DB, Johnston PG. *Clinical Cancer Res.* 2004; 10:2158. [PubMed: 15041737]
3. Goldwasser F, Bae I, Valenti M, Torres K, Pommier Y. *Cancer Res.* 1995; 55:2116. [PubMed: 7743511]
4. Rougier P, Bugat R, Douillard JY, Culine S, Suc E, Brunet P, Becouarn Y, Ychou M, Marty M, Extra JM, Bonnetterre J, Adenis A, Seitz JF, Ganem G, Namer M, Conroy T, Negrier S, Merrouche Y, Burki F, Mousseau M, Herait P, Mahjoubi M. *J. Clinical Oncology.* 1997; 15:251.
5. Rothenberg ML, Cox JV, DeVore RF, Hainsworth JD, Pazdur R, Rivkin SE, Macdonald JS, Geyer CE Jr, Sandbach J, Wolf DL, Mohrland JS, Elfring GL, Miller LL, Von Hoff DD. *Cancer.* 1999; 85:786. [PubMed: 10091755]
6. Garcia-Carbonero R, Supko JG. *Clinical Cancer Res.* 2002; 8:641. [PubMed: 11895891]
7. Traxler P, Allegrini PR, Brandt R, Brueggen J, Cozens R, Fabbro D, Grosios K, Lane HA, McSheehy P, Mestan J, Meyer T, Tang C, Wartmann M, Wood J, Caravatti G. *Cancer Res.* 2004; 64:4931. [PubMed: 15256466]
8. Ciardiello F, Caputo R, Damiano V, Caputo R, Troiani T, Vitagliano D, Carlomagno F, Veneziani BM, Fontanini G, Bianco AR, Tortora G. *Clin. Cancer Res.* 2003; 9:1546. [PubMed: 12684431]
9. Liu LF, Wang JC. *Proc. Natl. Acad. Sci. USA.* 1987; 84:7024. [PubMed: 2823250]
10. Wu HY, Shyy SH, Wang JC, Liu LF. *Cell.* 1988; 53:433. [PubMed: 2835168]
11. Wang JC, Caron PR, Kim RA. *Cell.* 1990; 62:403. [PubMed: 2165864]
12. Subramanian D, Rosenstein BS, Muller MT. *Cancer Res.* 1998; 58:976. [PubMed: 9500459]
13. Moisan F, Longy M, Robert J, Le Morvan V. *Br. J. Cancer.* 2006; 95:906. [PubMed: 16983402]
14. Ulukan H, Swaan PW. *Drugs.* 2002; 62:2039. [PubMed: 12269849]
15. Stewart L, Redinbo MR, Qiu X, Hol WG, Champoux JJ. *Science.* 1998; 279:1534. [PubMed: 9488652]

16. Covey JM, Jaxel C, Kohn KW, Pommier Y. *Cancer Res.* 1989; 49:5016. [PubMed: 2548707]
17. Hsiang YH, Lihou MG, Liu LF. *Cancer Res.* 1989; 49:5077. [PubMed: 2548710]
18. D'Arpa P, Beardmore C, Liu LF. *Cancer Res.* 1990; 50:6919. [PubMed: 1698546]
19. Tsao YP, D'Arpa P, Liu LF. *Cancer Res.* 1992; 52:1823. [PubMed: 1312900]
20. ěhová B, Rath R, Kope ková P, Kope ek J. *Int. J. Pharm.* 1992; 87:105.
21. Rath R, Kope ková P, ěhová B, Kope ek J. *J. Polymer Sci., Part A: Polym. Chem.* 1991; 29:1895.
22. Kope ková P, Rath R, Takada S, ěhová B, Berenson MM, Kope ek J. *J. Controlled Release.* 1994; 28:211.
23. Lu ZR, Gao SQ, Kope ková P, Kope ek J. *Bioconjugate Chem.* 2000; 11:3.
24. Sakuma S, Lu ZR, Kope ková P, Kope ek J. *J. Controlled Release.* 2001; 75:365.
25. Sakuma S, Lu ZR, Pecharová B, Kope ková P, Kope ek J. *J. Bioactive Compatible Polym.* 2002; 17:305.
26. Gao SQ, Lu ZR, Petri B, Kope ková P, Kope ek J. *J. Controlled Release.* 2006; 110:323.
27. Gao SQ, Lu ZR, Kope ková P, Kope ek J. *J. Controlled Release.* 2007; 117:179.
28. Leguizamo J, Quinn M, Takimoto CH, Liang MD, Ismail AS, Pang J, Dahut W, Grem JL. *Cancer Chemotherapy Pharmacology.* 2003; 52:333. [PubMed: 12819941]
29. Bartlett NL, Johnson JL, Wagner-Johnston N, Ratain MJ, Peterson BA. *Cancer Chemotherapy Pharmacology.* 2009; 63:793. [PubMed: 18648813]
30. Rubin E, Wood V, Bharti A, Trites D, Lynch C, Hurwitz S, Bartel S, Levy S, Rosowsky A, Toppmeyer D. *Clinical Cancer Res.* 1995; 1:269. [PubMed: 9815982]
31. Dahut W, Harold N, Takimoto C, Allegra C, Chen A, Hamilton JM, Arbuck S, Sorensen M, Grollman F, Nakashima H, Lieberman R, Liang M, Corse W, Grem J. *J. Clinical Oncology.* 1996; 14:1236.
32. Takimoto CH. *Clinical Cancer Res.* 2001; 7:229. [PubMed: 11234873]
33. McCarthy N, Sarosy G, Minasian L, Davis P, Tompkins A, Dyer V, Jones-Wells A, Smith J, Kohler D, Takimoto C, Fig WD, Kohn EC, Reed E. *Proc. Am. Soc. Clinical Oncology.* 1999; 18:363a.
34. Wilson WH, Little R, Pearson D, Jaffe ES, Steinberg SM, Cheson BD, Humphrey R, Kohler DR, Elwood P. *J. Clinical Oncology.* 1998; 16:2345.
35. Vokes EE, Ansari RH, Masters GA, Hoffman PC, Klepsch A, Ratain MJ, Sciortino DF, Lad TE, Krauss S, Fishkin PA, Golomb HM. *Ann. Oncology.* 1998; 9:1085.
36. Kindler HL, Avadhani A, Wade-Oliver K, Karrison T, Mani S, Vokes EE. *Investigational New Drugs.* 2004; 22:323. [PubMed: 15122080]
37. Gao SQ, Sun Y, Kope ková P, Peterson CM, Kope ek J. *Pharmaceutical Res.* 2008; 25:218.
38. Xiong HQ, Tran HT, Madden TL, Newman RA, Abbruzzese JL. *Clinical Cancer Res.* 2003; 9:2066. [PubMed: 12796369]
39. Supko JG, Malspeis L. *Cancer Res.* 1993; 53:3062. [PubMed: 8319213]
40. de Jonge MJ, Punt CJ, Gelderblom AH, Loos WJ, van Beurden V, Planting AS, van der Burg ME, van Maanen LW, Dallaire BK, Verweij J, Wagener DJ, Sparreboom A. *J. Clinical Oncology.* 1999; 17:2219.
41. Giovanella BC, Stehlin JS, Wall ME, Wani MC, Nicholas AW, Liu LF, Silber R, Potmesil M. *Science.* 1989; 246:1046. [PubMed: 2555920]
42. Magrini R, Bionde MR, Hanski ML, Nottter M, Scherubl H, Boland CR, Zeitz M, Hanski C. *Int. J. Cancer.* 2002; 101:23. [PubMed: 12209584]
43. Hsiang YH, Liu LF, Wall ME, Wani MC, Nicholas AW, Manikumar G, Kirschenbaum S, Silber R, Potmesil M. *Cancer Res.* 1989; 49:4385. [PubMed: 2545341]
44. McLeod HL, Douglas F, Oates M, Symonds RP, Prakash D, van der Zee AG, Kaye SB, Brown R, Keith WN. *Int. J. Cancer.* 1994; 59:607. [PubMed: 7960233]
45. Winkelman I, Nassl AM, Daniel H, Wenzel U. *Int. J. Cancer.* 2008; 122:2223. [PubMed: 18214853]

46. Dechat T, Shimi T, Adam SA, Rusinol AE, Andres DA, Spielmann HP, Sinensky MS, Goldman RD. Proc. Natl. Acad. Sci. USA. 2007; 104:4955. [PubMed: 17360326]
47. Ojima T, Iwahashi M, Nakamura M, Matsuda K, Nakamori M, Ueda K, Naka T, Ishida K, Primus FJ, Yamaue H. Int. J. Cancer. 2007; 120:585. [PubMed: 17096339]
48. Hilska M, Collan YU, Laine VJ O, Kossi J, Hirsimaki P, Laato M, Roberts PJ. Diseases Colon Rectum. 2005; 48:2197.
49. Modrak DE, Gold DV, Goldenberg DM, Blumenthal RD. Tumour Biol. 2003; 24:32. [PubMed: 12743424]
50. Boonpipattanapong T, Chewatanakornkul S. J. Clinical Gastroenterology. 2006; 40:592.
51. Kiyama T, Onda M, Tokunaga A, Nishi K, Mizutani T, Yoshiyuki T, Shimizu Y, Matsukura N, Tanaka N, Asano G. Jpn. J. Cancer Res. 1990; 81:58. [PubMed: 2108947]
52. Ward DG, Suggett N, Cheng Y, Wei W, Johnson H, Billingham LJ, Ismail T, Wakelam MJO, Johnson PJ, Martin A. Br. J. Cancer. 2006; 94:1898. [PubMed: 16755300]

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

Figure 2.

Orthotopic, human-colon HT29 carcinoma in the cecum of nude mice; all of the histological sections were stained with hematoxylin and eosin (H&E): A) The tumor grew beyond the limits of *muscularis propria* and spread towards the mucosa of the cecum two weeks after implantation. B) The tumor invaded the mucosa and kept growing as a large mass in the lumen three weeks after implantation. C) Normal cecum.

Figure 3.

Human-colon HT29 carcinoma heterotransplanted subcutaneously into nude mice: A) Growth inhibition after administration of $1.25 \text{ mg} \cdot \text{kg}^{-1}$ of 9-AC or 9-AC equivalent (P-9-AC) every other day by oral gavage. B) Growth inhibition after administration of $3.0 \text{ mg} \cdot \text{kg}^{-1}$ of 9-AC or 9-AC equivalent (P-9-AC) every other day by oral gavage. All of the mice treated with free 9-AC (triangles) died at day 14. C) Comparison of tumor sizes in nude mice bearing subcutaneous human-colon HT29 carcinoma xenografts (day 49). Left, tumor from nontreated mouse; right, tumor from mouse treated every other day with P-9-AC ($3.0 \text{ mg} \cdot \text{kg}^{-1}$ 9-AC equivalent). D) Relative bodyweights of mice bearing subcutaneous human colon HT29 carcinoma xenografts, treated with $3 \text{ mg} \cdot \text{kg}^{-1}$ of 9-AC or 9-AC equivalent (P-9-AC).

Figure 4. Survival curves of mice bearing human-colon carcinoma xenografts, treated with 9-AC and P-9-AC at a dose of $3 \text{ mg} \cdot \text{kg}^{-1}$ of 9-AC or 9-AC equivalent.

Figure 5. Relationship between CEA concentrations in serum and tumor sizes in mice bearing subcutaneous human-colon carcinoma xenografts ($r = 0.85$, $p = 0.0005$).