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Antitumor Activity of Polycarboxylic Acid Polymers

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ABSTRACT

We have synthesized several polyanionic polymers for biological evaluation and to probe the mechanism of macrophage activation. We have determined that, while the molecular weight of the polymers is toxicologically important, the hydrophlicity, chain rigidity, and surface charge play a significant role with respect to the degree of macrophage activation. Macrophages elicited with polyanionic polymers with hydrophobic groups were cytotoxic to Lewis lung carcinoma both in vitro and in vivo. However, macrophages stimulated by polymers with high carboxylic acid group density but little hydrophobicity did not demonstrate significant antitumor activity.

Naturally occurring polyanionic polymers are known to possess innate physiological properties. These natural macromolecules include polysaccharides, glycoproteins, and polynucleotides. The polyanionic functionality of these macromolecules are produced by sulfonate ($-SO_3^-$), phosphate ($-PO_4^-$), and carboxylate ($-COO^-$) groups. Several synthetic polyanions have become of interest to investigators for comparison of their physiological properties with those of naturally oc-

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curring polyanions. For example, polysulfonated polymers have been synthesized and compared to heparin and heparinoid molecules as anticoagulant agents. Recently, the evaluation of polycarboxylic acid macromolecules have become of interest for biological applications. These agents have been found by oncologists and virologists to produce prolonged protection against a number of diseases. Their possible clinical potential has established an impetus for assaying the fundamental role of polyanions in controlling host resistance to a variety of pathophysiology.

Synthetic polycarboxylic acid polymers were found to produce a broad spectrum of immunological effects [1]. They induce the production of interferon [2], modify the reticuloendothedial system [3], and display immunoadjuvant [4], antiviral [5], and antitumor activity [6].

The synthetic carboxylic acid polymers first investigated were poly(acrylic acid), poly(methacrylic acid), poly(ethylene-co-maleic anhydride), and oxidized polysaccharides. Subsequently, the copolymer of divinyl ether and maleic anhydride has shown significant biological activity [1]. Our interest has been to prepare a number of copolymers of maleic anhydride to study the effect of the molecular weight, structure, hydrophobicity, and hydrophilicity in relation to toxicity and antitumor activity.

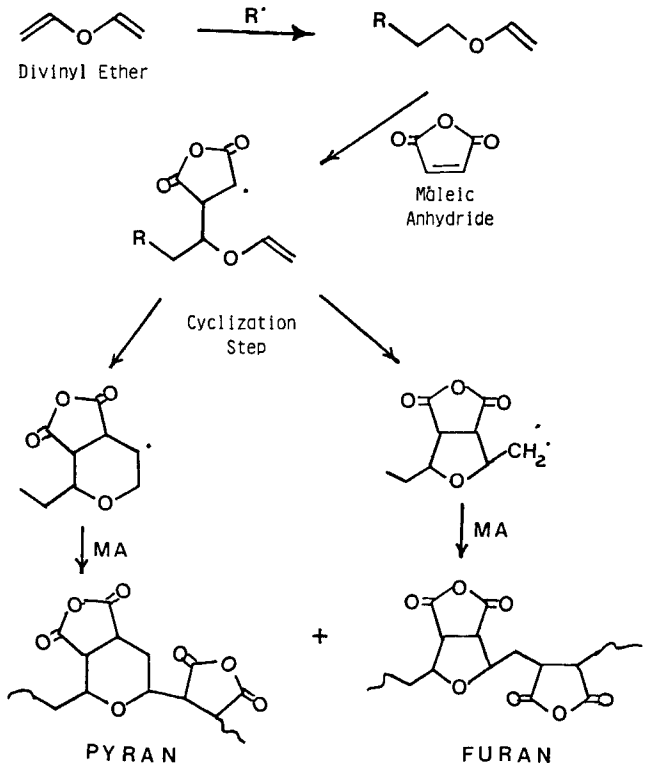
MALEIC ANHYDRIDE-DIVINYL ETHER COPOLYMER (MVE)

The copolymer obtained for maleic anhydride and divinyl ether is the most extensively investigated synthetic polycarboxylic acid polymer for biological activity [1]. In the literature it is referred to as pyran copolymer (due to its unique structure) and by the acronyms DIVEMA and MVE. The synthesis of MVE involves the combination of maleic anhydride and divinyl ether in a 2:1 ratio. The propagation occurs by means of a cyclopolymerization process, described by Butler [7], that can lead to the production of a 6-membered pyran ring in the polymer chain or a 5-membered furan ring as depicted in Scheme 1. Present studies indicate that there is approximately a 50/50 ratio of the two ring structures in the polymer chain [8].

MVE was designated by the National Cancer Institute as NSC 46015, and investigations showed that it exhibited a wide range of biological activities. It has antibacterial [9] and antifungal activity [10]; it stimulates immune response [11] and is an anticoagulant [12]. Most importantly, it has significant antitumor activity [13] and antiviral activity [14].

Originally, MVE, NSC 46015, was clinically too toxic in human patients for studies beyond phase 1. In animals, toxicity was manifested by enlarged livers and spleens, inhibition of microsomal enzymes, and sensitization to bacterial endotoxins.

A number of investigations have clearly demonstrated that the



SCHEME 1. Mechanism for pyran synthesis.

molecular weight of MVE is directly related to its biological activity and toxicity. Breslow [15] showed that the acute toxicity in mice increased with increasing molecular weight of the polymer. Kaplan [16] later reported that a low molecular weight sample of MVE with narrow polydispersity was less toxic and retained its activity against Lewis lung carcinoma. If, however, the molecular weight of MVE was below 5000, the drug was not only less toxic but ineffective biologically.

We confirmed and elaborated these findings by evaluating two low molecular weight fractions isolated from a broad molecular weight MVE polymer XA124-177 obtained from Hercules Co. These two fractions PM-10 (intrinsic viscisity 0.05) and PM-30 (intrinsic viscisity 0.08) were considerably lower in molecular size compared to parent material XA124-177 (intrinsic viscisity 0.021). The toxicologic, antitumor, and antiviral properties were investigated, and the results are listed in Table 1. As expected, the higher molecular weight parent pyran XA124-177 caused hepatosplenomegaly, swelling of the liver and the spleen, sensitization to endotoxin, and inhibition of microso-

TABLE 1. Pharmacologic Effects and MVE Molecular Weight

	Control	Pyran XA124-177	PM-10	PM-30
Intrinsic viscosity	-	0.21	0.05	0.06
Toxicologic properties:				
LD_{50}		74.0	120.0	115.0
Liver weight	5.4	7.8	5.1	5.9
Spleen weight	0.4	1.1	0.4	0.4
LD_{50} (endotoxin)	25.0	0.1	15.0	15.0
Hexobarbital sleeping time (min)	36.8	97.6	42.8	48.6
Antitumor and antiviral properties:				
Antitumor activity				
Lewis lung % inhibition	0.0	76.0	69.0	64.0
Antiviral (EMC) % protection	0.0	89.0	0.0	30.0

mal enzymes as measured by increased hexobarbitol sleeping times. However, the lower molecular weight PM-10 and PM-30 fractions showed much higher LD_{50} (lower acute toxicity) values than the parent MVE polymer, caused less hepatosplenomegaly, and did not significantly sensitize the animals to bacterial endotoxin. Inhibition of microsomal enzymes, measured by the hexobarbitol sleeping time, became almost insignificant. The activity against Lewis lung carcinoma for both fractions remained high and was comparable to the parent polymer. However, the antiviral activity against encephalomyocarditis virus was greatly decreased; the lowest molecular weight (PM 10) fraction showed no antiviral activity and the PM 30 fraction was only 30% effective compared to the whole polymer. This was not unexpected since antiviral activity with other agents was only experienced with large molecules (MW >50,000).

We investigated the effect of molecular weight and structure of several polyanionic polymers on biological activity [1,2]. Different molecular weight fractions of MVE, poly(acrylic acid-alt-maleic acid) (PAAMA), poly(maleic acid) (PMA), and poly(acrylic acid-co-3,6-endoxo-1,2,3,6-tetrahydrophthalic acid) (BCEP) were prepared. These were evaluated for activity against Lewis lung carcinoma and encephalomyocarditis virus (Table 2). Except for BCEP which inhibited tumor growth by only 30%, the other three polymers reduced tumor growth by approximately 75%. However, it was shown that inhibition of tumor growth did not necessarily lead to a concomitant increased life span (ILS). The greatest ILS was obtained with MVE copolymer (40%) and the least ILS was obtained with BCEP (10%). Therefore, it appears that the polymeric structure has a significant effect on biological activity. Furthermore, it was observed that all the higher molecular weight fractions (>30,000) showed antiviral activity whereas the lower molecular weight fractions were ineffective.

Acute toxicity studies indicate a significant increase in mortality with increase in molecular size of the polymers studied (Table 3). Endotoxin sensitization showed a similar dependence on molecular weight, particularly in the case of the more antitumor active polymers such as MVE and PAAMA.

Recently, Munson [17] reported that a mixture of 5-7% calcium and 93-95% sodium salts of MVE was much less toxic than the pure sodium salt. While the two salts showed differences in acute toxicity, little difference between the two salts was seen in the sensitization to bacterial endotoxin. It was also demonstrated in this study that toxicity and sensitization to bacterial endotoxin were molecular weight dependent.

TABLE 2. Antitumor and Antiviral Activity with Polymer Molecular Weight

Polymer[a]	MVE, %	PAAMA, %	PMA, %	BCEP, %
Inhibition of tumor size:				
Whole polymer	78	74	75	30
(Increased life span)	(40)	(33)	(15)	(<10)
1,000-10,000	89	-	74	22
10,000-30,000	84	80	72	-
30,000-50,000	74	-	76	-
50,000-100,000	70	78	74	30
Antiviral encephalomyocarditis protection:				
Whole polymer	89	90	30	25
1,000-10,000	0	0	0	0
10,000-30,000	30	29	0	-
30,000-50,000	58	54	<10	-
50,000-100,000	86	80	26	38

[a]Poly(divinylether-co-maleic anhydride) (MVE), poly(acrylic acid-co-maleic anhydride) (PAAMA), poly(maleic anhydride) (MA), poly(acrylic acid-co-3,6-endoxo-1,2,3,6-tetrahydrophthalic anhydride) (BCEP).

TABLE 3. Toxicologic Effects and Polymer Molecular Weight

Polymer	Pyran	PAAMA	PMA	BCEP
Polymer molecular weight and acute toxicity (LD_{50}):				
Whole polymer	74	110	120	150
1,000-10,000	120	-	160	>200
10,000-30,000	115	>200	150	-
30,000-50,000	95	-	140	-
50,000-100,000	84	180	135	180
Polymer molecular weight and endotoxin sensitization:				
Whole polymer	0.12	1.0	15	3.0
1,000-10,000	15	-	>20	>10
10,000-30,000	15	-	>20	>10
30,000-50,000	-	>20	>20	>3
50,000-100,000	-	>20	>20	>3

MACROPHAGE ACTIVATION BY CARBOXYLIC ACID POLYMERS

Macrophages may be "activated" in vivo to become cytotoxic to tumor cells in vitro. Activation is accomplished by stimulation of peritoneal macrophage by agents such as the bacteria C. parvum and Bacillus calmuette guerin (BCG). Normal peritoneal macrophages and those stimulated with most inflammatory agents are not cytotoxic. Recently we have been able to activate macrophages to tumoricidal capacity with several polyanionic copolymers [3] such as MVE (Fig. 1). A common finding among all these activated macrophage populations is that they are cytotoxic or cytostatic for tumor cells such as Lewis lung and Ehrlich ascites while they have no apparent effect on normal cell populations such as newborn mouse fibroblasts and fetal mouse fibroblasts. Therefore, polyanion activated macrophages possess the unique capability of discriminating between a normal cell and a tumor cell.

At the present time the basis for this discrimination is unknown, but it must be assumed that the activated macrophage can recognize a feature of a tumor cell that is inconsistent with that found on a

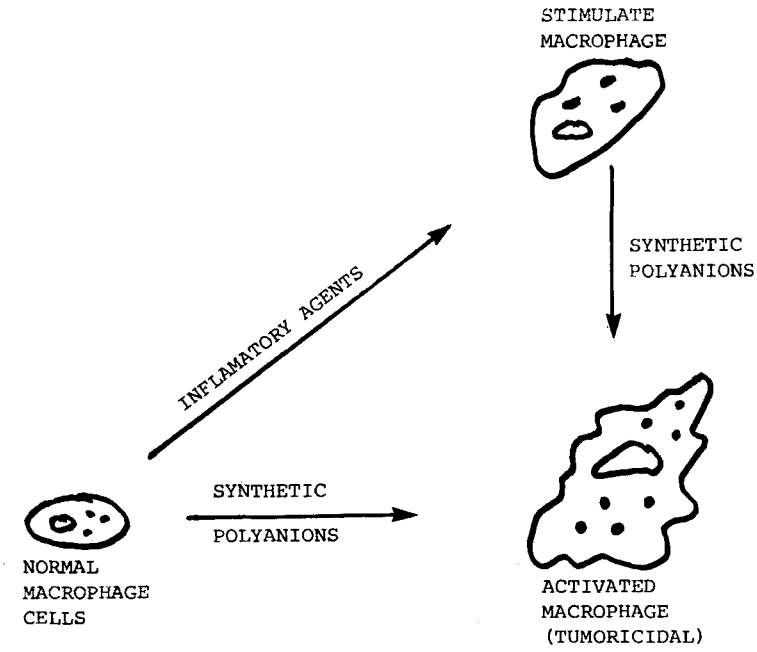


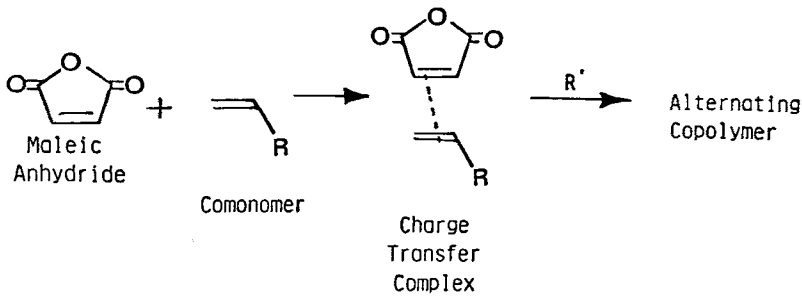
FIG. 1. Macrophage cell activation to tumoricidal activity.

normal cell. For some time now researchers have been trying to delineate singular differences between normal and tumor cells in a given system. Tumor cells have been found to exhibit differences in lecithin binding and agglutination, membrane microviscosity, enzyme activity, and cytochemical structure when compared to normal cells [18]. It appears, however, that in every instance these apparent differences could be duplicated in completely normal cells under the appropriate conditions. At the present time the mechanism by which activated macrophages recognize tumor cells remains to be elucidated. However, the consistency of the phenomenon and the potential value in the management of cancer renders it unique and is undergoing further study.

POLYMER STRUCTURAL EFFECTS ON MACROPHAGE ACTIVATION

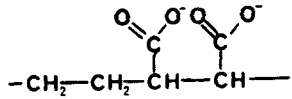
From earlier investigations it was ascertained that the features that seemed to play an important role in enhancing the antitumor activity of polymers were 1) a high carboxylic acid density along the polymer chain and 2) a rigid polymer chain and a lower molecular weight polymer with narrow polydispersity. Based on these facts, we investigated a series of copolymers of maleic anhydride.

Maleic anhydride is a unique monomer system in that it is an electron acceptor and forms charge-transfer complexes with many other monomer systems. On polymerization, the complexes form alternating copolymers [19]. This feature is essential if one is to consistently prepare a copolymer with repeating units of known structure (Scheme 2). By carefully selecting monomers, one can vary the lipophilicity and polar character of the resultant polymer. Consequently, maleic anhydride can afford copolymers of known structure and high carboxylic acid density with varying lipophilicity depending on the comonomer.

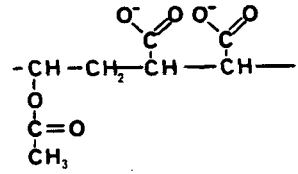


SCHEME 2. Maleic anhydride has a propensity to form alternating copolymers. This is due to the formation of a charge transfer intermediate.

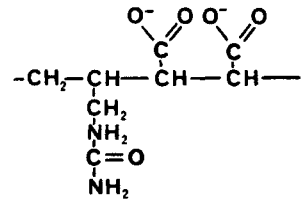
ETHYLENE-
MALEIC ANHYDRIDE
COPOLYMER (E-MA)



VINYL ACETATE-
MALEIC ANHYDRIDE
COPOLYMER (MAVA)



ALLYL-UREA-
MALEIC ANHYDRIDE
COPOLYMER (AV-MA)



4-METHYL-2-
PENTENONYL-
MALEIC ANHYDRIDE
COPOLYMER (MP-MA)

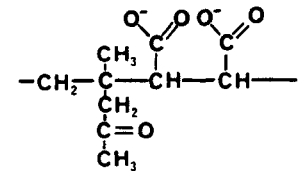
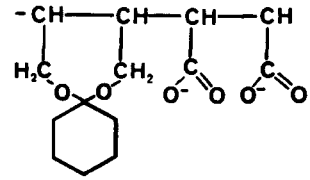


FIG. 2. Hydrophylic-type polymers evaluated for macrophage activation.

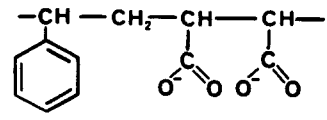
The molecular weight of polyelectrolyte polymers can be controlled by using conventional polymerization techniques such as: a) altering monomer concentrations, b) variation of initiator concentration, c) changing the temperature of the reaction mixture, d) adding chain transfer agents or inhibitors, and e) choice of solvent. Fractionation of the polymers was carried out with an Amicon Ultrafiltration apparatus. Molecular weight fractions from 1,000 to 10,000 and 10,000 to 30,000 were obtained by using appropriate filters. The polymer fractions were recovered by freeze-drying the filtrates.

We prepared and evaluated a series of polyanionic compounds (Figs. 2 and 3) which differ in molecular weight, structure, lipophilicity, chain rigidity, and surface charge for their ability to induce macrophages to a tumoricidal state of activation. There are generally three levels of activation attributed to peritoneal macrophage: 1) normal/resident/unstimulated, 2) inflammatory/stimulated–these cells are elicited with inflammatory agents, and 3) activated/tumoricidal–

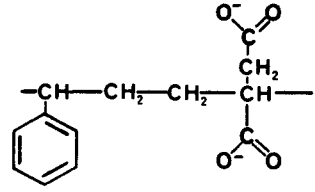
CYCLOHEXYL-1,3-
DIOXEPIN-
MALEIC ANHYDRIDE
COPOLYMER (CDA-MA)



STYRENE-
MALEIC ANHYDRIDE
COPOLYMER (LS-MA)



ITACONIC ACID-
STYRENE-
COPOLYMER (IAS)



α-ETHACRYLIC
ACID
HOMOPOLYMER (EA)

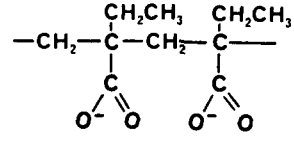


FIG. 3. Hydrophobic-type polymers evaluated for macrophage activation.

these cells are elicited with agents such as C. parvum or synthetic polyionic polymers (Fig. 1). These different states of macrophage activation were distinguished by specific morphological, functional, and biochemical criteria [20]. In order to determine the level of activation which was induced by our test polymers, we evaluated peritoneal exudate cells (PEC) which were activated by intraperitoneal injection of test polymers for their ability to kill Lewis lung tumor cells. In addition, we performed ectoenzyme analysis on lysates of these cell populations. The ectoenzyme profile consisted of 5'-nucleotidase, a marker enzyme for resident/unstimulated macrophages; alkaline phosphodiesterase, a marker enzyme for thioglycollate/inflammatory macrophages; and leucine aminopeptidase, a marker enzyme for activated/tumoricidal macrophages [21]. These

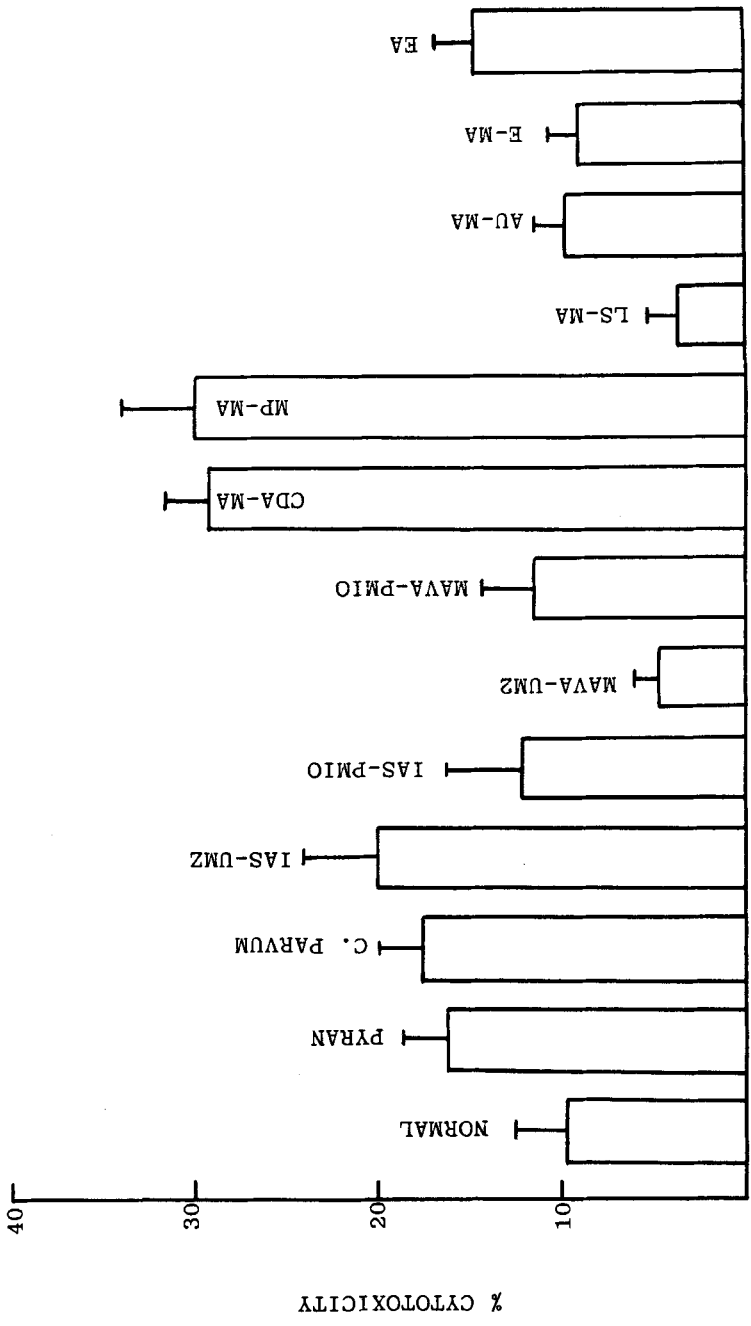


FIG. 4. Cytotoxicity of polycarboxylic acid polymer activated macrophages against Lewis lung tumor cells. E:T ratio = 20:1.

tests indicate that a new population of macrophages are being stimulated rather than the resident population in the case of MVE and C. parvum.

The ability of macrophages stimulated with test polymers to become activated to tumoricidal capacity was evaluated by ^{3}H release and morphological assays. Results from the ^{3}H release experiments are shown in Fig. 4. In general, a good correlation was obtained for the two assays of the test polymers. The polymers poly(itaconic acid-alt-styrene) (IAS), poly(cyclohexyl-1,3-dioxepin-co-maleic anhydride) (CDA-MA), and poly(4-methyl-2-pentenone-co-maleic anhydride) (MP-MA) demonstrated the greatest capacity for induction of tumoricidal macrophages in both assays. Each of these polymers possess a lipophilic group, enhanced surface charge properties, and chain rigidity.

Survival studies performed in mice which had received Lewis lung cells subcutaneously indicate that CDA-MA and MP-MA are the most effective antitumor agents in vivo. CDA-MA enhanced survival at all dosages and MP-MA was most effective at 100 mg/kg. Mice which received either of these agents did not exhibit any overt toxic effects. IAS administration results in a reversal of the dose response. This is probably due to direct toxic effects of the compound. Only 2 out of 5 mice which received 100 mg/kg of IAS survived 3 days after administration. Poly(styrene-co-maleic anhydride) was ineffective in prolonging the survival time of tumor innoculated mice. CDA-MA (50 and 100 mg/kg) and MP-MA (100 mg/kg) were more effective in inhibiting tumor growth than MVE or C. parvum at previously determined optimal doses.

These data point to basic differences between the cellular response and pharmacologic efficacy between the structurally modified anionic polymers and MVE. These data give precedence for further synthesis and evaluation of other polyanionic polymers.

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