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Polymer conjugates of acridine-type anticancer drugs with pH-controlled activation

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ABSTRACT

Acridines are potent DNA-intercalating anticancer agents with high in vivo anticancer effectiveness, but also severe side effects. We synthesized five 9-anilinoacridine-type drugs and their conjugates with biocompatible water-soluble hydrazide polymer carrier. All of the synthesized acridine drugs retained their in vitro antiproliferative properties. Their polymer conjugates were sufficiently stable at pH 7.4 (model of pH in blood plasma) while releasing free drugs at pH 5.0 (model of pH in endosomes). After internalization of the conjugates, the free drugs were released and are visible in cell nuclei by fluorescence microscopy. Their intercalation ability was proven using a competitive ethidium bromide displacement assay.

1. Introduction

9-Anilinoacridines are potent DNA intercalators and strong topoisomerase II inhibitors used as anticancer agents.[1–3] Amsacrine (m-AMSA) was the first 9-anilinoacridine clinically employed for the treatment of leukemia and lymphoma (see Fig. 1).[2,4] Since that, an enormous effort was made to investigate their structure–activity relationship.[5–7] Consequently, several 9-anilinoacridine derivatives have shown superior antileukemic activity and a broader spectrum of antitumor activity as compared to amsacrine. Among them, 3-(9-acridinylamino)-5-hydroxymethylaniline[8] (AHMA, **1**, Fig. 1) has greater antitumor efficacy against murine leukemia and solid tumors than amsacrine, and its blood circulation time is prolonged because it is less sensitive to deactivation by oxidation to the corresponding quinone.[8–10] Furthermore, it was proven that functionalization of AHMA at certain positions, especially at positions 4 and 5 of the acridine ring, can even potentiate its activity.[11,12] Despite their remarkable antitumor effects and preferable accumulation in tumor tissue, 9-anilinoacridines suffer from severe systemic toxicity and side effects.[13–15]

Polymer conjugates of anticancer drugs with tumor-specific activation are widely studied due to their ability to deliver anticancer drugs into the tumor tissue while lowering their concentration elsewhere in the organism.[16–18] Furthermore, the optimal polymer carrier delivers the drug in the inactive polymer-bound form into the solid tumor tissue, where the free active drug needs to be released. Solid tumors spontaneously accumulate biocompatible polymers, polymer micelles, liposomes and nanoparticles with sizes <200 nm because of the leaky nature of the newly formed tumor vasculature and the poor or missing lymphatic drainage system in the solid tumor tissue.[19] This so-called Enhanced Permeation and Retention (EPR) effect is relatively universal for many solid tumors and allows increased accumulation of the polymers when compared to the surrounding tissue by an order of magnitude or higher. In addition, this accumulation may be further improved, for example, by ligand-mediated targeting.[19,20]

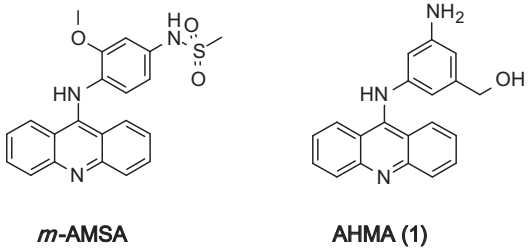

Figure 1. Structures of *m*-AMSA and AHMA (**1**).

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A hydrolytically labile hydrazone bond is a popular option for binding anticancer drugs, such as doxorubicin,[21–23] to carriers to achieve pH-controlled drug release.[24] The hydrazone bond is susceptible to hydrolysis under mildly acidic conditions (e.g., at pH ca 6.5, typical for tumor interstitial space, or even at pH typical for endosomes after internalization, which may be as low as ca 5.0), but it is relatively stable towards hydrolysis at neutral pH (e.g., in blood plasma at pH 7.4). The rate of free active drug release by hydrolysis of the hydrazone bond at different pH values can be fine-tuned by changing the steric hindrance, electron density and conjugation of the substituent with the π-system on the –C=N– part of the hydrazone bond.[25] The structure of the acyl substituent on the hydrazone bond also influences the release kinetics but significantly less.[21] However, because the original ketone is a part of the drug after hydrolysis, one must be cautious to not disrupt the drug's biological activity. The conversion of a 9-anilinoacridine's side phenyl ring amino group to an amide with various acids, including the oxo-acids (e.g., levulinic acid), does not limit its biological activity.[8] This property makes 9-anilinoacridines ideal for fine-tuning of their release rate to achieve an optimal release profile (slow release at pH 7.4 and fast release at pH 5.0).

In this paper, the results of the study of the relationship between the structure of 9-anilinoacridine substituents and the hydrolytic stability of the hydrazone bond between the drug and the polymer carrier are given. We synthesized conjugates of five oxo-group-containing 9-anilinoacridine-type amides with poly[*N*-(2-hydroxypropyl)methacrylamide-*co*-N^1-(6-hydrazino-6-oxohexyl)methacrylamide] with drugs bound to the polymer carrier via hydrolytically labile hydrazone bonds, and we tested the drugs' in vitro release profiles and in vitro biological properties. To the best of our knowledge, no such polymer derivatives of acridine drugs have been described to date.

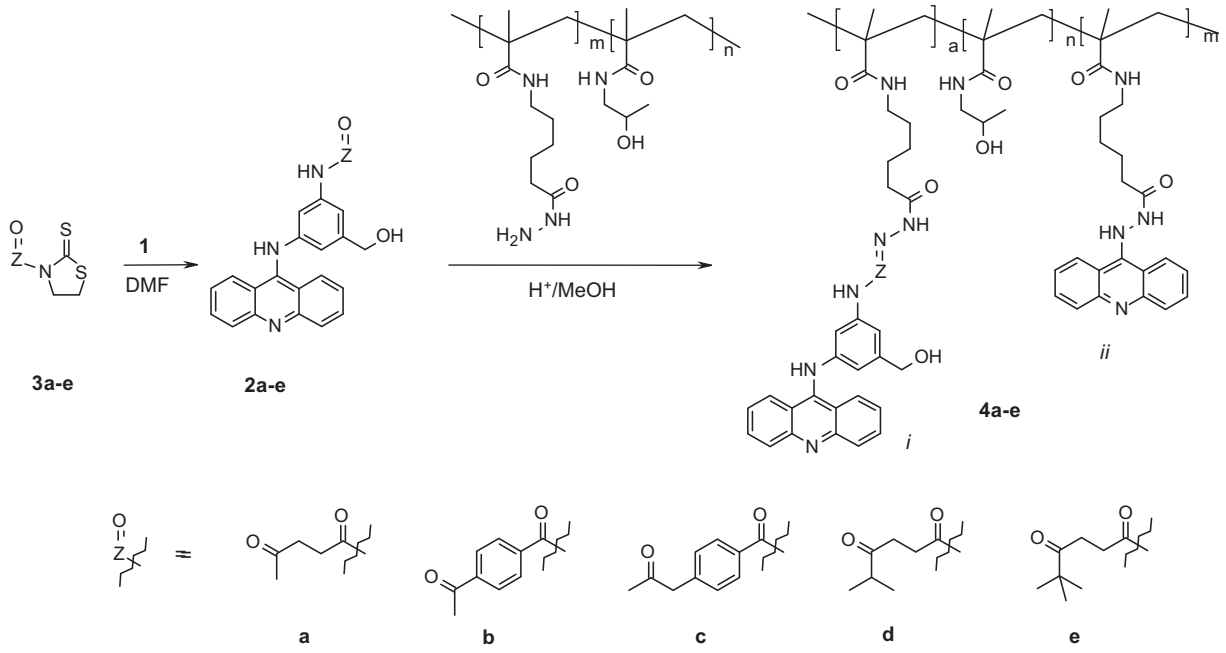
2. Results and discussion

2.1. Chemistry

The oxo moiety-containing 9-anilinoacridines **2a–e** were synthesized by selective acylation of AHMA with thiazolidine-2-thione-activated carboxylic acids **3a–e** in outstanding yields, 82–90% (Scheme 1). This activation was chosen due to the good reactivity of thiazolidine-2-thione amides with amines, while being rather stable towards alcoholysis.[31] The respective oxo-derivatives **2a–e** were linked to pHPMA-MAAcap hydrazide to produce conjugates **4a–e**. This polymer precursor was chosen because of its good biological behavior when used as a doxorubicin carrier.[32] Using this method, one can attach 0.54–6.2 wt.% drug to the polymer, depending on the steric availability of the oxo-group (Table 1).

However, part of the acridine was also bound in a non-cleavable manner as the product of a side reaction. In this case, the hydrazide group of the polymer substituted the aniline group at position 9 of the acridine ring. When the oxo-derivative was more sterically hindered, the hydrazone bond formed slower and more of the uncleavable acridine-polymer product was produced (Table 1). To clarify this process, we treated the sterically hindered acridine derivative **2e** with the hydrazide monomer **5**. Using this reaction, we isolated almost entirely product of the unwanted acridine nucleophilic substitution **6** and no hydrazone derivative **7** (Scheme 2). A similar reaction (nucleophilic substitution of the aniline part of the 9-anilinoacridines) was described in the stability study of cytostatic 9-anilinoacridines with various amines and thiols in aqueous solution modeling the physiological milieu (the typical half-lives of 9-anilinoacridines were ca 1 h with thiols and several hours with various amines at 37 °C).[33–35] Although hydrazides are not strong nucleophiles as compared to thiols, we assume that the non-cleavable fraction of the acridine is bound to the polymer in the same way. Therefore, during further drug release experiments, we only took into account the hydrolytically cleavable part of the bound drug (i.e., the amount cleaved by 0.1 M HCl was equal to 100%). This strategy is relevant from a biological point of view because the polymer bearing the drug bound via a non-cleavable linkage is most likely to be biologically inert, as described for doxorubicin.[36]

For the biological studies, we synthesized the conjugate with the best release profile (see below) bearing the acridine drug bound only via hydrazone bond using a different synthetic strategy (Scheme 2). We obtained conjugate **8** with M_w = 21.5 kDa and acridine **2e** content of 6.3% wt. In this case, all of the acridine drug was bound via a hydrazone bond, as proven by cleavage with 0.1 M HCl

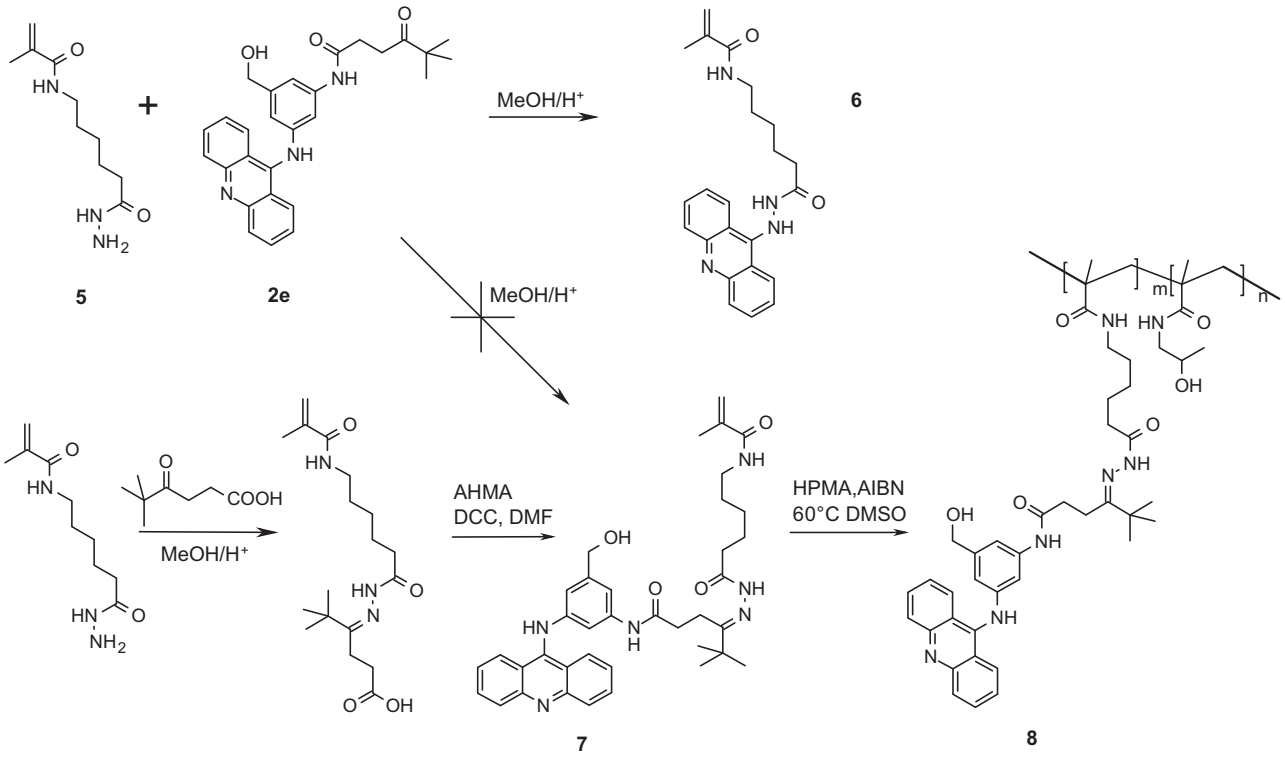


Scheme 1. Scheme of synthesis of **4a–e**.

Table 1
Yields of conjugation of 9-anilinoacridines to polymer

Polymer	Method of synthesis[a]	M_w (kDa)	M_w/M_n	AHMA-a. (%wt)[b]	**4** Isomer ratio (*i*/*ii*)[c]
4a	A	21.9	2.91	5.0	95/5
4b	A	20.7	2.24	4.8	96/4
4c	A	20.0	2.19	6.2	91/9
4d	A	17.8	2.14	2.1	85/15
4e	A	17.6	2.25	0.54	43/57
8	B	21.5	1.75	6.3	—[d]

[a] A, reaction of hydrazide polymer with oxo-acyl acridine derivatives; B, radical copolymerization.
[b] Total acridine content.
[c] Molar ratio of hydrolytically cleavable/stable bound acridine derivative (Scheme 2).
[d] All the drug bound via a hydrazone bond.



Scheme 2. Scheme of alternative synthesis of polymer conjugate **8**.

after which the polymer did not absorb light at wavelengths over 280 nm.

2.2. In vitro drug release profiles of the conjugates

The drug release from conjugates **4a**–**e** at pH 7.4 (a model of the pH in blood where the conjugate should be as stable as possible) and at pH 5.0 (a model of the pH in late endosomes where the drug should be quickly released) was followed. As shown in Figs. 2 and 3, the release rate of the anilinoacridine derivatives is strongly pH-dependent. A relatively fast rate was observed at pH 5.0, and a slower to negligible rate was observed at pH 7.4. Therefore, in some cases, the basic stability requirements for polymer–drug conjugates are fulfilled.

There are basically two main effects in the structure-release rate relationship: conjugation with the aromatic ring and steric effects. Figure 2 displays the effects of conjugation of the hydrazone bond with the aromatic ring. The conjugates **4a** and **4b**, where the hydrazone bond is not conjugated with an aromatic ring, behave similarly. They release their 9-anilinoacridine derivatives very quickly at pH 5.0 (quantitatively within 2 h) and at a moderate rate

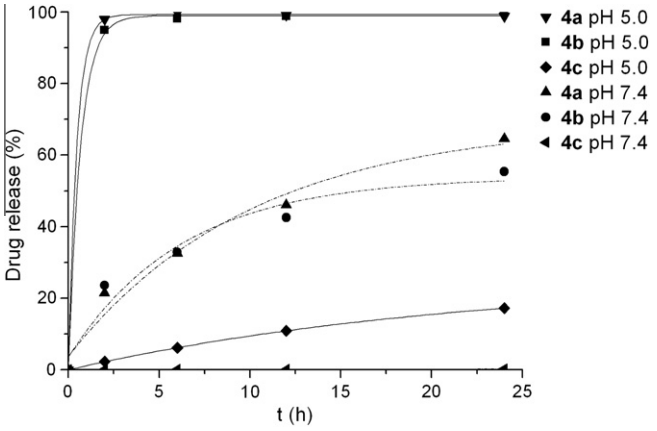


Figure 2. The release of 9-anilinoacridine derivatives **2a**–**c** from conjugates **4a**–**c** at different pH (calculated as hydrolytically cleavable amount of drug = 100%).

at pH 7.4 (conjugate **4a**: 64% anilinoacridine released within 24 h, conjugate **4b**: 55% anilinoacridine released within 24 h). The

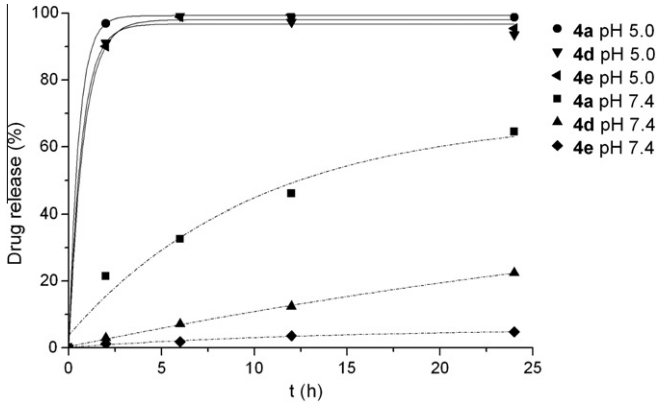


Figure. 3. The release of 9-anilinoacridine derivatives **2a** and **2d–e** from conjugates **4a** and, **4d–e** at different pH (calculated as hydrolytically cleavable amount of drug = 100%).

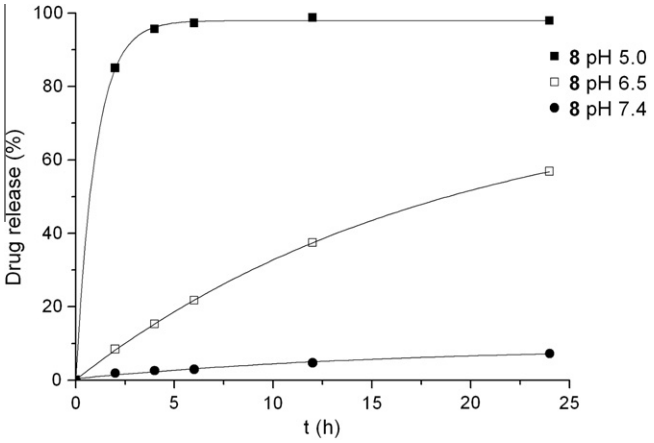


Figure 4. The release of the 9-anilinoacridine derivative **2e** from its conjugate **8** at different pH.

slightly slower release rate observed with conjugate **4b** compared to conjugate **4a** at pH 7.4 is probably caused by the steric hindrance caused by the benzyl group in the proximity of the hydrazone bond.[25] On the contrary, conjugate **4c** contains a hydrazone bond conjugated with the phenyl aromatic ring and released the drug slowly even at pH 5.0 (17% after 24 h), and it did not release any drug at pH 7.4. The most plausible explanation is that the conjugation stabilizes the hydrazone bond by delocalization of its electrons into the aromatic ring, which dramatically slows down the rate of hydrolysis.[37]

The effect of steric hindrance next to the hydrazone group on its release rate was studied using conjugates **4a**, **4d** and **4e**, differing only by their substituents adjacent to the original oxo-group, which were methyl (**4a**), *i*-propyl (**4d**) and *t*-butyl (**4e**), respectively. Steric hindrance of the hydrazone increases in the order **4a** < **4d** < **4e**. One can clearly see from Figure 3 that steric hindrance does not influence the drug release rate at pH 5.0 (it is nearly quantitative within the initial 2 h in all cases) but has a dramatic effect at pH 7.4. A greater steric hindrance induces a slower release rate at pH 7.4. This observation allows us to fine-tune the release rate and create a conjugate that is almost entirely stable at the pH of blood (7.4) but can quickly release the drug after internalization into the target cell at pH 5.0. As we have shown from an in vitro cytotoxicity assay (see below), the acyl substituent has only a small effect on cytotoxicity; therefore, one can use it to modify the release rate. The *t*-butyl group-containing conjugate **4e** has optimal release rate in this study.

However, strong steric hindrance of the oxo-group by the *t*-butyl group also slows the formation of conjugate **4e**, which leads to side reactions, namely, the nucleophilic substitution at the 9 position of the acridine ring with hydrazide. To overcome this problem, we synthesized the HPMA copolymer conjugate of **2e** (with the best release profile) using a procedure that avoided these side reactions (Scheme 2). The product formed, conjugate **8**, contained all acridine drug bound via an acid-cleavable hydrazone bond. As shown in Fig. 4, this conjugate is fairly stable at pH 7.4 (7% drug released during 24 h) and should not quickly release the drug during its transport to the target tumor tissue. In a slightly acidic environment at pH 6.5 (a model of pH in tumor interstitial space), the drug was released considerably faster (57% drug released during 24 h), whereas at pH 5.0 (a model of pH in the endosomal environment), the drug was released very quickly and quantitatively.

2.3. In vitro biological studies

The affinity of the acridine drugs to DNA often correlates with their antiproliferative activity.[38] Therefore, the relative intercalation strength of the drug into the double helix of DNA was measured using a DNA–ethidium bromide (EtBr) displacement test.[28] In this experiment, the mixture of calf-thymus (CT) DNA and EtBr in HEPES buffer was titrated with a solution of AHMA (**1**) or drug derivatives **2a, 2d** and **2e**. The concentration of drug that produces a 50% drop in the fluorescence of the DNA–EtBr complex (CC_{50}, see Table 2) is approximately inversely proportional to its DNA affinity. Our results show that the intercalation strength of the synthesized oxoacridines, **2a, 2d** and **2e**, (22.8–36.6 μmol/L) is only slightly lower than that of the parent compound, AHMA (**1**, 21.3 μmol/L).

Furthermore, we tested the antiproliferative activity of the synthesized derivatives in several human cell lines (MCF-7—breast cancer, HepG-2–hepatocellular liver carcinoma and PC-3—prostate adenocarcinoma; see Table 2 for the IC_{50} values). The cytotoxicities of the 9-anilinoacridines, expressed as IC_{50} values, were in the range of 0.6–6.2 μmol/L, which is in accordance with reported literature data for other AHMA derivatives.[8,11,12] The cell lines differ in their sensitivity towards AHMA (**1**), **2a**, **2d** and **2e**. The cell line HepG-2 is considerably more sensitive to the tested compounds than the remaining two cell lines. Compound **2a** is the most cytotoxic of the compounds tested. Acyl substituent on the AHMA amino group thus possesses a moderate effect on cytotoxicity. The differences in IC_{50} values are within one order of magnitude but are statistically significant (analysis of variance, ANOVA, on level $\alpha = 0.01$). Our most important observation is that changing the acyl group to optimize the release profile does not compromise antiproliferative activity. Compound **2e** was chosen to optimize the polymer conjugate synthesis because it fulfills both of the basic requirements; it is sufficiently cytotoxic and has a favorable release profile from the polymer hydrazone at different pH values (minimal release at pH 7.4 and relatively fast release at pH 5.0). The cytotoxicity of the conjugates was not tested because we

Table 2
DNA binding and antiproliferative activity of 9-anilinoacridines

Compound	CC_{50}[a], μmol/L	IC_{50}[b], μmol/L		
		MCF-7	HepG-2	PC-3
1 (AHMA)	21.3 ± 0.6	4.5 ± 0.6	0.6 ± 0.4	3.3 ± 0.5
2a	22.8 ± 0.4	2.9 ± 0.4	1.4 ± 0.6	2.3 ± 0.7
2d	36.6 ± 0.4	4.8 ± 0.4	2.1 ± 0.6	2.3 ± 0.7
2e	30.7 ± 0.8	6.2 ± 1.0	1.6 ± 0.6	2.2 ± 0.6

[a] Acridine drug concentration in μmol/L that reduces fluorescence of DNA–ethidium bromide complex (c_{DNA}:c_{EtBr} = 1.26:1) to 50% (CC_{50} ± standard deviation).
[b] Concentrations causing 50% inhibition in the MTT test (IC_{50} ± standard deviation) in μmol/L.

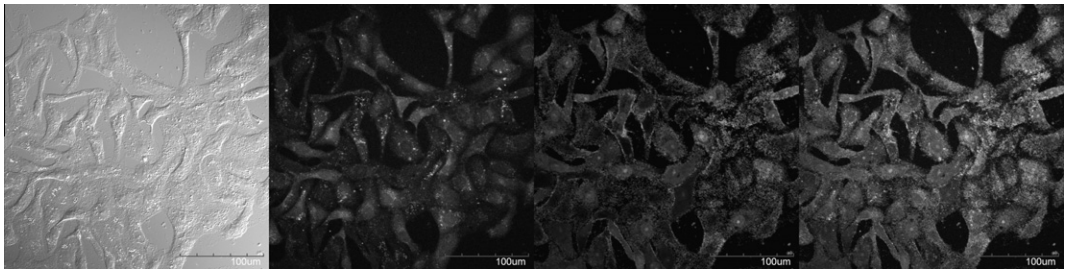


Figure 5a. The cellular internalization of 9-anilinoacridine **2e** (c = 5 μg/mL). The images are sequentially shown according to the channels used (left to right): brightfield, Hoechst 33342 dye (cell nuclei stained), acridine fluorescence, merged channels of Hoechst 33342 dye and acridine fluorescence.

showed by HPLC that free drug in its original form is released from the conjugates after incubation in media. Therefore, the data on in vitro cytotoxicity of the conjugates may be misleading due to significantly different concentrations of the drug released into the media during incubation with the cells (pH 7.4) compared to the in vivo situation. This is because in an in vivo situation, the system is opened, i.e. the released drug is being continuously removed by internalization into cells or diffusion out of the tumor tissue. In addition, pH in tumor tissue is generally slightly acidic, but varies according to the exact site in the tumor within 1–1.5 pH units, which has dramatic effect on the drug release rate (see above) and therefore the published IC_{50} values of hydrazone conjugates, typically one order of magnitude higher than the IC_{50} values of free drugs,[21–23] generally do not correspond with the in vivo antitumor effectivity.

The intracellular fate of the 9-anilinoacridines and their conjugates was studied by confocal microscopy. The free 9-anilinoacridine derivative **2e**, which is significantly cytotoxic due to intercalation with DNA, penetrates into cells and subsequently into cell nuclei (Fig. 5a). A portion of the acridine-related fluorescence was observed in vesicles inside the cells. The fluorescence of **2e** belonging, with high probability, to its polymer conjugate **8**, was clearly visible within intracellular vesicles of the cells (Fig. 5b). However, its fluorescence in cell nuclei was less than that observed from cells incubated with the same amount of free drug. This result is probably due to a portion of **2e** that was released from the conjugate and intercalated in DNA as well as portion of conjugate internalized into cells and subsequently released in endosomes. The results are also influenced by the fact that the fluorescence intensity of acridines greatly decreases after intercalation.[14]

3. Conclusions

New types of biodegradable polymer-conjugates of anilinoacridines were synthesized. In these conjugates, the acridine drug derivatives were connected to the water-soluble polymer (poly[*N*-(2-hydroxypropyl)methacrylamide-*co*-N^1-(6-hydrazino-6-oxohexyl)methacrylamide]) via a pH-labile hydrazone bond. The dependence of the drug release on the linker structure was examined. The best release profile, where the conjugate was almost completely stable at physiological pH (7.4) but sufficiently labile at the pH of late endosomes (pH 5.0), showed the conjugate containing a linker with the strongest steric hindrance of the original oxo-group. However, this conjugate could not be easily synthesized by reaction of the ketone linker-bearing acridine drug with the hydrazide group-containing polymer because of unwanted side reactions. Therefore, the acridine drug-containing monomer was synthesized first, followed by its radical copolymerization. All of the synthesized acridine drugs retain their in vitro antiproliferative activity (the free intercalators have IC_{50} values in the range of 0.6–6.8 μmol/L in MCF-7, HepG-2 and PC3 tumor cell lines), intercalate into DNA and readily penetrate into the cell, as shown by confocal microscopy.

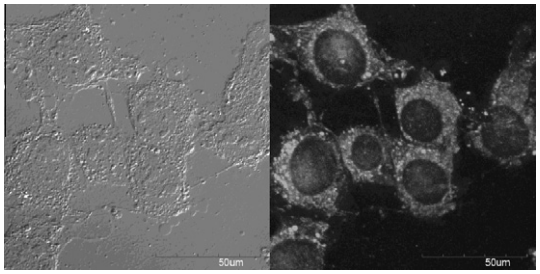


Figure 5b. The cellular internalization of polymer conjugate **8** (c = 5 μg (9-anilinoacridine **2e** equivalent)/mL). The images are sequentially shown according to the channels used (left to right): brightfield, acridine fluorescence.

4. Experimental part

The 3-(9-acridinylamino)-5-hydroxymethylaniline (AHMA, **1**) was prepared from 9-chloroacridine and 3-amino-5-(hydroxymethyl) aniline in 92% yield, according to the literature procedure.[8] The *p*-oxopropylbenzoic acid (**9b**) was obtained from Rieke Metal. The 5-methyl-4-oxohexanoic acid (**9d**) was synthesized in 68% yield, according to the literature procedure.[26] The poly[*N*-(2-hydroxypropyl)methacrylamide-*co*-1-*N*-(6-hydrazino-6-oxohexyl)-2-methylacrylamide] (pHPMA-MAAcap hydrazide, weight average molecular weight M_w = 17.5 kDa, polydispersity $I = M_w/M_n$ = 1.87, where M_n is number-average molecular weight, 7.5 mol% hydrazide monomer unit) was synthesized by radical copolymerization of *N*-(2-hydroxypropyl)methacrylamide with N^1-(6-hydrazino-6-oxohexyl)-2-methylacrylamide (**5**) using azobis(isobutyronitrile) as an initiator according to reference.[27] All other chemicals were purchased from Sigma–Aldrich Ltd. (Prague, Czech Republic) and were used without further purification. Analyses were performed on a HPLC chromatograph (Shimadzu, Japan) using a reverse-phase column (Chromolith Performance RP-18e 100 × 4.6 mm) and UV detection. A mixture of water and acetonitrile was used as the eluent with a gradient of 0–100 vol.% and a flow rate of 2 mL/min. The melting point temperatures were determined with a Kofler's block (VEB Analytik Dresden, Germany). NMR spectra were measured with a Bruker Avance MSL 300 MHz NMR spectrometer (Bruker Daltonik, Germany). The molecular weights of the polymers were determined by gel permeation chromatography (GPC) using an HPLC Shimadzu system equipped with a GPC column (TSKgel G3000SWxl 300 × 7.8 mm, 5 μm), UV/VIS, refractive index (RI) Optilab®-rEX and multiangle light scattering (MALS) DAWN EOS (Wyatt Technology Co., USA) detectors using a methanol and sodium acetate buffer (0.3 M, pH 6.5) mixture (80:20 vol.%, flow rate of 0.5 mL/min). UV/VIS spectra were measured with a SPECORD 205 Spectrometer (Analytik Jena AG, Germany).

4.1. Synthesis of dimethyl pivalylmethylmalonate

Sodium methoxide (615 mg, 11.4 mmol) in methanol (5 mL) was added dropwise to the solution of dimethyl malonate (1.3 mL, 11.4 mmol) in methanol (50 mL). After stirring for 30 min, bromopinacolone (2 g, 11.4 mmol) was added dropwise and the mixture was refluxed for 2 h. The solution was partitioned between water and diethyl ether. The organic phase was separated, dried with anhydrous magnesium sulfate and evaporated under reduced pressure. The crude product was purified by column chromatography (silica, hexane/ethyl acetate 4:1) to obtain 1.28 g (49%) of the title compound as colorless oil. R_f (hexane/ethyl acetate 4:1) = 0.56; ^{1}H NMR (300 MHz, *CDCl*$_3$) δ ppm 3.88 (t, *J* = 7.18, 1H), 3.74 (s, 6H), 3.13 (d, *J* = 7.18 Hz, 2H), 1.16 (s, 9H); ^{13}C NMR (75 MHz, *CDCl*$_3$) δ ppm 212.6, 169.6, 52.9, 46.8, 44.0, 36.3, 26.5; MS (ESI+) *m*/*z* 230.26 (calcd): 253.24 $[M+Na]^+$, Anal. calcd for $C_{11}H_{18}O_5$: 57.38% C, 7.88% H; found: 57.36% C, 7.90% H.

4.2. Synthesis of 5,5-dimethyl-4-oxohexanoic acid (pivalylpropionic acid, 9e)

Dimethyl pivalylmalonate (1.2 g, 5.2 mmol) and KOH (1 g, 17.8 mmol) were refluxed in 50% aqueous methanol (10 mL) for 2 h. The reaction mixture was then neutralized with 1 M HCl and the resulting malonic acid derivative was precipitated by excess of aqueous calcium chloride as described by Hill.[39] The white solid was filtered off, dissolved in 1 M HCl, extracted in diethyl ether and evaporated. The resulting malonic acid derivative was decarboxylated by heating at 145 °C for 2 h without solvent. After cooling, the crude product was recrystalized from petroleum ether to give 640 mg (78%) of the title compound **9e** as white needles. Mp = 66–69 °C; ^{1}H NMR (300 MHz, *CDCl*$_3$) δ ppm 2.80 (t, *J* = 6.54 Hz, 2H), 2.60 (t, *J* = 6.38 Hz, 2H), 1.15 (s, 9H); ^{13}C NMR (75 MHz, *CDCl*$_3$) δ ppm 213.9, 179.1, 43.8, 31.2, 28.0, 26.4; MS (ESI+) *m*/*z* 158.20 (calcd): 181.08 $[M+Na]^+$; Anal. calcd for $C_8H_{14}O_3$: 60.74% C, 8.92% H; found: 60.71% C, 8.95% H.

4.3. General procedure for synthesis of thiazolidine-2- thione (TT) activated oxoacids 3a–e

A mixture of the respective oxoacid **9a–e** (2 mmol) and thiazolidine-2-thione (262 mg, 2.2 mmol) in dichloromethane (3 mL) was cooled to 0 °C in an ice-water bath. Then, *N*,*N*′-dicycloehexylcarbodiimide (DCC, 515 mg, 2.5 mmol) in dichloromethane (2 mL) was added. The reaction mixture was allowed to warm to ambient temperature and stirred for 2 days. The precipitated *N*,*N*′-dicyclohexylurea was filtered off and the filtrate was evaporated under reduced pressure. The crude product was purified by column chromatography (silica, hexane/ethyl acetate 4:1) yielding activated acid derivatives **3a–e**.

4.3.1. 1-(2-Thioxo-thiazolidin-3-yl)-pentane-1,4-dione (3a)

Synthesized from levulinic acid (**9a**) as yellow solid in 84% yield. R_f (hexane-ethyl acetate 4:1) = 0.45; ^{1}H NMR (300 MHz, *CDCl*$_3$) δ ppm 4.50 (t, *J* = 7.54 Hz, 2H), 3.39 (t, *J* = 6.02 Hz, 2H), 3.26 (t, *J* = 7.54 Hz, 2H), 2.77 (t, *J* = 5.92 Hz, 2H), 2.15 (s, 3H); ^{13}C NMR (75 MHz, *CDCl*$_3$) δ ppm 206.9, 201.6, 173.7, 56.0, 37.9, 33.2, 29.9, 28.5; MS (ESI+) *m*/*z* 217.31 (calcd): 218.22 $[M+H]^+$; Anal. calcd for $C_8H_{11}NO_2S_2$: 44.22% C, 5.10% H, 6.45% N; found: 44.24% C, 5.18% H, 6.40% N.

4.3.2. 1-[4-(2-Thioxo-thiazolidine-3-carbonyl)-phenyl]-propan-2-one (3b)

Synthesized from *p*-(2-oxopropyl)-benzoic acid (**9b**) as yellow solid in 67% yield. R_f (hexane/ethyl acetate 4:1) = 0.52; ^{1}H NMR (300 MHz, *CDCl*$_3$) δ ppm 7.63 (d, *J* = 8.34 Hz, 2H), 7.19 (d, *J* = 8.43 Hz, 2H), 4.47 (t, *J* = 7.24 Hz, 2H), 3.69 (s, 2H), 3.41 (t, *J* = 7.24 Hz, 2H), 2.11 (s, 3H); ^{13}C NMR (75 MHz, *CDCl*$_3$) δ ppm 205.2, 202.0, 171.0, 139.4, 132.4, 130.0, 129.5, 56.6, 50.8, 29.8, 29.6; MS (ESI+) *m*/*z* 279.38 (calcd): 280.25 $[M+H]^+$; Anal. calcd for $C_{13}H_{13}NO_2S_2$: 55.89% C, 4.69% H, 5.01% N; found: 55.79% C, 4.64% H, 4.96% N.

4.3.3. 1-[4-(2-Thioxo-thiazolidine-3-carbonyl)-phenyl]-ethanone (3c)

Synthesized from *p*-acetylbenzoic acid (**9c**) as yellow solid in 70% yield. R_f (hexane-ethyl acetate 4:1) = 0.54; ^{1}H NMR (300 MHz, *CDCl*$_3$) δ ppm 7.90 (d, *J* = 8.60 Hz, 2H), 7.68 (d, *J* = 8.61 Hz, 2H), 4.51 (t, *J* = 7.24 Hz, 2H), 3.45 (t, *J* = 7.24 Hz, 2H), 2.57 (s, 3H); ^{13}C NMR (75 MHz, *CDCl*$_3$) δ ppm 202.0, 197.3, 170.4, 139.5, 137.9, 129.4, 128.2, 56.3, 29.9, 26.9; MS (ESI+) *m*/*z* 265.35 (calcd): 266.26 $[M+H]^+$; Anal. calcd for $C_{12}H_{11}NO_2S_2$: 54.32% C, 4.18% H, 5.28% N; found: 54.17% C, 4.21% H, 5.25% N.

4.3.4. 5-Methyl-1-(2-thioxothiazolidin-3-yl)hexane-1,4-dione (3d)

Synthesized from 5-methyl-4-oxohexanoic acid (**9d**) as yellow oil in 86% yield. R_f (hexane/ethyl acetate 4:1) = 0.52; ^{1}H NMR (300 MHz, *CDCl*$_3$) δ ppm 4.54 (t, *J* = 7.53 Hz, 2H), 3.44 (t, *J* = 5.88 Hz, 2H), 3.30 (t, *J* = 7.53 Hz, 2H), 2.85 (t, *J* = 5.91 Hz, 2H), 2.71–2.59 (m, *J* = 6.92 Hz, 1H), 1.12 (d, *J* = 6.96 Hz, 9H); ^{13}C NMR (75 MHz, *CDCl*$_3$) δ ppm 212.9, 201.6, 174.0, 56.0, 40.8, 34.8, 33.2, 28.5, 18.3; MS (ESI+) *m*/*z* 245.36 (calcd): 246.23 $[M+H]^+$; Anal. calcd for $C_{10}H_{15}NO_2S_2$: 48.95% C, 6.16% H, 5.71% N; found: 48.85% C, 6.20% H, 5.66% N.

4.3.5. 5,5-Dimethyl-1-(2-thioxothiazolidin-3-yl)hexane-1,4-dione (3e)

Synthesized from 5,5-dimethyl-4-oxohexanoic acid (**9e**) as yellow oil in 73% yield. R_f (hexane/ethyl acetate 4:1) = 0.60; ^{1}H NMR (300 MHz, *CDCl*$_3$) δ ppm 4.49 (t, *J* = 7.53 Hz, 2H), 3.37 (t, *J* = 5.88 Hz, 2H), 3.25 (t, *J* = 7.53 Hz, 2H), 2.85 (t, *J* = 5.78 Hz, 2H), 1.12 (s, 9H); ^{13}C NMR (75 MHz, *CDCl*$_3$) δ ppm 214.3, 201.6, 174.1, 56.1, 44.0, 33.2, 31.6, 28.6; MS (ESI+) *m*/*z* 259.39 (calcd): 260.27 $[M+H]^+$; Anal. calcd for $C_{11}H_{17}NO_2S_2$: 50.94% C, 6.61% H, 5.40% N; found: 50.81% C, 6.63% H, 5.76% N.

4.4. General procedure for the synthesis of AHMA-amides 2a–e

3-(9-Acridinylamino)-5-hydroxymethylaniline (AHMA, **1**, 158 mg, 0.5 mmol) and the respective TT-activated oxo-acid **3a–e** (0.6 mmol) were stirred in *N*,*N*-dimethylformamide (DMF) at room temperature overnight. The solvent was evaporated under reduced pressure, and the residue was purified by column chromatography (silica, chloroform-methanol 4:1) followed by crystallization from ethanol.

4.4.1. 3-(9-Acridinylamino)-5-hydroxymethylaniline *N*-levulinate (2a)

Activated oxo-acid **3a** was used. Yield 79% of orange crystals. R_f (chloroform/methanol 4:1) = 0.38; mp (ethanol) = 198–201 °C; ^{1}H NMR (300 MHz, *DMSO-d*$_6$) δ ppm 9.75 (s, 1H), 8.06 (brs, 2H), 7.42 (s, 4H), 7.25 (s, 1H), 7.14 (s, 3H), 6.83 (1H), 5.07 (br s, 1H), 4.34 (s, 2H), 2.63 (t, *J* = 6.05 Hz, 2H), 2.44 (t, *J* = 6.13, 2H), 2.04 (s, 3H); ^{13}C NMR (75 MHz, *DMSO-d*$_6$) δ ppm 207.4, 170.1, 154.6, 150.7, 144.4, 142.8, 140.3, 137.6, 131.2, 127.1, 124.4, 120.5, 116.7, 109.9, 106.3, 63.0, 37.6, 30.1, 29.7; MS (ESI+) *m*/*z* 413.48 (calcd): 414.37 $[M+H]^+$; Anal. calcd for $C_{25}H_{23}N_3O_3$: 72.62% C, 5.61% H, 10.16% N; found: 72.59% C, 5.67% H, 10.28% N.

4.4.2. 3-(9-Acridinylamino)-5-hydroxymethylaniline *N*-(*p*-oxopropylbenzoate) (2b)

Activated oxo-acid **3b** was used. Yield 81% of orange crystals. R_f (chloroform/methanol 4:1) = 0.48; mp (ethanol) = 218–221 °C; ^{1}H NMR (300 MHz, *DMSO-d*$_6$) δ ppm 10.11 (s, 1H), 8.01 (s, 2H), 7.81 (d, *J* = 8.28 Hz, 2H), 7.63 (s, 4H), 7.48 (s, 1H), 7.35 (s, 1H), 7.25 (d, *J* = 8.29 Hz, 2H), 7.08 (s, 2H), 6.64 (s, 1H), 5.16 (br s, 1H), 4.40 (s, 2H), 3.81 (s, 2H), 2.10 (s, 3H); ^{13}C NMR (75 MHz, *DMSO-d*$_6$) δ ppm 205.5, 165.3, 151.4, 147.2, 144.5, 142.9, 140.3, 138.6, 135.0, 133.7, 133.1, 132.3, 129.6, 127.6, 120.5, 119.1, 113.8, 111.6, 107.2, 62.7, 49.2, 29.6; MS (ESI+) *m*/*z* 475.55 (calcd): 476.36 [M+H]$^+$; Anal. calcd for $C_{30}H_{25}N_3O_3$: 75.77% C, 5.30% H, 8.84% N; found: 75.72% C, 5.31% H, 8.80% N.

4.4.3. 3-(9-Acridinylamino)-5-hydroxymethylaniline *N*-(*p*-acetylbenzoate) (2c)

Activated oxo-acid **3c** was used. Yield 74% of orange crystals. R_f (chloroform/methanol 4:1) = 0.49; mp (ethanol) = 236–239 °C; ^{1}H NMR (300 MHz, *DMSO-d*$_6$) δ ppm 10.25 (s, 1H), 8.00 (br s, 6H), 7.39 (s, 4H), 7.28 (s, 2H), 7.08 (s, 1H), 6.44 (s, 1H), 5.14 (br s, 1H), 4.43 (s, 2H), 2.58 (s, 3H); ^{13}C NMR (75 MHz, *DMSO-d*$_6$) δ ppm 197.6, 164.5, 153.8, 150.2, 149.4, 144.5, 143.9, 139.9, 138.8, 131.3, 130.2, 129.5, 128.1, 127.9, 124.4, 120.7, 111.4, 110.5, 107.8, 62.9, 26.9; MS (ESI+) *m*/*z* 461.52 (calcd): 462.36 [M+H]$^+$; Anal. calcd for $C_{29}H_{23}N_3O_3$: 75.47% C, 5.02% H, 9.10% N; found: 75.33% C, 5.06% H, 9.04% N.

4.4.4. 3-(9-Acridinylamino)-5-hydroxymethylaniline *N*-(5-methyl-4-oxohexanoate) (2d)

Activated oxo-acid **3d** was used. Yield 82% of orange crystals. R_f (chloroform/methanol 4:1) = 0.43; mp (ethanol) = 166–169 °C; ^{1}H NMR (300 MHz, *DMSO-d*$_6$) δ ppm 9.91 (s, 1H), 7.99 (s, 2H), 7.65 (s, 4H), 7.27 (s, 1H), 7.15 (s, 3H), 6.58 (s, 1H), 5.15 (s, 1H), 4.36 (s, 2H), 2.69 (t, *J* = 6.41 Hz, 2H), 2.57 (m, *J* = 6.92 Hz, 1H), 2.45 (t, *J* = 6.40 Hz, 2H), 0.95 (d, *J* = 6.92 Hz, 6H); ^{13}C NMR (75 MHz, *DMSO-d*$_6$) δ ppm 213.5, 170.9, 151.9, 145.3, 142.5, 141.7, 141.0, 133.4, 126.9, 122.7, 121.4, 119.8, 117.2, 112.7, 109.4, 63.3, 40.9, 35.0, 30.6, 18.7; MS (ESI+) *m*/*z* 441.53 (calcd): 442.38 [M+H]$^+$; Anal. calcd for $C_{27}H_{27}N_3O_3$: 73.45% C, 6.16% H, 9.52% N; found: 73.29% C, 6.16% H, 9.47% N.

4.4.5. 3-(9-Acridinylamino)-5-hydroxymethylaniline *N*-(5,5-dimethyl-4-oxohexanoate) (2e)

Activated oxo-acid **3e** was used. Yield 90% of orange crystals. R_f (chloroform/methanol 4:1) = 0.49; mp (ethanol) = 145–148 °C; ^{1}H NMR (300 MHz, *DMSO-d*$_6$) δ ppm 9.91 (s, 1H), 7.97 (s, 2H), 7.64 (s, 4H), 7.27 (s, 1H), 7.14 (s, 3H), 6.56 (s, 1H), 5.16 (s, 1H), 4.36 (s, 2H), 2.75 (t, *J* = 6.39 Hz, 2H), 2.45 (t, *J* = 6.41 Hz, 2H), 1.03 (s, 9H); ^{13}C NMR (75 MHz, *DMSO-d*$_6$) δ ppm 214.1, 170.4, 151.2, 144.7, 141.3, 140.5, 132.6, 126.4, 122.0, 119.3, 119.2, 116.7, 112.4, 111.8, 108.6, 62.7, 43.3, 31.1, 30.1, 26.3; MS (ESI+) *m*/*z* 455.56 (calcd): 456.39 [M+H]$^+$; Anal. calcd for $C_{28}H_{29}N_3O_3$: 73.82% C, 6.42% H, 9.22% N; found: 73.75% C, 6.40% H, 9.21% N.

4.5. *N*-[5-(*N*′-Acridin-9-yl-hydrazinocarbonyl)-pentyl]-2-methyl-acrylamide (6)

9-Anilinoacridine **2e** (227 mg, 0.5 mmol) and 6-methacrylamidohexanohydrazide (**5**, 107 mg, 0.5 mmol) were stirred with 50 μL of acetic acid in methanol (2 mL) at room temperature overnight. The solvent was evaporated under reduced pressure, and the residue was purified by column chromatography (silica, chloroform/methanol 9:1) to give 72 mg (37% yield) of **6** as an orange solid. R_f (chloroform-methanol 9:1) = 0.56; ^{1}H NMR (300 MHz, *DMSO-d*$_6$) δ ppm 10.52 (br s, 1H), 8.26–6.97 (m, 10H), 5.58 (s, 1H), 5.23 (t, *J* = 1.50, 1H), 3.07 (dd, *J* = 12.90, 6.74 Hz, 2H), 2.23 (t, *J* = 7.24 Hz, 2H), 1.79 (s, 3H), 1.58–1.16 (m, 6H); ^{13}C NMR (75 MHz, *DMSO-d*$_6$) δ ppm 169.1, 167.3, 145.9, 140.8, 140.1, 138.5, 133.4, 126.0, 120.9, 120.4, 118.6, 38.8, 34.3, 29.0, 26.3, 25.1, 18.7; MS (ESI+) *m*/*z* 390.49 (calcd): 391.30 [M+H]$^+$; Anal. calcd for $C_{23}H_{26}N_4O_2$: 70.75% C, 6.71% H, 14.35% N; found: 70.53% C, 6.75% H, 14.29% N.

4.6. 5,5-Dimethyl-4-{[6-(2-methyl-acryloylamino)-hexanoyl]-hydrazono}-hexanoic acid [3-(acridin-9-ylamino)-5-hydroxy methyl-phenyl]-amide (7)

Pivalylpropionic acid (**9e**, 158 mg, 1 mmol), 6-methacrylamidohexanohydrazide (**5**, 214 mg, 1 mmol) and 2,5-di-*tert*-butylhydroquinone (5 mg) were stirred with 50 μL of acetic acid in methanol (2 mL) at 60 °C overnight. The mixture was evaporated under reduced pressure, dissolved in DMF (3 mL) and cooled to 0 °C. *N*,*N*′-Dicyclohexyl carbodiimide (DCC, 309 mg, 1.5 mmol) and *N*,*N*-dimethylaminopyridine (10 mg) were added, and the mixture was stirred while cooling. After 2 h, 3-(9-acridinylamino)-5-hydroxymethylaniline (AHMA, **1**, 316 mg, 1 mmol) was added. The reaction mixture was allowed to warm to ambient temperature and stirred overnight. The solvents were evaporated under reduced pressure, the residue was extracted with dichloromethane and the suspension was filtered. The filtrate was evaporated and purified on preparative TLC (silica, chloroform/methanol/triethylamine 9:1:0.2) to give the title compound **7** as an orange solid in 26% yield. R_f (chloroform/methanol/triethylamine 9:1:0.2) = 0.40; ^{1}H NMR (300 MHz, *DMSO-d*$_6$) δ ppm 10.01 (br s, 1H), 8.03–7.31 (m, 11H), 6.63 (s, 1H), 5.56 (s, 1H), 5.26 (s, 1H), 5.22 (t, 1H), 4.34 (s, 2H), 3.25 (t, *J* = 6.84 Hz, 2H), 2.87 (t, *J* = 6.50 Hz, 2H), 2.52 (t, *J* = 6.54 Hz, 2H), 2.29 (t, *J* = 6.43 Hz, 2H), 2.07 (s, 3H), 1.65–1.41 (m, 6H), 1.08(s, 9H); ^{13}C NMR (75 MHz, *DMSO-d*$_6$) δ ppm 177.4, 171.2, 169.8, 166.7, 150.0, 149.1, 147.6, 143.5, 140.6, 140.2, 132.6, 125.9, 121.7, 119.3, 117.4, 116.8, 113.5, 112.2, 108.7, 62.4, 40.6, 39.8, 35.8, 32.5, 27.0, 26.3, 24.4, 23.9, 22.7, 18.5; MS (ESI+) *m*/*z* 650.83 (calcd): 651.69 [M+H]$^+$; Anal. calcd for $C_{38}H_{46}N_6O_4$: 70.13% C, 7.12% H, 12.91% N; found: 70.040% C, 7.09% H, 12.87% N.

4.7. Conjugation of 2a-e with pHPMA-MAAcap hydrazide

A solution of pHPMA-MAAcap hydrazide (40 mg), AHMA derivative **2a–e** (10 mg) and glacial acetic acid (80 μL) in methanol (800 μL) was stirred at room temperature overnight. The polymer was then isolated by gel filtration on a Sephadex LH-20 column (60 mL bed volume) with methanol as the eluent, and the polymer-containing fractions were collected and concentrated under reduced pressure. The solution was poured into an excess of diethyl ether, and the precipitated polymer conjugate was filtered off and dried under vacuum. A yield of 40-45 mg (80–90%) of **4a–e** was obtained.

4.8. Synthesis of polymer 8 by radical copolymerization

Monomer **7** (12 mg) was mixed with *N*-(2-hydroxypropyl)-methacrylamide (88 mg) and azobis(isobutyronitrile) (AIBN, 20 mg) in dry dimethylsulfoxide (0.6 mL), introduced under argon into a polymerization ampoule and sealed. The reaction mixture was stirred under argon at 60 °C overnight. The polymer was then precipitated with diethyl ether and purified by gel filtration on a Sephadex LH-20 column (60 mL bed volume) using methanol as the eluent. A yield of 55 mg (55%) of polymer **8** was obtained.

4.9. Determination of drug content

The molar ratio of the cleavable (i) *versus* non-cleavable (ii) acridine drug bound to the polymer was determined by size exclusion

chromatography (SEC) on a TSK3000 column with UV detection at 408 nm after hydrolysis with a 0.1 M HCl solution. It was calculated as a wagered ratio of polymer peak to low molecular weight peak area at 408 nm using the equation (1), where I_p is the area under the polymeric peak representing the non-cleavably bound acridine content, I_a is the area under the low molecular weight peak representing the hydrazone-bound anilinoacridine, ε_n is the molar absorption coefficient of the non-cleavable monomer derivative **6** at 408 nm (8761 L mol^{-1} cm^{-1}), ε_h is the molar absorption coefficient of the hydrazone monomer **7** at 408 nm (8490 L mol^{-1} cm^{-1}).

$$i : ii = \frac{I_p}{\varepsilon_n} : \frac{I_a}{\varepsilon_h}$$

Because the UV–VIS absorption spectra and molar absorptivities of the acridine derivatives **2a–e** do not substantially differ from that of **7**, the same ε_h value was used for all acridine conjugates. The total amount of acridine drug in the conjugate was determined using the sum of the wagered peak areas (i and ii in (1)).

4.10. In vitro drug release study

The drug release profiles of the polymer conjugates were evaluated by size exclusion chromatography (SEC) at pH 5.0 and 7.4 (also, at pH 6.5 for conjugate **8**) using 10 mM acetate buffer (pH 5.0 or 6.5) or phosphate buffer saline (pH 7.4). The conjugate (0.1 mg) was dissolved in the appropriate buffer (1 mL) and placed into a thermostatted autosampler of the SEC instrument (37 °C). After 0, 2, 6, 12 and 24 h, 20 μL of the conjugate solution was injected directly on the TSK3000 column. The drug release was determined from the integral areas of the low molecular weight (free drug) and high molecular weight (polymer conjugate) peaks. After the last measurement (24 h), the solutions were analyzed by reverse-phased HPLC chromatography to confirm that only the anilinoacridine derivatives **2a–e** were released.

4.11. In vitro ethidium bromide displacement assay

The ethidium bromide displacement assay was performed using a solution of 1.26 μM(bp) calf thymus (CT) DNA (concentration determined spectrophotometrically, ε_{260} = 12,824 M(bp)$^{-1}$ cm^{-1}) and 1 μM ethidium bromide in HEPES buffer (10 mM HEPES, 76 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid, pH = 7.0).[27] This solution was titrated with a solution of the measured drug in the same buffer to determine the drug concentration, CC_{50}, which reduced the fluorescence (excitation 546 nm, emission 595 nm) of the DNA–ethidium bromide complex by half. None of the compounds showed fluorescence emission at 595 nm. All experiments were carried out in triplicate.

4.12. In vitro cytotoxicity assay and cellular internalization

In vitro cytotoxicity of the 9-anilinoacridine derivatives was assessed with human cancer cell lines. Ninety-six-well flat-bottomed microplates (NUNC, Roskilde, Denmark) were seeded with MCF-7 (breast cancer), HepG-2 (hepatocellular liver carcinoma) and PC-3 (prostate adenocarcinoma) cancer cells (5 × 104 cells/well). The tested samples (in triplicate) were then added to the wells to obtain the desired drug concentration (0.1–2660 ng mL^{-1} free drug equivalent). The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air for 72 h. The cell viability was then evaluated by a standard MTT test according to a reference[29] on a plate reader UT-2100C (MRC, Israel).

Internalization of the acridine derivatives and conjugates into murine MCF-7 cells was assayed by confocal microscopy using the intensive acridine fluorophore caused fluorescence in analogy as described for the doxorubicin conjugates.[30] The cell nuclei were also visualized using the Hoechst 33342 nucleic acid dye. Statistical evaluation of the differences (analysis of variance, ANOVA) was performed on level α = 0.01 using Microcal Origin program, version 5.0 (Microcal Software Inc., Northampton, MA, U.S.A.).

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