Poly (maleic anhydride – *alt* –vinyl acetate) Conjugates with Alkylating Agents: II. Organotropic Effects and Antitumoral Activity

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A series of poly(maleic anhydride-alt-vinyl acetate) conjugates with alkylating agents were evaluated regarding antitumoral activity, biodistribution and organotropic effects. The synthesized conjugates exhibits a significant antitumoral activity expressed as ATR (average tumor retention). In addition the obtained macromolecular compounds show a prolonged half-life in sanguine serum and accumulation in tumoral tissue. The last effect is supposed to be enhanced by EPR (enhanced permeation and retention) effect. The analyzed compounds also exhibit some organotropic effects such as the increase of liver, spleen and kidney weight. The LD_{50} values of the alkylating agents and conjugated ones proved that the coupling reaction decrease the intrinsic toxicity of the alkylating agents.

Keywords: poly(maleic anhydride-alt-vinyl acetate), antitumoral activity, EPR effect

Polymers are introduced into a cell by endocytosis rather than by permeation through the plasma membrane or by means of transport proteins [1]. Since polymers follow a different cellular uptake mechanism than low molecular weight chemical entities, drug uptake by cells can be modulated by conjugating the reactive reagent to an appropriate polymeric carrier. Conjugation of cytotoxic compounds to polyanionic polymers such as maleic anhydride (MA) copolymers has been actively explored with the aim to improve solubility, plasma half-time, toxicity and the targeting capacity of the drug [2].

On the other hand, the MA copolymers formed in the presence of radical inhibitors are in 1:1 ratio and in an alternating sequence [3]. Finally, the choice of the MA copolymers as carrier of alkylating agents is determined by the fact that they exhibit many biological activities. For example, MA copolymers act as mitotic inhibitors and their functional role in neoplasmic processes as well as their immunology and resistance to viruses have been reported [4-7].

A major problem associated with most chemotherapeutics is the side effects produced by these polymeric drugs. Our preliminary data [6-8] showed that the conjugation of low molecular weight compounds to an anionic polymer system reduce side effects determined by polymeric support or by conjugated compound.

The purpose of the present work is to investigate the chemical structure dependency on the biological behaviour parameters and antitumor activity of three poly(MA-*alt*-VA) conjugates with alkylating agents synthesized in the first part of this series [9].

Materials and Methods

The unmodified poly(MA-*alt*-VA) and the conjugates I-III were synthesized in our laboratory using the procedures described in the first part of this series [9]. The structures of these compounds are given in formulas I-IV.

Biological tests

Poly(MA-*alt*-VA) and the mustard conjugates I-III were studied *in vivo* on Wistar rats weighting 100-130g (±15 g) that were obtained from the Oncology Institute, Cluj-Napoca, Romania. Before treatment, the animals were quarantined for 2 weeks and ear tagged with the study identification number. The rats were weighted and divided in five groups of 20 rats each. They were sampled using randomization and stratification methods to ensure that the average weight and variance of weight in each group of the treatment was as similar as possible. The animals were treated with poly(MA-*alt*-VA), conjugates I-III or placebo daily for 14 days. During the quarantine and study periods, the rats were housed four per metal cage under a 12 h light\dark cycle at approximately 25°C. They were given proper care, feed (Purina Rodend Chow) and acidified water (*p*H=6.0) *ad libitum*. The rats were observed twice a day and weighted twice a week.

Experimental carcinosarcoma Walker solid tumors were administrated by subcutaneous injection of cell suspensions according to Pollak's modification of the published procedures [10] to afford reproducibility and suitability for routine screening.

To each experimental group 40,150 and 200 mg\day\kg body of the studied compounds were administrated daily for 14 days by intraperitoneal *(ip)* injection starting 14 days after tumor inoculation. The substances were administrated as 1% aqueous methylcellulose suspensions. A 0.9% aqueous NaCl control was administrated in the same manner.

The animals were sacrificed by chloroform anesthesia and decapitation. After sacrifice, the standard methodology was applied for the determination of tumor weights. The antitumor activity was evaluated by the calculation of average tumor regression (ATR, %):

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samples	Concentration\ μg [a]		
	serum	liver	Solid tumor
A[b]	<0.1	<0.1	<0.1
Poly(MA-*alt*-VA)	0.5	0.1	1.2
Conjugate I	0.5	0.1	1.9
Conjugate II	0.4	<0.1	1.6
Conjugate III	0.6	<0.1	2.1

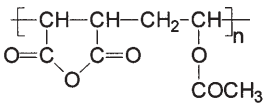
[a] Determined 8h after administration

[b] Tri(β-chloroethyl)amine

$$ATR(\%) = M_C\text{-}M_T \backslash M_C \times 100 \quad (1)$$

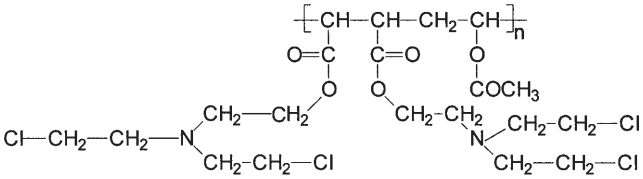
where M_C represents the average tumor weight of the control sample and M_T is the average tumor weight of the treated samples.

The ATR and DL_{50} values listed in table 1 and table 2 contain the values of organotropic effects.



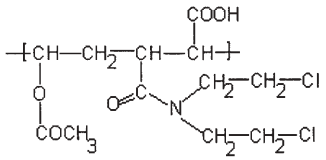
Poli(MA-alt-VA)

formula I



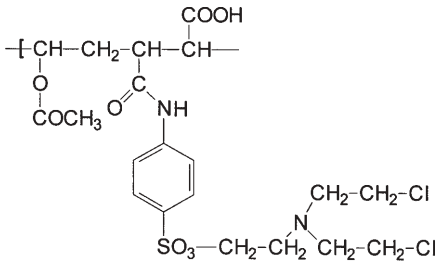
Conjugate I

formula II



Conjugate II

formula III



Conjugate III

formula IV

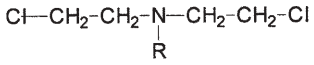
Poly(MA-*alt*-VA), conjugates I-III and compounds a,b (table 2) concentrations in rat liver, sanguine serum and solid tumor were determined by the method suggested by Markaverich [11] and by Brown [12]. The samples were obtained on the last day of the treatment.

The liver from three adults rats (10g) were perfused with saline, weighted, homogenized in HPLC grade water and boiled for 60 min. The homogenate was centrifuged (40,000 g for 30 min) and the supernatant collected and evaporated under nitrogen atmosphere to 10 mL. A Varian HPLC instrument equipped with a PL gel column was employed with tetrahydrofuran as mobile phase. The quantitative evaluation of the elution curves where the peak heights vs. sample concentration were plotted.

Serum volumes of 0.1 mL were extracted with 1.0 mL of 2% hexane in butanol. The samples were centrifuged for 10 min and the supernatant fluid was transferred to 2 mL conical vials and then evaporated at 55°C under nitrogen. Dried samples were dissolved in 50 mL of HPLC solvent and aliquots were used for HPLC determination. The obtained results are listed in table 3.

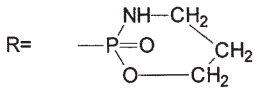
Results and discussion

The alkylating agents are a well-defined category of antitumoral drugs due to the fact that contain or can form an alkyl group that can be covalently linked with certain cell components. The DNA alkylation determines the antitumoral activity of the majority of the alkylating agents; nitrogen (7th position) and oxygen (6thposition) atoms of guanine represents the targets of the DNA alkylation [13]. Mustard derivatives represent a category of alkylation agents that can be represented by the typical structure given in formula V.

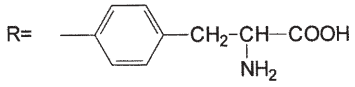


formula v

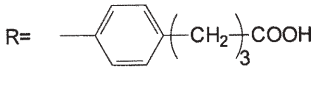
Where:



cyclophosphamide



melphalan



clorambucil

In the case of the conjugates I-III:

R= $-CH_2-CH_2-$

conjugate I

R= — H

conjugate II

R= $-C_6H_4-SO_3-CH_2-$

conjugate III

The mustard derivatives are well-known for their antitumor activity and, unfortunately, for their marked aggressiveness toward the normal cells as well [14-16]. Their high toxicity always represents a major setback for chemotherapy treatment of malignant tumors and, consequently, extended studies on the synthesis of new compounds with higher antitumoral activity and lower toxicity were pursued [17-20]. One approach was to covalently bind mustard type moieties onto macromolecular carriers whose chemical structure could significantly influence the toxicity and solubility of newly obtained compounds [21].

Antitumoral activity

Regarding *in vivo* evaluation of the antitumoral activity of the synthesized compounds, the data listed in table 1 show the influence of the comonomer structure. If we consider that MA copolymers and related conjugates are direct effectors [5], it is obvious that the *in vivo* antitumoral effect will depend on the macromolecules capacity of accessing the region of interest from the organism subjected to the treatment. Such a property is determined by several structure parameters, as of the comonomers structure and the hydrophilic\hydrophobic ratio in the structure of the molecule. This assertion suggests that the presence of the long spacer containing the phenyl nuclei in the structure of conjugate III enhances the macromolecule capacity of accessing the tumoral tissues and thus, the ATR values (table 1).

In addition, the data listed in table 1 show that the variation of the conjugates toxicity and their antitumoral activity has similar tendencies.

Effect of polymers on internal organ weights

Administration of MA copolymers caused significant variation on certain organ weights without significant modification of the body weight [6, 22]. The data given in Table 2 indicate that both mustard derivatives and their conjugates with poly(MA-*alt*-VA) induced significant weight increases in the liver, spleen and particularly the kidney.

As an organ that represents the site of differentiated immunocompetent lymphocytes, in which lymphocytes clonal expression occurs under the influence of antigen, the spleen weight change was 40%. For poly(MA-*alt*-VA), the spleen weight decreased significantly compared with the reference sample-the decrease correlated with that of leukocytes (unpublished results). For conjugates I,II and III, the spleen weight increases visibly, correlated with the increase in the number of leukocytes (unpublished results).

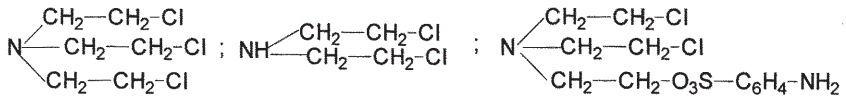
For the unmodified MA copolymer and conjugates I-III, hepatic hypoglycemia was observed, which indicated aerobic glycolysis indicating interference by these compounds in the process of pyruvic acid's transformation in lactic acid. The data listed in table 2 indicate that the insertion of mustard derivatives as side groups onto MA copolymer also increased organotropic effects.

Table 1

ART AND DL_{50} VALUES OF THE ANALYZED SAMPLES

Sample	Dose Mg\kg body	ATR %	DL_{50} [a] Mg\kg body
a*	40 150 200	-	50
b**	40 150 200	-	70
c***	40 150 200	-	40
Poly(MA-*alt*-VA)	40 150 200	10 17 22	370
Conjugate I	40 150 200	22 25 33	300
Conjugate II	40 150 200	31 34 41	270
Conjugate III	40 150 200	36 40 44	150

*



a) Mode of administration: intraperitoneal (ip).

Table 2
ORGANOTROPIC EFFECTS ON THE COMPOUNDS TAKEN IN THE STUDY

Sample*	Liver (%)	Spleen (%)	Kidney (%)
A°	+22.4-31.0	+24.0	+32.0
Poly(MA-*alt*-VA)	Unmodified	-32.0	+42.0
Conjugate I	+27.0 to +62.0	+40.9 to 90.0	+72.0
Conjugate II	+24.7 to +49.0	+35.0 to +110.9	+55.0
Conjugate III	+19.7 to +70.0	+31.7 to 140.0	+82.0

*All data have been calculated versus the control sample: + and – express weight increases and decreases, respectively
a) tri(β-chloroethyl)amine

Biodistribution

The data listed in table 3 are the variations in the polymer concentration in the blood serum, the liver, and the solid tumor determined 8h after administration. The liver is one of the major organs responsible for the removal of foreign substances from the blood. Liver capillaries, so-called sinusoidal capillaries, are characterized by an absence of basement membranes and a sieve-like structure through which macromolecules and particles with nanometer diameters can pass. Thus, in the liver, substances can traffic between the blood compartment and the interstitial space. Furthermore, the liver has large surface area and has reticuloendothelial system (RES) cells including Kupffer cells. These cells routinely take up foreign substances, particularly vesicles, nano and even micron sized particles. Thus in terms of both structure and functional, the liver works as a blood filtering system. Consequently, the polymeric drugs need to be structured so as to avoid being recognized by the liver in order to achieve prolonged circulation in the blood. The higher concentration of polymer conjugate in the blood serum 8 h after administration (table 3) and the lower concentration in liver indicate that these compounds may be stable in the blood and the liver uptake may be less significant for these compounds.

It is well known that tumor masses are located outside of the vasculature system [23]. Therefore, after extravagation, the polymeric drugs must enter the tumor mass. To ascertain whether polymeric drugs can effectively penetrate into the interstitial tissue of tumors from the blood, one needs to understand the unique features of the tumor vasculatures.

In inflammatory conditions the permeability of the blood vessels is greatly increased by factors acting on endothelial cells and opening the tight intercellular junctions. These factors include agents such as bradykinin, histamine, prostaglandins, and tumor necrosis factor. This has also been shown to be the case with certain microbial infections, where the bradykinin-generating cascade is activated and hence are at least two substances known to be involved in modulation of vascular permeability. The first is vascular permeability factor (VPF) a protein produced by a range of cancer cells and also by pituitary follicular cells [25, 26] and the second is bradikinin (or kinin) that is an endogenous vasoactive peptide and it acts on smooth muscle cells inducing an enlarged group between endothelial cells. It has been shown that fluid accumulation in tumors and the leakage of plasma protein out of the blood vessels is a result of kinin action. In addition to VPF and bradykinin, interleukin-2, prostadlandins, tumor necrosis factor, and other agents are also known to influence the vascular permeability of tumors, although currently many questions into their regulation and mechanism of action remain unanswered. It is now well established that macromolecules greater than 15KDa circulating for extended periods in the bloodstream show substantial tumor accumulation. This effect has been extensively studied and reported by Maeda [27-29] and recently by Uglea [19, 20, 30, 32]; the process is called "enhanced permeability and retention (EPR) effect".

Based on the data listed in table 3, the polymer-conjugate accumulation in the solid tumors is evidenced. This process is influenced by several parameters, such as the chemical structure of the monomers, molecular weight,

Table 3
VARIATION OF THE CONCENTRATION OF THE STUDIED COMPOUNDS IN SERUM, LIVER AND SOLID TUMORS

samples	Concentration\ μg [a]		
	serum	liver	Solid tumor
A[b]	<0.1	<0.1	<0.1
Poly(MA-*alt*-VA)	0.5	0.1	1.2
Conjugate I	0.5	0.1	1.9
Conjugate II	0.4	<0.1	1.6
Conjugate III	0.6	<0.1	2.1

[a] Determined 8h after administration

[b] Tri(β-chloroethyl)amine

and the ratio between the hydrophilic and hydrophobic parts of the polymer structure [32]. In addition good blood and tissue compatibility, and neutral or slightly negative electric charge appears to be optimal characteristics of the macromolecular support since polycationic polymers are rapidely captured by the first pass effect and also during circulation [33]. The reason for this is that the endothelial surfaces of the blood vessels are covered as chondroitin sulfate, heparin sulfate, and glycocalyx. The presence of bulky and hydrophobic side groups, such as the p-sulfonated anilide substituents in conjugates I and III enhanced the EPR effect more than conjugate II which did not have this substituent in its structure (table 3).

Conclusions

The data obtained for the synthesized conjugates show antitumor effects that are dependent on the macromolecular structure. The *in vivo* evaluation of the synthesized conjugates shows that the presence of the long spacer containing the phenyl group in the structure of conjugates in this study enhanced the antitumor effect. In addition, the accumulation on the studied macromolecules in the solid tumors is explained by the presence of the EPR effect.

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