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Synthesis, characterization, and in vitro and in vivo evaluation of a novel pectin–adriamycin conjugate

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ABSTRACT

Adriamycin (ADM) has been widely used in the treatment of many types of solid malignant tumor. However, cardiotoxicity, multidrug resistance and a short half-life in vivo are significant problems that limit its clinical application. To resolve these problems, a novel pectin–adriamycin conjugate (PAC) was synthesized by attaching ADM to low-methoxylated pectin via an amide linkage. The ADM content and weight-average molecular weight (Mw) of PAC were greater than 25% (w/w) and 50,360 g/mol, respectively. PAC was highly stable in plasma, but 33.2% of ADM was released from PAC after incubation for 30 h with lysosomes derived from rat liver. PAC was distributed uniformly in the cytoplasm of most A549 cells and accumulated in the nucleus of a few A549 cells after incubation for 30 h. At concentrations equivalent to 0.125–1.000 μg of ADM/mL, PAC did not inhibit the growth of either A594 or B16 cells to the same extent as free ADM or a mixture of ADM and pectin. Interestingly, at all concentrations, PAC inhibited the growth of 2780cp cells in vitro significantly more effectively than ADM or the mixture of ADM and pectin. The anticancer effect of PAC in vivo was evaluated with C57BL/6 mice bearing pulmonary metastases of B16 cells. Compared with ADM and the mixture of ADM and pectin, PAC suppressed tumor growth significantly and prolonged the mean survival time of the B16-inoculated mice. PAC has great potential for development as a tumor targeting polymer-drug.

1. Introduction

Anticancer drugs have been used extensively in cancer therapy over five to six decades. Most anticancer drugs are small molecules that penetrate into all cell types by diffusion. One of their main drawbacks is that they cannot be targeted within the body, which both weakens their anticancer effects and results in serious toxic and side effects. To improve the distribution of these drugs in the body and their anticancer effects, polymer-drug conjugates have been introduced over the last two decades and have been established as an effective drug delivery system.[1–3] Conjugating low molecular weight drugs with high molecular weight polymers insures that they can only enter cells by endocytosis. Conjugates that are internalized are transferred into lysosomes, and digested by lysosomal enzymes, which releases the attached small anticancer drugs.[4] Polymer-drug conjugates accumulate preferentially in solid tumors due to the enhanced permeability and retention (EPR) effect. It is widely accepted that the EPR effect can be attributed to the high vascular density of the tumor, increased permeability of the tumor vessels, defective tumor vasculature, and defective or suppressed lymphatic drainage in the tumor interstitium.[5]

Adriamycin (ADM) has been effective against many types of solid malignant tumor in extensive clinical applications. However, it can induce severe toxic and side effects, such as bone marrow depression, nephrotoxicity, hepatotoxicity, and baldness, together with effects on the heart and gastrointestinal tract. In addition, multidrug resistance and the short half-life of ADM in vivo limit its clinical application.[6–9] Conjugating ADM with polymers such as carboxymethylpullulan,[10] *N*-(2-hydroxypropyl)methacrylamide copolymer,[3,11,12] poly(D,L-lactic-co-glycolic acid),[13] poly-(L-lysine citramide),[14] D-α-tocopheryl polyethylene glycol 1000 succinate[15] and polyethylene glycol[16] can improve its anticancer activity and decrease toxic and side effects. However, the drug-loading efficiency of these polymer-drug conjugates is comparatively low, which limits their application. Recently, dendritic polymers have emerged as potentially ideal drug delivery vehicles because they are manipulated easily and provide a high density of functional groups.[17–19] However, although the dendrimers have the capability to be conjugated with high amounts of drug molecules, most of the reports imply that only 4–5 molecules are conjugated to each molecule of polymer.[20] The low reproducibility of synthesis of the branched polymers is also a

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drawback.[3] Therefore, a polymer is required that has a high density of functional groups and can be used to prepare polymer-drug conjugates with high drug-loading efficiency.

Pectin, which is a natural water-soluble polysaccharide, is an anionic biopolymer.[21] Due to its good biocompatibility, pectin has been studied as a vehicle material for drug delivery and has even been used as a blood substitute.[22,23] The characteristic structure of pectin is as follows. The basic pectin backbone comprises a linear chain of α-(1→4)-linked D-galacturonic acid; this is referred to as homogalacturonan. In portions of this backbone, galacturonic acid can be replaced by (1→2)-linked L-rhamnose, and then side-chains that contain various neutral sugars can branch off from the rhamnose residues. This type of pectin is called rhamnogalacturonan I. A third structural type of pectin is rhamnogalacturonan II; it occurs less frequently and is a complex, highly-branched polysaccharide.[24–26] The majority of pectin consists of homogalacturonan domains. There is an abundance of carboxyl and hydroxyl groups on the α-D-galacturonic acid residues of homogalacturonan. The carboxyl groups of pectin can be condensed with the amino groups of drugs through dehydration to yield pectin–drug conjugates of high drug-loading efficiency. Furthermore, the amide linkage of the conjugates can be hydrolyzed easily by lysosomal enzymes. Therefore, pectin seems a good polymer for conjugation with anticancer drugs to improve tumor targeting properties.

In the study reported herein, a modified low-methoxylated pectin was conjugated with ADM to give a pectin–adriamycin conjugate (PAC). PAC was synthesized and prepared in such a way such that very little free ADM was released into the blood during intravenous application, and it was designed to enter cells easily by internalization and to target solid tumors by the EPR effect. The synthesis, characterization, pharmaceutics, and in vitro and in vivo evaluation of the targeting of PAC were studied successively.

2. Results and discussion

2.1. Synthesis of PAC

2.1.1. Preparation of low-methoxylated pectin

High-methoxylated pectin was treated sequentially by alkaline hydrolysis, dialysis, and ultrafiltration by centrifugation to prepare low-methoxylated pectin. Gel permeation chromatography (GPC) revealed that the weight-average molecular weight (Mw) and the number-average molecular weight (Mn) of the pectin were 43,930 g/mol and 10,750 g/mol, respectively. The polydispersity of the pectin was 4.088. The results suggested that the modified low-methoxylated pectin had a broad molecular weight distribution.

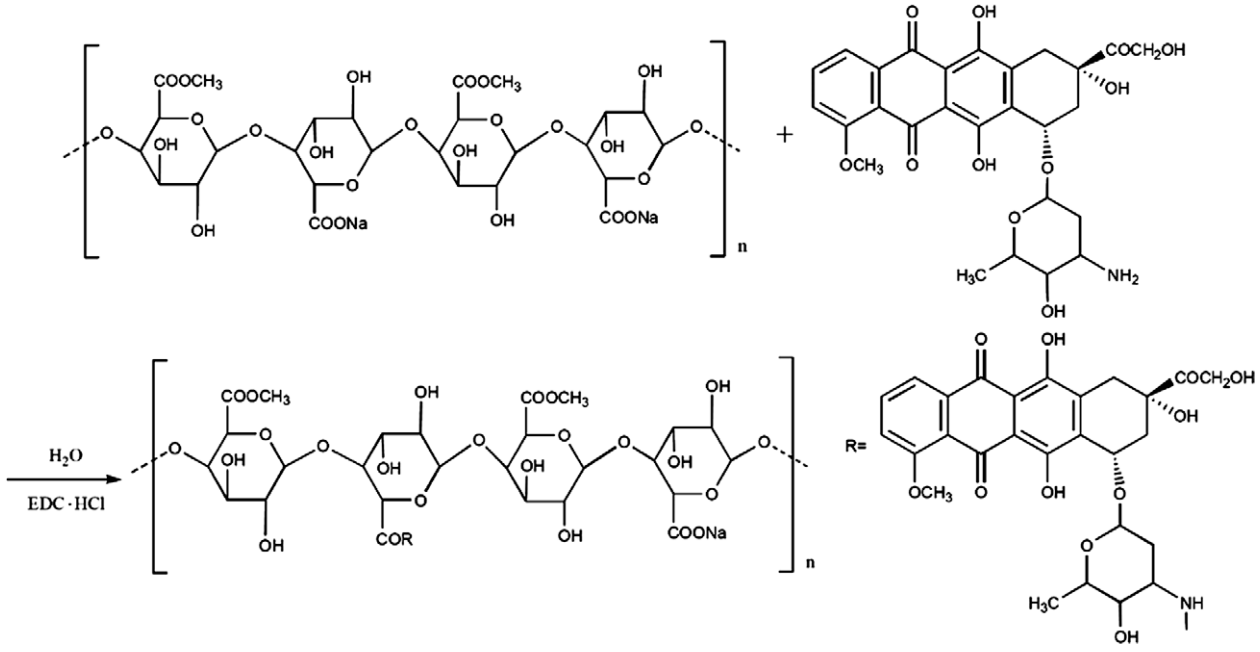
2.1.2. Synthesis of PAC

The low-methoxylated pectin was conjugated with ADM in the presence of 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC·HCl) in aqueous solution to give PAC via the route shown in Scheme 1. The pH of pectin in solution is 3.0 or lower depending on its concentration. However, at pH <3.5, pectin forms a hydrogel, mainly by hydrophobic interaction and the formation of hydrogen bonds,[27,28] and this gel state interferes with the conjugation process. To eliminate gel formation during the synthesis process, the pH of the pectin solution was adjusted to 7.0 before ADM·HCl and EDC·HCl were added. PAC obtained by this method was water-insoluble, which improved its stability in the circulatory system. To purify PAC further, free ADM was removed by dialysis because it passes easily through a semi-permeable membrane.

The Mw and Mn of PAC were 50,360 g/mol and 24,040 g/mol, respectively. The polydispersity of PAC was 2.095. It was reasonable that the Mw of PAC was higher than that of the low-methoxylated pectin because a considerable amount of ADM became linked to the pectin during the conjugation. Furthermore, the polydispersity of PAC decreased obviously compared with that of low-methoxylated pectin. The lower value of polydispersity suggested that the molecular distribution of the conjugate was more homogeneous than that of the pectin.

2.2. Characterization of PAC

The structure of PAC was characterized by ultraviolet–visible (UV–vis) spectroscopy, Fourier Transform Infrared (FT-IR) spectroscopy, ^{13}C solid state nuclear magnetic resonance (ssNMR) spectroscopy, and X-ray Diffraction (XRD).



Scheme 1. Synthesis of PAC. ADM was conjugated with low-methoxylated pectin in the presence of EDC·HCl in aqueous solution to yield PAC.

It can be seen from Figure 1 that in the UV spectra, the maximum absorption of ADM, the mixture of ADM and pectin, and PAC was at 479.5 nm, 479.5 nm, and 498.0 nm, respectively. Pectin did not absorb in the UV spectrum. The obvious red shift of PAC suggested that a covalent conjugate had been formed between ADM and the low-methoxylated pectin.

The infrared spectra of PAC, pectin, mixture of ADM and pectin, and ADM were shown in Figure 2. The spectra revealed typical bands for functional groups at 1611.4 cm^{-1} (overlap of the amide I and amide II bands) and 1283.0 cm^{-1} (amide III band), which indicated the formation of an amide linkage between ADM and the low-methoxylated pectin.[29] A major absorption at around 3400 cm^{-1} was attributed to stretching of hydroxyl groups and an absorption at 1740 cm^{-1} was caused by C=O stretching vibration of methyl-esterified carboxyl groups.[30]

In the ^{13}C ssNMR spectra of high-methoxylated pectin (Fig. 3a), the peak at 171.5 ppm and 53.8 ppm were the resonances of C=O and OCH_3 of methyl ester. In spectra of low-methoxylated pectin (Fig. 3b), only one peak was observed at 182.5 ppm which was corresponded to ionic carboxyl group since the saponification of the pectin led to the disappearance of the signals at 171.5 ppm and there were no signals of methyl ester as observed in the initial high-methoxylated pectin.[31] In Figure 3c, the ^{13}C ssNMR spectra of PAC contained four carbonyl peaks: δ 176.8 ppm, free carboxyl of pectin; δ 161.6 ppm, amide between pectin and adriamycin;[32] δ 186.2 ppm and δ 156.8 ppm were corresponded to adriamycin.[33]

The results of the ^{13}C ssNMR, FT-IR, and UV–vis spectroscopy demonstrated that ADM was linked covalently to the low-methoxylated pectin. Furthermore, given that dehydration is part of the conjugation process, we deduced that the amide linkage predominated in the conjugate. Taking into account the results of GPC (data not shown), no obvious cross-linking was found between pectin molecules in the conjugate.

XRD (data not shown) analyzes indicated that PAC was an amorphous powder with a few microcrystallite structures.

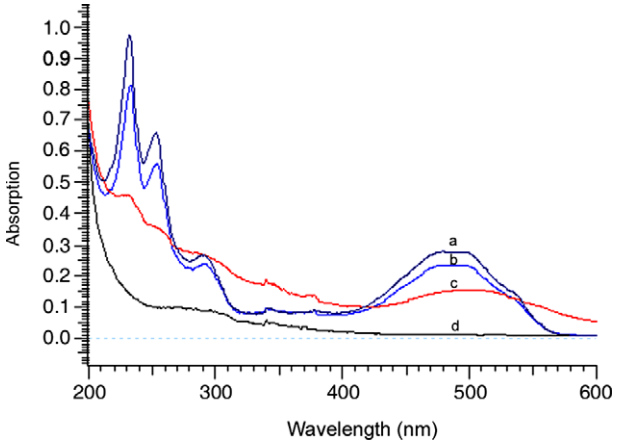


Figure 1. UV–vis spectra of (a) ADM (12 μg/mL), (b) mixture of ADM (10 μg/mL) and pectin (30 μg/mL), (c) PAC (40 μg/mL), and (d) pectin (30 μg/mL). Pectin did not absorb in the UV spectrum. The maximum absorption of (a), (b), and (c) was at 479.5 nm, 479.5 nm and 498.0 nm, respectively. The obvious red shift of PAC suggested that a covalent conjugate had been formed between ADM and pectin.

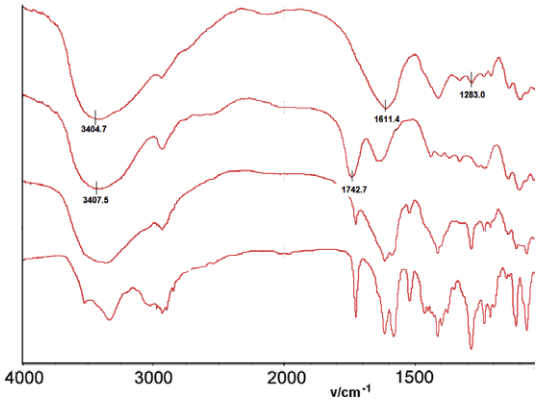


Figure 2. IR spectra of (a) PAC, (b) pectin, (c) mixture of ADM and pectin, and (d) ADM. The IR spectra revealed typical bands for functional groups at 1611.4 cm^{-1} (overlap of the amide I and amide II bands) and 1283.0 cm^{-1} (amide III band), which indicated the formation of an amide linkage between ADM and the pectin. A major absorption at around 3400 cm^{-1} was attributed to stretching of hydroxyl groups and an absorption at 1740 cm^{-1} was caused by C=O stretching vibration of methyl-esterified carboxyl groups.

2.3. Determination of ADM content

The ADM content of PAC was determined by measuring the absorbance at 480 nm as described previously.[10] The regression equation, $A = 0.0196C - 0.0058$ ($r^2 = 0.9997$), was used in which C is the concentration of the ADM solution and A is absorbance. The ADM contents of the three batches of PAC were 25.8%, 25.1%, and 25.3% (w/w), respectively. The results suggested that the low-methoxylated pectin was an excellent carrier for drug delivery due to a high drug-loading efficiency and good reproducibility in the synthesis of the conjugate.

In the synthesis of polymer–drug conjugates, various spacers have been incorporated along with the polymers and copolymers to decrease the effect of crowding and steric hindrance and increase the drug-loading efficiency.[20,34] However, even without the incorporation of a spacer, the drug content of PAC was greater than 25%. In the low-methoxylated pectin, methoxyl groups are located between the carboxyl groups, which provide good steric spacing for the conjugation.

2.4. Preparation of a PAC nanoparticle suspension

PAC is intended to be applied intravenously. Therefore, the conjugate was prepared as a nanoparticle suspension using a microfluidizer. The particle size distribution of the PAC suspension is shown in Figure 4. The results indicated that the particle size distribution of the PAC nanoparticle suspension was relatively narrow with the main peak at 152 nm and a Z-average diameter of 126 nm. This is the optimal particle size to avoid trapping of particles via the reticuloendothelial system and renal clearance.[35–38] In addition, this size of particle enables PAC to target cancer sites passively via the EPR effect.[35–38] The zeta-potential of the PAC nanoparticle suspension was measured to be −28.7 mV. The morphology of PAC was shown in Figure 6. From Figure 6, the PAC nanoparticles were regularly spherical.

2.5. Drug release experiments

In order to obtain preliminary information about the potential application of PAC as a tumor targeting drug delivery system, the conjugate was subjected to hydrolysis at 37 ± 0.5 °C in phosphate buffered saline (PBS) at pH 7.4 and pH 5.0, human plasma, and lysosomal enzymes derived from rat liver. The ADM release profiles are shown in Figure 5a, b, and c, respectively.

Only 1.7% and 7.3% of ADM were released from PAC in PBS at pH 7.4 and pH 5.0, respectively, after incubation for 120 h. This suggested that the conjugate was highly stable in PBS at both values of pH. Analogously, only 1.3% of ADM was released from PAC after incubation in plasma for 30 h, which indicated that the conjugate was also highly stable in plasma.

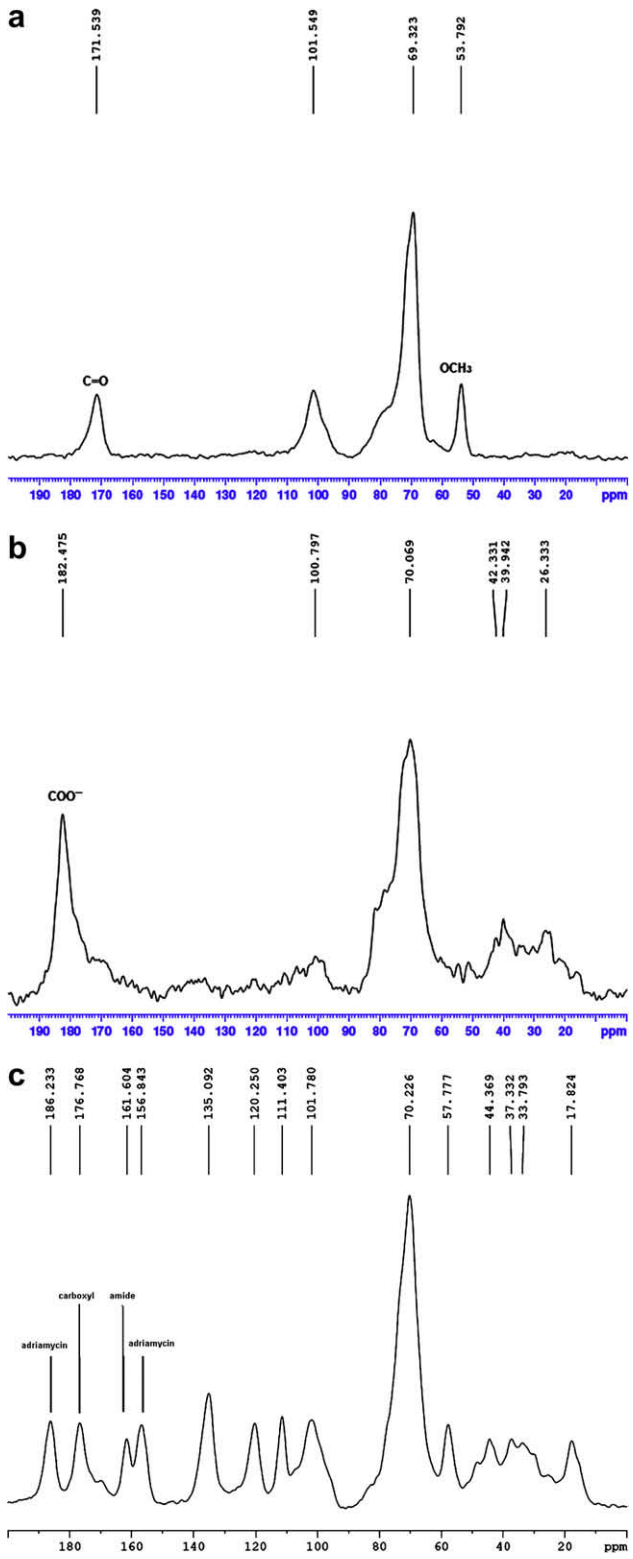


Figure 3. ^{13}C ssNMR spectrum of (a) high-methoxylated pectin, (b) low-methoxylated pectin, (c) PAC. In Figure 3c, the peak at δ 161.6 ppm corresponded to the formation of an amide linkage between ADM and the low-methoxylated pectin.

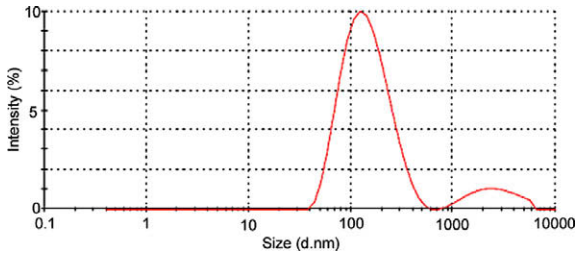


Figure 4. Particle size distribution of the PAC suspension. The distribution was relatively narrow with the central peak at 152 nm and a mean diameter of 126 nm.

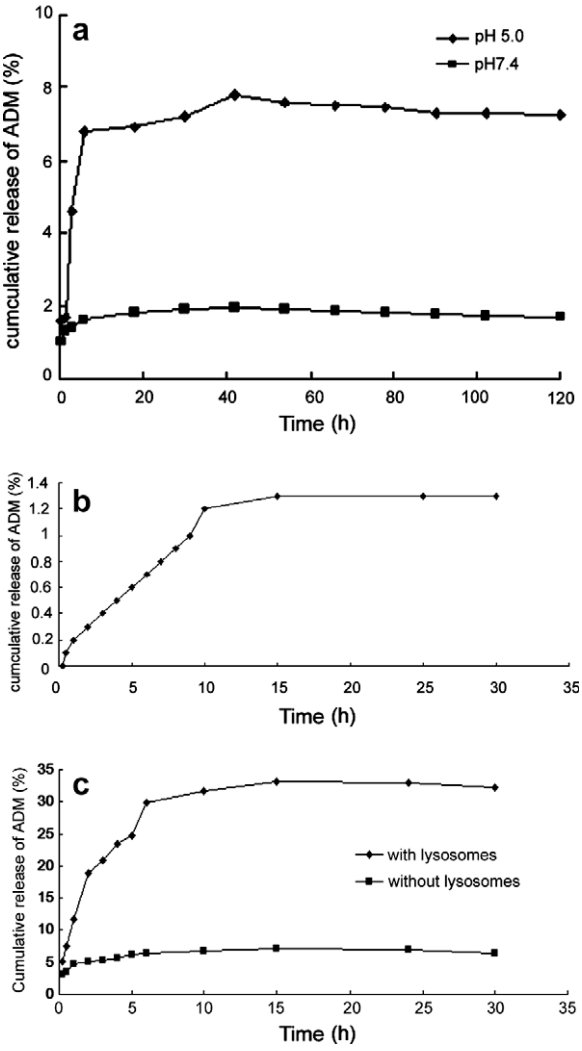


Figure 5. Release of ADM from PAC incubated (a) in PBS (both pH 7.4 and pH 5.0) at 37 ± 0.5 °C, (b) in human plasma, and (c) in medium containing lysosomes at 37 ± 0.5 °C. The maximum amounts released were (a) 1.7% (pH 7.4) and 7.3% (pH 5.0), (b) 1.3%, and (c) 33.2%, which suggested that PAC was stable in the blood and ADM could be released in lysosomes.

In contrast, 33.2% of ADM was released from PAC mixed with lysosomal enzymes after incubation for 30 h. In particular, during the first 5 h, ADM was released rapidly from PAC in the presence of lysosomes (24.7% was released) whereas only 6.2% was released in the negative control. Furthermore, the majority of drug release occurred during the first 15 h, and after 24 h the release of ADM declined slowly, which was thought to be due to the degradation of ADM and inactivation of the lysosomal enzymes. The same trends were observed in the negative control. Overall, in the presence of lysosomes, the amount of ADM released from PAC was six times greater than that of the negative control.

Therefore it is feasible that PAC will exist stably in the circulatory system over a long period and that free drug will be released in lysosomes after internalization by tumor cells.[20,36–38]

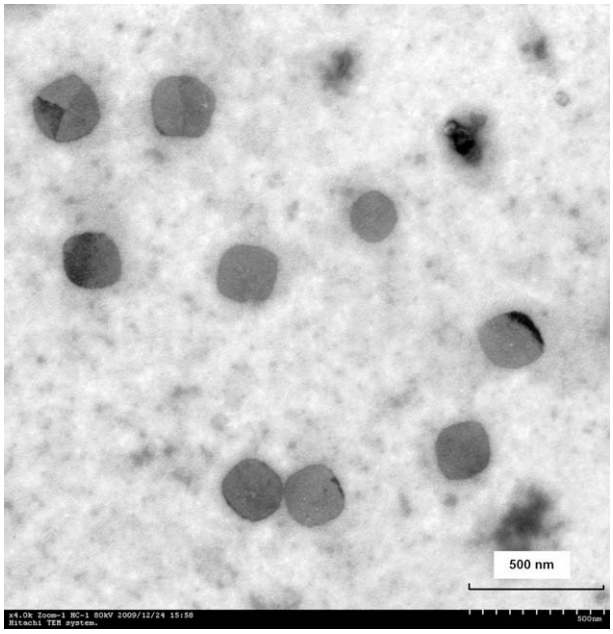


Figure 6. TEM image of PAC nanoparticles. The PAC nanoparticles were regularly spherical.

2.6. Intracellular localization of PAC and ADM

The intracellular localization of PAC and ADM were compared to investigate the mechanism of cytotoxicity of PAC. ADM is autofluorescent, therefore the intracellular localization was analyzed by inverted fluorescence microscopy. In the majority of A549 cells incubated with PAC for 24 h, the red fluorescence was distributed uniformly in the cytoplasm, but in a few cells the red fluorescence had accumulated in the nucleus (Fig. 7A). In contrast, in A549 cells incubated with free ADM for 24 h, the red fluorescence had accumulated in the nucleus with minimal staining of the cytoplasm (Fig. 7B), which was consistent with the results of previous studies.[39,40] On the other hand, the fluorescence was invisible in both the cytoplasm space and the nucleus for A549 cells treated with PAC under 4 °C for 24 h, in which condition endocytosis of cells was inhibited. The results suggest that PAC has been internalized by A549 cells.

2.7. Biodistribution of PAC in Kunming mice bearing colon carcinoma C26 cells

The fluorescence intensities of the administered dose of PAC in tissues at different times after iv administration were summarized in Tables 1 and 2. The accumulation of PAC in tissues at 2 h post injection can be ranked in the following order: lung > liver > spleen > kidney > tumor > heart. However, the accumulation of ADM in tissues at 2 h can be ranked in the following order: liver > spleen > lung > tumor > heart > kidney. More PAC than ADM accumulated in the tumor for all samples collected at the scheduled times.

2.8. In vitro cytotoxicity

The in vitro cytotoxicity of PAC was examined in A549, B16, and 2780cp cells.

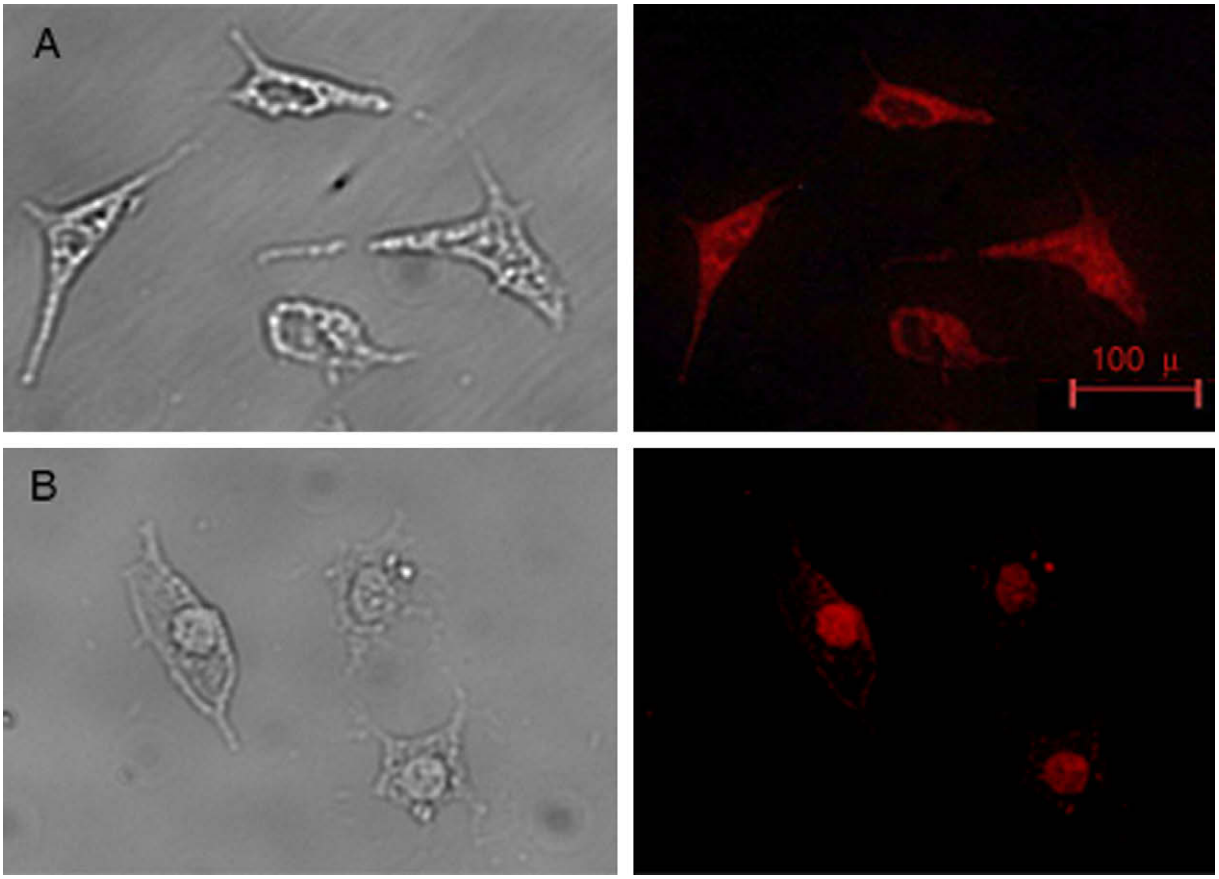


Figure 7. Intracellular localization by fluorescence microscopy of PAC in A549 cells incubated with the conjugate for 24 h. (A) In cells treated with PAC at a concentration equivalent to 2 μg of ADM/mL, most of the red fluorescence was distributed uniformly in the cytoplasm, although a little accumulated in the nucleus. (B) In cells treated with free ADM, most of the red fluorescence had accumulated in the nucleus and very little was present in the cytoplasm. Scale bar = 100 μm.

Table 1
Fluorescence intensities of PAC in tissues

Sample	Time (iv)				
	2 h	4 h	6 h	24 h	48 h
Heart	22.28 ± 5 93	24.85 ± 933	28.32 ± 7.34	13.02 ± 5 36	5.31 ± 1.78
Liver	59.32 ± 1003	55.83 ± 3.99	49.34 ± 3.56	30.32 ± 6.75	12.47 ± 1.42
Sjleen	33.56 ± 8.38	23.79 ± 437	14.38 ± 2.21	10.09 ± 2.14	9.33 ± 1.52
Lung	47.44 ± 13.29	50.48 ± 20.92	54.37 ± 10.83	34.37 ± 6.64	13.29 ± 5.91
Kidrey	31.23 ± 2.26	30.45 ± 4.32	35.84 ± l.28	19.93 ± 1.53	10.34 ± 3.37
Tumor	24.08 ± 6 79	29 56 ± 2.77	36.75 ± 12.36	20.76 ± 4.43	15.77 ± 3.59

Table 2
Fluorescence intensities of ADM in tissues

Sample	Time (iv)				
	2 h	4 h	6 h	24 h	48 h
Heart	25.08 ± 2.11	23.46 ± 3.74	15.66 ± 5.96	7.02 ± 1.36	2.16 ± 0.67
Liver	38.71 ± 4.43	36.53 ± 4.56	25.32 ± 3.27	18.06 ± 4.28	10.54 ± 2.86
Sjleen	26.59 ± 1.01	28.85 ± 0.84	16.63 ± 0.22	12.22 ± 0.32	4.88 ± 0.07
Lung	35.67 ± 0.97	29.29 ± 2.71	24.27 ± 1.16	9.05 ± 0.23	5.37 ± 0.21
Kidrey	28.77 ± 0.41	20.38 ± 1.15	14.74 ± 0.65	5.12 ± 0.41	3.75 ± 0.16
Tumor	22.53 ± 0.45	26.57 ± 1.33	12.72 ± 1.22	8.55 ± 0.61	3.01 ± 0.38

As shown in Figure 8a, b and c, PAC obviously inhibited the growth of A549 cells less than ADM or the mixture of ADM and pectin over a concentration range equivalent to 0.125–1.000 μg of ADM/mL (p <0.05). However, at a concentration equivalent to 2 μg of ADM/mL, PAC inhibited the growth of A549 cells by 58.1%, which was very similar to the level of inhibition by ADM (59.7%, p >0.05) and the mixture of ADM and pectin (55.7%, p >0.05). PAC inhibited the growth of B16 cells slightly less than ADM or the mixture of ADM and pectin over the concentration range equivalent to 0.125–1.000 μg of ADM/mL. Moreover, at a concentration equivalent to 2 μg of ADM/mL, PAC inhibited the growth of B16 cells by 72.5%, which was less than ADM (85.9%) or the mixture of ADM and pectin (84.2%). These results can be explained by the fact that endocytosis is a much slower internalization process than simple diffusion. In addition, in vitro experiment showed only 33.2% of ADM was released from PAC mixed with lysosomal enzymes after incubation for 30 h. Therefore, a much higher concentration of PAC outside the cell is required to produce the same intracellular effect as the free drug.[20]

Interestingly, PAC inhibited the growth of 2780cp cells at all concentrations from 0.125 to 2.000 μg of ADM/mL and the inhibition was dose-dependent. The inhibition rates were 27.2%, 48.6% and 59.1% at 0.125, 0.500 and 2.000 μg of ADM/mL, respectively. On the other hand, the inhibition rates of mixture of ADM and pectin on 2780cp cells were only 8.2%, 26.1% and 49.4%, respectively, at the same gradient concentrations. Similarly, the inhibition rates of ADM on 2780 cells were 2.9%, 24.7% and 46.0%, respectively, at the same gradient concentration. Therefore, PAC inhibited the growth of 2780cp cells more effectively than did the mixture of ADM and pectin or ADM alone (p <0.05 at all concentrations). This result suggests that PAC may partly overcome the drug resistance of 2780cp cells through a pathway different to that used by ADM.[36,41]

Inhibition rate of A549 cells versus incubation time is shown in Figure 9. This figure involves a comparison of PAC with ADM at same concentration (2 μg/mL). Free ADM showed a higher inhibition rate than PAC at all observed time. But PAC also displayed a significant inhibition rate to A549 cells, and this effect had a time-dependent relationship.

Although there were differences among the different types of cell, PAC showed satisfactory anti-tumor activities against A549, B16, and 2780cp cells in vitro and the effects were dose-dependent and time-dependent.

2.9. In vivo anti-tumor activities

2.9.1. Therapeutic effect of PAC by macro and histological examination

In the untreated control group, the volume of the lung was obviously increased, compared to that of the treated mice, countless black tumor nodules were seen on the surface of the lung, and the mean weight of the lung was 0.89 ± 0.15 g. In the ADM group, both the volume and weight of the lung were also increased, compared to that of uninoculated mice, and black tumor nodules were seen on the surface of the lung. However, the effects were not as severe as those in the untreated group. In contrast, in the PAC group, the volume of the lung was not increased obviously, the mean weight of the lung was only 0.29 ± 0.12 g, and black tumor nodules were seldom seen on the surface of the lung (Table 3).

As shown in Figure 10, in slices of lung tissue from a mouse in the untreated control group that died on day 20 after inoculation, nearly all of the alveoli were replaced by masses of tumor cells. Slices of lung tissue from the ADM group showed a number of nodules of tumor cells scattered through the normal alveoli. However, in the PAC group, only a few nodules of tumor cells were seen.

2.9.2. Evaluation of the therapeutic effect of PAC by survival

None of the mice in the untreated control group were alive on day 20 post injection of tumor cells. In the ADM group, the survival rate declined sharply after 20 days and no animals remained alive on day 30 (Fig. 11). In contrast, in the PAC group, 80% of the mice survived to day 40 post inoculation and 20% were still alive at the close of the study on day 60. These statistically significant results (P = 0.0019) correlated well with the in vitro data. No overt toxicity was observed in animals treated with the therapeutic doses of PAC or ADM.

3. Conclusion

A novel PAC with a high ADM content was synthesized successfully by conjugating ADM with low-methoxylated pectin. PAC was highly stable in plasma and PBS at pH 7.4 or 5.0. Interestingly, ADM was easily released by incubation with lysosomal enzymes. PAC entered A549 cells cultured in vitro and became distributed rapidly in the cytoplasm. PAC was able to suppress the growth of A549, B16, and 2780cp cells significantly. We proposed that PAC could partly overcome drug resistance through a pathway different to

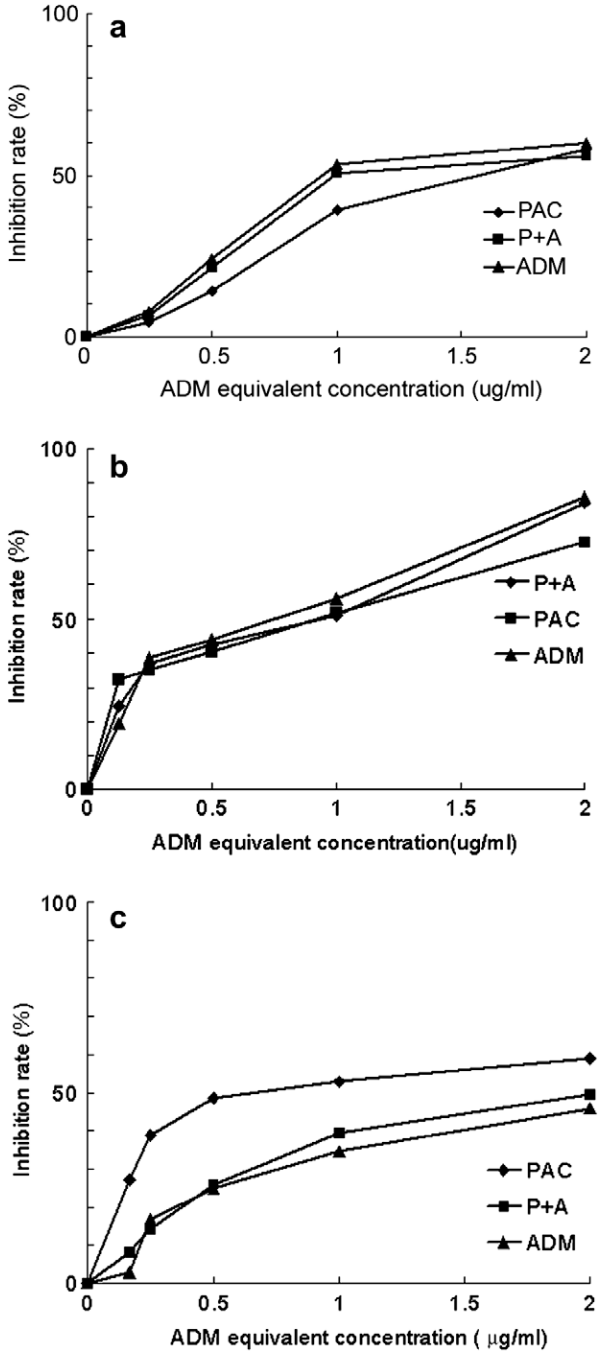


Figure 8. Effects of PAC and ADM on the growth of (a) A594 cells, (b) B16 cells, and (c) 2780cp cells. At concentrations equivalent to 0.125–1.000 μg of ADM/mL, PAC inhibited the growth of A594 and B16 cells less than ADM or the mixture of ADM and pectin (p <0.05). However, at a concentration equivalent to 2 μg of ADM/mL, PAC inhibited the growth of these cells to the same extent as ADM and the mixture of ADM and pectin (p >0.05). Interestingly, PAC inhibited the growth of 2780cp cells in vitro significantly more than ADM or the mixture of ADM and pectin at all concentrations (p <0.05).

that used by ADM. In vivo studies showed that PAC had a good therapeutic effect on pulmonary metastasis of melanoma (B16 cell line) in C57BL/6 mice and the mean survival time of the mice treated with PAC was prolonged remarkably compared with the survival of those treated with the free drug.

In conclusion, PAC is a potential candidate for development as a polymer prodrug for tumor targeting in cancer therapy.

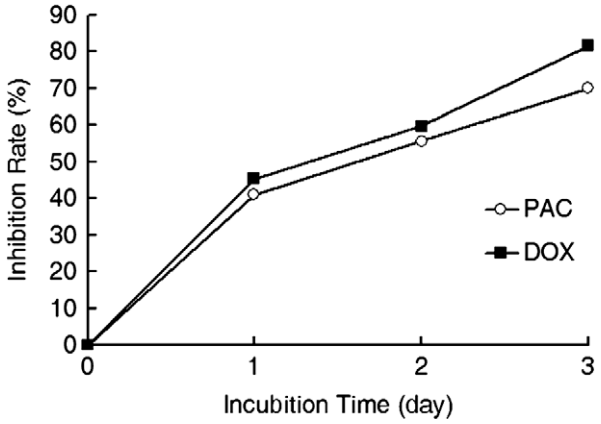


Figure 9. Effects of incubation time on the growth of A549 cells treated with PAC or ADM. A comparison of PAC with ADM at same concentration (2 μg/mL). Free ADM showed a higher inhibition rate than PAC at all observed time. PAC also displayed a significant inhibition rate to A549 cells, and this effect had a time-dependent relationship.

Table 3
Mean weights of lung in the PAC and control groups (n = 10)

Group	Weight ± S.D. (g)
PAC	0.29 ± 0.12***
ADM	0.69 ± 0.09#
Untreated control	0.89 ± 0.15

Note: #, compared to untreated control group, P <0.01; ∗, compared to untreated control group, P <0.01; ∗∗, compared to ADM group, P <0.01. In the untreated control group, the weight of the lung was increased obviously compared with that of the treated mice. In the ADM group, the weight of the lung was also increased, compared with that of mice in the PAC group. However, the effects were not as severe as those in the untreated group.

4. Experimental

4.1. Materials

4.1.1. Chemicals

High-methoxylated pectin from oranges (Mw = 120,000), with a degree of esterification of 58.5%, was purchased from Shangrao Fuda Pectin Co., Ltd (Jiangxi, China). Adriamycin hydrochloride was obtained from Haikou Manfangyuan Chemical Co., Ltd (Haikou, China). 1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC·HCl) was provided by Astatech (Chengdu) Pharmaceutical Co., Ltd (Chengdu, China). The dialysis bag (molecular weight cut-off: 7000) was purchased from Greenbird Science & Technology Co., Ltd (Shanghai, China). Amicon Ultra-15 Centrifugal Filter Units with Ultracel-50 membrane (molecular weight cut-off: 50,000) were supplied by Millipore (Bedford, MA, USA). PVP K_{30} was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). All other chemicals and reagents were of analytical grade.

4.1.2. Cells

The human lung adenocarcinoma epithelial cell line A549 and the human ovarian cell line 2780cp, which were donated by the State Key Laboratory of Biotherapy of Sichuan University, were maintained in RPMI 1640 medium (**Invitrogen**, **Carlsbad**, **USA**) containing 10% fetal bovine serum and 100 μg/mL penicillin and streptomycin at 37 ± 0.5 °C in a humidified atmosphere with 5% CO_2. The murine melanoma cell line B16 was purchased from Shanghai Institute for Biological Science and maintained under the same conditions.

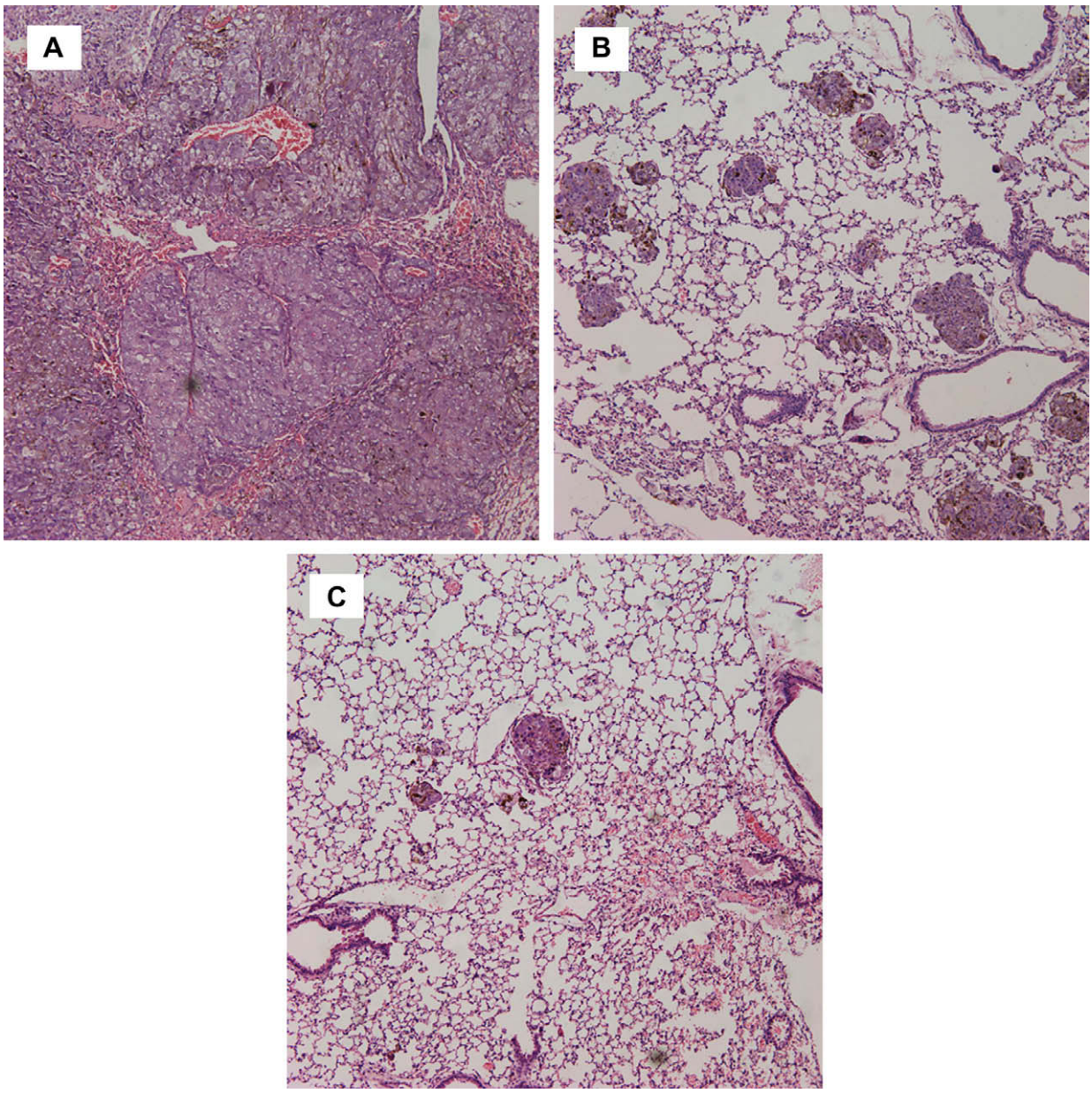


Figure 10. Histological comparison of lung metastases from melanoma in three groups of C57BL/6 mice (original magnification ×200). (a) In the untreated group, the slices of lung tissue showed that nearly all of the alveoli were replaced by masses of tumor cells. (b) In the ADM group, a number of nodules of tumor cells were scattered through the normal alveoli. (c) In the PAC group, only a few nodules of tumor cells were seen in the slices of lung tissue.

4.1.3. Animals

Female C57BL/6 mice, an inbred strain, that were aged 6–7 weeks (20 ± 3 g) were purchased from the West China Animal Experimental Center of Sichuan University. The mice were housed five per cage with a central air conditioning and ventilation system. The animal room was maintained at 20 °C. Food and water were given ad libitum. All animal studies were conducted in accordance with Animal Care and Use Committee Guidelines **published by State Science and Technology Committee of the People's Republic of China**. The experimental protocol was approved by the Committee on Animal Research of Sichuan University (Chengdu, Sichuan). Animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals of Sichuan University.

4.2. Synthesis of PAC

4.2.1. Preparation of low-methoxylated pectin

High-methoxylated pectin (10 g) was dissolved in distilled water (1000 mL), and the pH of the solution was adjusted to 12.5 with 5 mol/L NaOH. The reaction mixture was stirred for 8 h at 65 °C; then the pH was adjusted to 7.0 with 0.5 mol/L HCl and the solution concentrated to 500 mL in a rotary evaporator. The mixture was dialyzed against water in a dialysis bag (molecular weight cut-off: 7000) for 24 h. After that, the dialysate was concentrated and dried under vacuum at 60 °C to give low-methoxylated pectin sodium salt with a wide range of molecular weight. The pectin salt was dissolved in distilled water (1%, w/w). The solution was centrifuged through an Amicon Ultra-15 Centifugal Filter Unit with

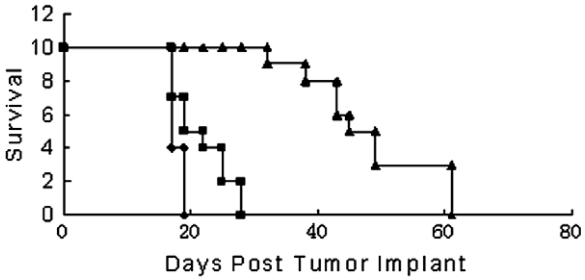


Figure 11. Comparison of survival between the PAC group, the ADM group and the untreated group. None of the mice in the untreated control group were alive on day 20 post injection of tumor cells. In the ADM group, the survival rate declined sharply after 20 days and no animals remained alive on day 30. In contrast, in the PAC group, 80% of the mice survived to day 40 post inoculation and 20% were still alive at the close of the study on day 60. These statistically significant results ($P = 0.0019$) correlated well with the in vitro data. No overt toxicity was observed in animals treated with the therapeutic doses of PAC or ADM.

a molecular weight cut-off of 50,000 at 3000 rpm to obtain low-methoxylated pectin sodium salt.

4.2.2. Determination of the molecular weight of the low-methoxylated pectin

The molecular weight and molecular weight dispersity of the low-methoxylated pectin were determined using a Waters high performance liquid chromatographic (HPLC) system equipped with a 515 pump (Waters, Milford, MA, USA) and a DAWN HELEOS multiangle light-scattering detector (Wyatt Technologies, Santa Barbara, CA, USA). Chromatographic separation was carried out on three columns (Shodex SB-805, SB-804, SB-803; Shodex, Tokyo, Japan).

PAC (10 mg) was added to $NaNO_3$ (2 mL, 0.2 mol/L), incubated overnight, and the supernatant was used as the sample for determination of the molecular weight. The injection volume of the sample was 200 μL. The mobile phase was $NaNO_3$ (0.2 mol/L) with 0.02% NaN_3 as an antibacterial agent, and the flow rate was 0.5 mL/min. During the assay, the column temperature was 40 °C.

4.2.3. Preparation of PAC

Low-methoxylated pectin (1 g, carboxyl content 2.4 mmol/g) was dissolved in 100 mL of distilled water with stirring at 50 °C. Adriamycin hydrochloride (0.5 g) was dissolved in 100 mL of distilled water by ultrasonic oscillation at room temperature. The reaction mixture was prepared by mixing the two solutions and adding EDC·HCl (1 g). The reaction was allowed to proceed for 8 h at 50 °C with stirring and protection against light. Upon completion of the reaction, the mixture was dialyzed in a dialysis bag (molecular weight cut-off: 7000) against distilled water for 24 h. The solvent was removed under reduced pressure at 50 °C and the resulting red solid was dried under vacuum to give a water-insoluble pectin–adriamycin conjugate (1.2 g). ^{13}C ssNMR (Bruker Avance III, 400 MHz, 299.1 K): 186.2, 176.8, 161.6, 156.8, 135.1, 120.3, 111.4, 101.78, 70.2, 57.8, 44.4, 37.3, 33.8, 17.8 ppm; IR (KBr) ν: 3404.7, 2935.8, 1611.4, 1411.3, 1329.6, 1283.0, 1235.6, 1209.6, 1140.8, 1097.4, 1071.3, 1012.2, 950.0, 889.6, 832.7, 763.2, 637.0, 535.9 cm^{-1}. The synthetic procedure was repeated three times to give three batches of product.

4.3. Determination of the molecular weight of PAC

The molecular weight and molecular weight dispersity of PAC were determined using the method described in Section 4.2.2.

4.4. Determination of the ADM content of PAC

4.4.1. Preparation of the ADM stock solution

Adriamycin hydrochloride (10.7 mg) was dissolved in 50 mL of a dilute solution of hydrochloric acid (pH 2.0) and diluted to a final volume of 100 mL (equivalent to 100 μg of ADM/mL). The stock solution had to be freshly prepared before use because an aqueous solution of ADM is not stable at room temperature.

4.4.2. Linearity of ADM

Aliquots of the ADM stock solution were diluted in a dilute solution of hydrochloric acid (pH 2.0) to obtain five different concentrations, which corresponded to 10, 20, 30, 40, and 50 μg of ADM/mL. The absorbance of these solutions was determined by UV spectroscopy at 480 nm. The regression equation was constructed from concentration versus absorbance.

4.4.3. Determining the ADM content

PAC (10 mg) was dissolved in 80 mL of dilute hydrochloric acid (pH 2.0) and diluted to a final volume of 100 mL to give a working solution. The absorbance of the solution was determined by UV at 480 nm. The drug content was calculated by linear regression.

4.5. Preparation of solutions for in vitro and in vivo evaluation

4.5.1. Preparation of the blank solution

1000 mg of PVP K_{30} and 6 mL of glycerol were dissolved in an appropriate volume of normal saline, and diluted to 100 mL to obtain a blank solution, which was used as a negative control in the following experiments.

4.5.2. Preparation of the ADM solution

Adriamycin hydrochloride (214 mg), PVP K_{30} (1000 mg), and glycerol (6 mL) were dissolved in an appropriate volume of normal saline and diluted to 100 mL to obtain a solution that was equivalent to 2 mg of ADM/mL. This solution was used as a positive control in the following experiments.

4.5.3. Preparation of the solution containing a mixture of ADM and pectin

Adriamycin hydrochloride (214 mg), low-methoxylated pectin (576 mg), PVP K_{30} (1000 mg), and glycerol (6 mL) were dissolved in an appropriate volume of normal saline and diluted to 100 mL to obtain a solution that was equivalent to 2 mg of ADM/mL. This solution was used as a further positive control in the following experiments.

4.5.4. Preparation of a nanoparticle suspension of PAC

PAC (776 mg, 25.8% ADM content) was mixed with PVP K_{30} (1000 mg) and glycerol (6 mL) and ground in a mortar for 30 min. The ground mixture, which had a mean particle size of 12 μm, was added to normal saline to a final volume of 100 mL. It was then pulverized and dispersed at 120 MPa pressure by using a microfluidizer (Model 7-200D; Langfang Tongyong Machinery Manufacturing Co., Ltd, Langfang, China) to give a PAC nanoparticle suspension (equivalent to 2 mg of ADM/mL), which was used in the following experiments. The particle size distribution and zeta-potential were measured using a Malvern Zetasizer Nano-ZS instrument (Malvern Instruments, Herrenberg, Germany).The morphology of PAC was observed by transmission electron microscopy (Hitachi H-7650, Japan).

The prepared PAC nanoparticle suspension was aliquotted, sterilized with γ-rays, and stored at −20 °C. Before use, the stock solutions were diluted to appropriate concentrations.

4.6. In vitro investigation of drug release

The release of PAC was investigated in PBS at pH 5.0 and 7.4 at 37 ± 0.5 °C, in human plasma, and in lysosomal enzymes derived from rat liver.

4.6.1. Study of hydrolysis in buffer solutions at pH 5.0 and 7.4

A mixture that contained 5 mL of the PAC nanoparticle suspension and 5 mL of PBS was sealed in a dialysis bag (molecular weight cut-off: 7000). The dialysis bag was incubated in 50 mL of PBS at 37 ± 0.5 °C with gentle shaking (50 rpm). At designated time points, 0.2 mL of the incubation mixture were withdrawn and an equal volume of fresh medium was added. Free ADM was determined by HPLC (Agilent 1100 series; Agilent Technologies, Diegem, BE). The chromatographic conditions were as follows: column: Phenomenex Luna C_{18}; temperature: 30 °C; mobile phase: 7:4:6 methanol/acetonitrile/phosphate buffer (25 mM $NH_4H_2PO_4$/30 mM H_3PO_4) (v/v); flow rate: 0.8 mL/min; wavelength: 480 nm.

4.6.2. Study of hydrolysis in human plasma

PAC nanoparticle suspension (corresponding to 10 mg of PAC and 4.75 μM ADM) was added to 10 mL of human plasma. The sample was incubated at 37 ± 0.5 °C with gentle shaking (50 rpm). At various time intervals, 0.5 mL of the incubation mixture were removed to a polypropylene tube and 0.5 mL of water, 0.2 mL of 1 mol/L Na_2CO_3/$NaHCO_3$ (pH 9.8) and 2.5 mL of $CHCl_3$/MeOH (3:1) were added to the sample and mixed.[10] The organic layer was evaporated and free **ADM** was determined by HPLC using the method described in Section 4.6.1.

4.6.3. Study of hydrolysis in lysosomal enzymes

A mixture of rat liver lysosomes was isolated by differential centrifugation according to the methods described by Zhang and Lian[42] and Win-Aung et al.[43]

Two aliquots of the PAC nanoparticle suspension described in Sections 4.1.1 and 4.5.4 (each corresponding to 10 mg of PAC, 4.75 μM ADM) were added to 10 mL of PBS (pH 5.5) that contained 20 mg of the mixture of lysosomes and 10 mL of PBS (pH 5.5) as a negative control, respectively. The free ADM was separated and determined by the methods described in Sections 4.6.1 and 4.6.2.

4.7. Intracellular localization of PAC in A549 cells

The intracellular localization of PAC was determined by treating the cells with PAC at an equivalent concentration of ADM that was identical to its concentration in the cytotoxicity study. ADM was applied as a control. A549 cells were seeded in 96-well plates at a concentration of 5×10^3 cells/well. Each well of the plates contained 0.1 mL of culture medium. The cells were incubated for 24 h at 37 ± 0.5 °C in a humidified atmosphere with 5% CO_2 to permit cell adhesion. Then, the cells were incubated in 0.1 mL of culture medium that contained PAC. The concentration of PAC in the culture medium was equivalent to 2 μg of ADM/mL. After 24 h of incubation at 37 ± 0.5 °C or 4 ± 0.5 °C, the cells were washed twice with PBS. Cells were fixed with 3% paraformaldehyde for 10 min at room temperature and washed again with PBS, then imaged with a Zeiss Axiovert 200 inverted fluorescence microscope (Zeiss, Jena, Germany) through a 20× objective. Images were acquired with Zeiss AvioVision software. The above-mentioned procedure was followed for the ADM control.

4.8. Biodistribution of PAC in Kunming mice bearing colon carcinoma C26 cells

Colon carcinoma C26 cells (2.0×10^6/100 μL) were implanted subcutaneously in the Kunming mice. Experiments were not initiated until a consistent growth rate and a minimum tumor volume of 15 mm^3 was achieved. The mice were given PAC via caudal vein injection. A aliquot of 50 μL of drug was administered to each mouse. The concentration of the drugs was equivalent to 2 mg of ADM/mL. The mice were sacrificed at 2, 4, 6, 12, 24 h (free drug) and at 2, 4, 6, 24 and 48 h (PAC) after administration of the drug. The tissues, including heart, lung, liver, spleen, kidney and tumor, were harvested without perfusion. Tissue samples (500 mg) were homogenized on ice in 5 mL homogenization buffer (PBS containing 30% HCl), after centrifugation at 3500 rpm for 5 min at 4 °C, then 100 μL supernatant was collected and the fluorescence intensity of the supernatant were analyzed by SpectraMax M5 microplate reader (Molecular devices corp. USA).

4.9. In vitro cytotoxicity studies

The effects of PAC on the growth of A549, B16, and 2780cp cells were evaluated in vitro by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were incubated in 96-well plates as described in Section 4.1.2. Then, the cells were treated with the PAC nanoparticle suspension at different concentrations, which were equivalent to 0.125, 0.25, 0.5, 1.0, and 2.0 μg of ADM/mL. Culture medium (100 μL) containing PAC was added to each well. After 48 h of exposure, the PAC medium was removed, the cells were washed with PBS (0.1 mL), and fresh medium was added. The cells in each well were then incubated in culture medium that contained 0.02 mL of a 5 mg/mL solution of MTT for 4 h. After the medium had been removed, dimethyl sulfoxide (0.15 mL) was added to each well. Absorbance at 570 nm (maximum) was measured with a Power Wave X Microplate ELISA Reader (Bio-TeK Instruments, Winoski, VT, USA). The rate of inhibition was calculated according to the following formula: Growth inhibition (%) = (1 − Absorbance of experimental group/Absorbance of blank control group) × 100%. In these studies, ADM and a mixture of ADM and pectin were used as positive controls, whereas the blank solution was used as the negative control. The concentration of ADM was identical in PAC, ADM, and the mixture of ADM and pectin.

To investigate the time-effect relationship of PAC in inhibiting the growth of A549 cells, the cells were incubated for several days (1, 2, 3 days) with PAC or ADM, the concentrations were equivalent to 2.0 μg of ADM/mL.

4.10. In vivo anti-tumor activity of PAC

4.10.1. Mouse modeling

To model pulmonary metastasis of melanoma, murine melanoma B16 cells (5.0×10^5/100 μL) were implanted intravenously into the vena caudalis of C57BL/6 mice.

4.10.2. Evaluation of the therapeutic effect of PAC by macro and histological examination

Thirty C57BL/6 mouse models of pulmonary metastasis of melanoma were divided randomly into three groups with ten mice in each group. Drug therapies were begun on day 4 after inoculation of B16 cells via caudal vein injection. A aliquot of 50 μL of drug was administered to each mouse once every four days, four times in total. The three groups were treated with either the PAC nanoparticle suspension or ADM (positive control), or remained untreated (negative control). The concentration of the drugs was equivalent to 2 mg of ADM/mL.

All mice were sacrificed by decapitation on day 20 when two mice in the negative control group died. The therapeutic effects of PAC were evaluated by macro observation and histological examination of the lung. For macro examination, the volume and weight of the lung were measured and metastatic nodules in the

lung were observed. For histological examination, slices of lung tissue were subjected to hematoxylin and eosin (H&E) staining and observed by using an Olympus BX 60 microscope (Olympus, Tokyo, Japan).

4.10.3. Evaluation of the therapeutic effect of PAC by survival

The protocol described in Section 4.9.2 was repeated, except that the drugs were administered a total of five times. Survival of animals post inoculation was recorded. Kaplan–Meier statistical analysis was based on differences in survival time for the pulmonary metastasis of melanoma model.

4.11. Statistical analysis

Results were expressed as the mean ± S.D. of three experiments. Statistical significance in the differences of the means was evaluated by (one-way) Student's *t*-test for the single or multiple comparisons of experimental groups, respectively. The difference was considered statistically significant when $P \leqslant 0.05$.

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