Journal of Inclusion Phenomena and Macrocyclic Chemistry (2005) 53:191–196
DOI 10.1007/s10847-005-2021-z

Original Research Article

On the Mechanism of Action of Dibenzo-18-crown-6 Diacyl-Derivatives on Malignant Tumors

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(Received: 3 November 2004; in final form: 9 February 2005)

Key words: carcinosarcoma Walker-256, crown ether, sarcoma-45

Abstract

Ca^{2+} permeable channels have been registered in bilayer membranes formed from phospholipids of sarcoma-45 and sarcocarcinoma Walker-256. On the basis of data obtained, it is supposed that the channels are proteins located in the plasmalemmal membrane of tumor cells. Diacetyl-dibenzo-18-crown-6 was found to suppress Ca^{2+} currents through the channels. In *in vivo* experiments diacetyl-dibenzo-18-crown-6 inhibited the sarcoma-45 and carcinosarcoma Walker-256 tumors growth, whereas divaleril-dibenzo-18-crown-6, which is known to be a Ca^{2+} ionophore, acted as a growth-stimulating agent. The mechanism of the tumor growth inhibition by diacetyl-dibenzo-18-crown-6 is supposed to consist in cytostatic action of the compound, presumably, by blocking Ca^{2+} channels of tumor cells.

Introduction

Today's biology seems to have no more complex problem than understanding the causes that underlie the process of cancer transformation of a cell. It is all received that ''transformed cell'' concept can be defined using a number of features and properties, any of which can be habitual to a normal cell. One of such a feature is the high Ca^{2+} permeability of the cancer cells plasma membranes. Ca^{2+} ions are cellular function regulators: they mediate action of ''second messengers'': cAMP, diacyl glycerol, inositol tri-phosphate and many others. Besides that, Ca^{2+} influences the membrane permeability and resting potential. Many authors showed high permeability of the plasma membranes of malignant tumor cells [1–3]. It was shown that ionophore A23187 failed to accelerate the Ca^{2+} accumulation rate and total Ca^{2+} in tumor cells [4]. Thus, Ca^{2+} uptake in transformed cells goes at maximal rate. All physiological functions, which are switched on by Ca^{2+} entry seem to be completely activated. The anomaly high requirements in tumor cells of Ca^{2+} correlates with data obtained from investigations of the cells' metabolism [5] (higher permeability of plasma membranes for Ca^{2+}, high Ca^{2+} capacitance of mitochondria, tolerance of oxidative phosphorylation to the high Ca^{2+}), as well as with phenomenology of cancer on the level of the whole organism (hypercalcemia) [6–8]. Thus, investigation of mechanisms of Ca^{2+} transport in tumor cells is of great theoretical and practical interest which results from pressing needs of medicine giving rise to searches for new anticancer agents either natural or synthetic.

Crown compounds are being applied to diagnostics of human cancer and intensive investigations are being carried out aimed at obtaining therapeutic agents [9, 10]. The crown ethers' enormous potential that results from their unique physical and chemical properties do not let one have any doubts of prospective and successful development of this field.

Experimental

Planar lipid bilayer membranes (BLM) were formed [11] on the hole (d = 200 μm) in the wall of a two-chamber Teflon cell. For measurements of single channel characteristics, the voltage clamp method was used. Single channel currents were registered using a current-to-voltage converter. Visualization of currents was made by means of a X-Y recorder.

Extraction of phospholipids from tumor tissue was carried out as follows: after removing, tumor material was put into an ice-cold medium containing (in mM) 150 KCl, 10 Tris-HCl, pH 7.4. All the further

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operations were conducted at 0 °C. Tumor tissue was homogenized, phospholipids were extracted as [12]. Chloroform–methanol mixture was added to the homogenate so that the chloroform–methanol–water ratio of 1:2:0.8 was obtained. The extract was diluted with 1 volume of water and 1 volume of chloroform. Then the chloroform layer containing phospholipids was evaporated at 30 °C. Phospholipid extraction was conducted twice. Phospholipids of the obtained summary fraction of tumor tissue lipids were precipitated with cold acetone (−30 °C) as described in [13].

In order to obtain phospholipids enriched by membrane components of a given cell organelle, tumor material was fractionated by means of separative centrifugation. Freshly removed tumor material was homogenized in 10 volumes of a medium, containing (in mM) 300 saccharose, 10 Tris-HCl, pH 7.2 and then fractionated. All fractions were reprecipitated in media containing (in mM) 120 KCl, 5 Tris-HCl, pH 7.2 at appropriate magnitude of the separation factor. All operations were conducted at −4 °C. Phospholipids of the obtained fractions enriched by corresponding organelles were extracted as described above.

Diacyl-derivatives of dibenzo-18-crown-6 (Figure 1) were synthesized as [14] and kindly given by A.K. Tashmukhamedova (National University of Uzbekistan). The crown ethers being hydrophobic, their liposomal preparations were used. Crown ethers were dissolved in a small volume of chloroform followed by addition of egg lecithin obtained as [15]. Chloroform having been evaporated, physiological solution was added to the phospholipid residue (0.25–10 mg phospholipids). The suspension of multi-lamellar liposomes was sounded at 44 kHz and maximal resonance for 10 min at 0 °C. Unloaded liposomes (without crown ethers) for control groups of animals were prepared analogically.

Experiments were conducted on wild-type male rats weighed 120–160 g. Sarcoma-45 (S-45) and carcinosarcoma Walker-256 (W-256) were grafted hypodermally on hip as [16]. Experiments with sarcoma-45 were carried out comprised of three series (with 10–12 animals in a series): in I and II series, liposomal preparations of crown ethers and unloaded liposomes were applied intratumorally, whereas in III – intraperitoneally. The regimes and doses used are shown in Table 1. On finishing the experiment animals were killed and tumors were weighed.

One series of experiment was conducted on W-256 (36 animals). The animals were chosen whose tumor linear sizes were 17.0 ± 4.0 mm on 7th day after the tumor grafting. The preparation was applied intratumorally. The regimes and doses used are shown in Table 2. In the course of experiments, the tumor growth was assessed. All experiments were continued until the natural death of all animals occurred.

Results and discussion

Ca^{2+} conductance of sarcoma-45 and carcinosarcoma Walker-256 BLM

In order to ascertain the causes of abnormally high Ca^{2+} permeability of malignant tumors, we investigated Ca^{2+} conductance of BLM made from phospholipids of S-45 and W-256.

BLM composed of S-45 phospholipids were different in electric properties as compared with those from normal cells' phospholipids. In the presence of Ca^{2+} in membrane bathing solutions, step-wise changes of transmembrane current were observed [17]. As seen in Figure 2 (upper traces), at the same voltage clamped and all the rest experimental conditions being identical, the number of opened channels varied, however the levels of maximal integral membrane conductions were constant. The changes of clamped voltage led to a decrease in number of open channels and, as a result, to a reduction of integral conductance. The pronounced voltage dependence of the channel functioning was experimentally observed. Figure 3b illustrates current–voltage curves of BLM measured at different Ca^{2+} concentrations. It is seen that the current–voltage characteristics are linear in the used Ca^{2+} concentrations range. Formation of channels was observed at a concentration of 1×10^{-3} M. An increase in Ca^{2+} up to 5×10^{-3} M led to widening of the channels amplitude

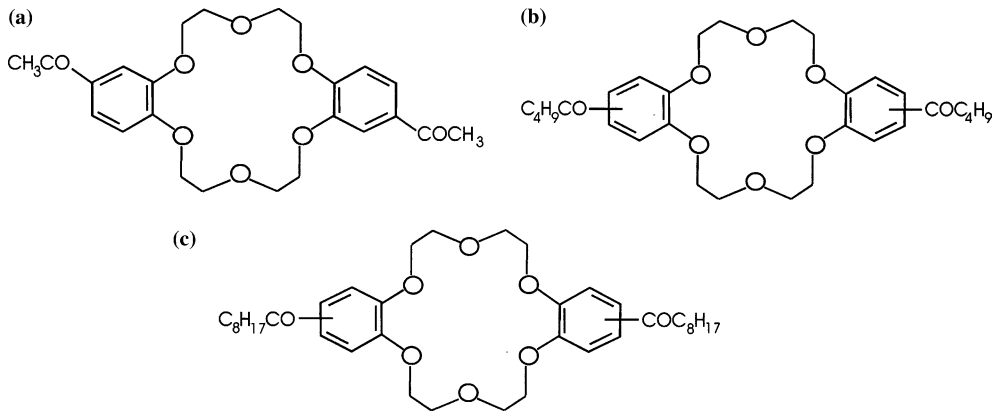

Figure 1. Diacyl-derivatives of DB18C6: 4',5''-diacetyl-DB18C6 **(a),** 4',4''(5'')-divaleryl-DB18C6 **(b),** 4',4''(5'')-dinonanoyl-DB18C6 **(c)**.

Table 1. Effects of diacyl-derivatives of DB18C6 on the growth rate of sarcoma-45

Series	Group	Regime and dose of application	Duration of experiment, days	Amount of animals died*, %	Average weight of tumor	Inhibition of the tumor growth rate	P %
I Intratumoral application	1	Liposomal preparation of Diacetyl-DB18C6 on 1, 5, 9, 22 days after the tumor grafting. The summary dose is 40 mg/kg	27	0	1.25 ± 0.64	87	99
	2	Divaleryl-DB18C6 in the same regimes and doses	27	0	16.18 ± 9.22	+75	99
	3	Dinonanoyl-DB18C6 in the same regimes and doses	27	0	9.79 ± 1.10	0	99
	4	Control. Unloaded liposomes in the same regime. The summary dose of phospholipids is 400 mg/kg	27	0	9.62 ± 3.17	–	–
II Intratumoral application	5	Liposomal preparation of Diacetyl-DB18C6 on 12, 13, 15 days after the tumor grafting. The summary dose is 10 mg/kg	35	20	18.30 ± 6.35	28	99
	6	Control. Unloaded liposomes in the same regime. The summary dose of phospholipids is 100 mg/kg	35	40	25.50 ± 11.92	–	–
III Intraperitoneal application	7	Liposomal preparation of Diacetyl-DB18C6 on 12, 13, 15 days after the tumor grafting. The summary dose is 10 mg/kg	18	0	6.86 ± 2.07	0	–
	8	Control. Unloaded liposomes in the same regime. The summary dose of phospholipids is 3500 mg/kg	18	0	5.56 ± 2.09	–	–

*Animals having died by the day of the tumor weighing.

Table 2. The life duration of rats with carcinosarcoma Walker-256 on intratumoral application of diacetyl-DB18C6 liposomal preparations

Group	Regime and dose of application	Average diameter of the tumor to 13th day after the grafting	Beginning of the animals deaths, days after the grafting	The death of the last animal, days after the grafting	Average life duration, days
1	Liposomal preparation of Diacetyl-DB18C6 on 10–14 days after the tumor grafting. The summary dose is 350 mg/kg	35.2 ± 2.2	15	31	21.0 ± 6.5
2	Control. Unloaded liposomes in the same regime. The summary dose of phospholipids is 3500 mg/kg	36.0 ± 5.1	16	24	18.0 ± 4.0
3	Control. Intact animals	37.0 ± 0.3	16	22	19.0 ± 4.5

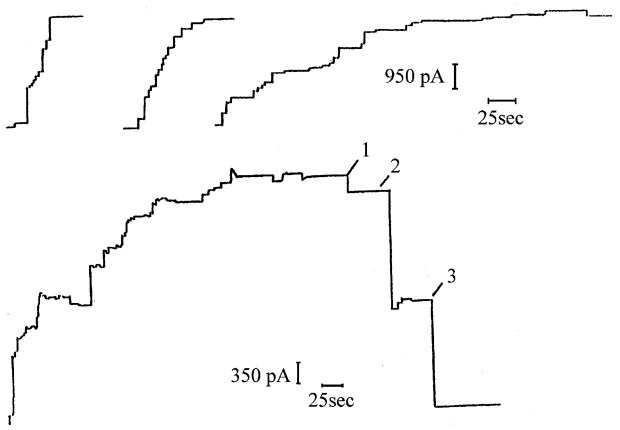

Figure 2. Recordings of Ca^{2+} currents through BLM composed from S-45 phospholipids (upper traces). Blockage of Ca^{2+} currents though S-45 BLM caused by application of diacetyl-DB18C6 in concentrations (in mM) 5×10^{-6} (1), 2×10^{-5} (2), 2×10^{-4} (3) (lower traces). Bath solutions contained CaCl 3×10^{-3} mM, Tris-HCl 25 mM, pH 7.4, $U_{clamped} = 50$ mV.

diversity. Two main channel types depending on their conductance were registered: those multiple to 35 pA and 90 pA. Besides that, step-like changes of trans-membrane currents being not multiple to those of the main types were rarely observed (Fig 3a). A shift of pH from pH 7.4 to pH 8.6 caused disappearance of 90 pA multiple channels. The presence of both the channel types under conditions when Ca^{2+} is changed and constant pH, as well as independence of current–voltage characteristics from Ca^{2+} changes suggest that the observed disappearance of one channel type is only connected with the alkalization.

It was found that diacetyl-dibenzo-18-crown-6 (DB18C6) ("trans-isomer"), when applied symmetrically to both sides of BLM at a concentration of 2×10^{-4} M caused complete suppression of Ca^{2+} currents through channels (Figure 2, lower traces). When one-sidedly administered, the compound did not cause the suppression at negative voltages. Reversion or an increase in voltage resulted in block of channels. Such voltage-dependence of the blocking suggests positively charged Ca^{2+} -crown ether complexes participating in this process. Seemingly, the "cork" type of blocking is realized as channels with minimal conductance first become blocked, which does not let any higher conducting structures to assemble and, as a result, the full block of Ca^{2+} channel currents occurs. It is unlikely that a complex of diacetyl-DB18C6 with Ca^{2+} having Stocks radius of 6.3 Å [15] can block channels with higher conductance.

Ca^{2+} permeability of BLM formed from W-256 phospholipids was different from normal cells formed BLM. Two-sided application of Ca^{2+} (4×10^{-3} M) into bath solutions caused step-like changes of trans-membrane currents (Figure 4). The amplitudes of minimal discrete steps were 7 pA. All other current

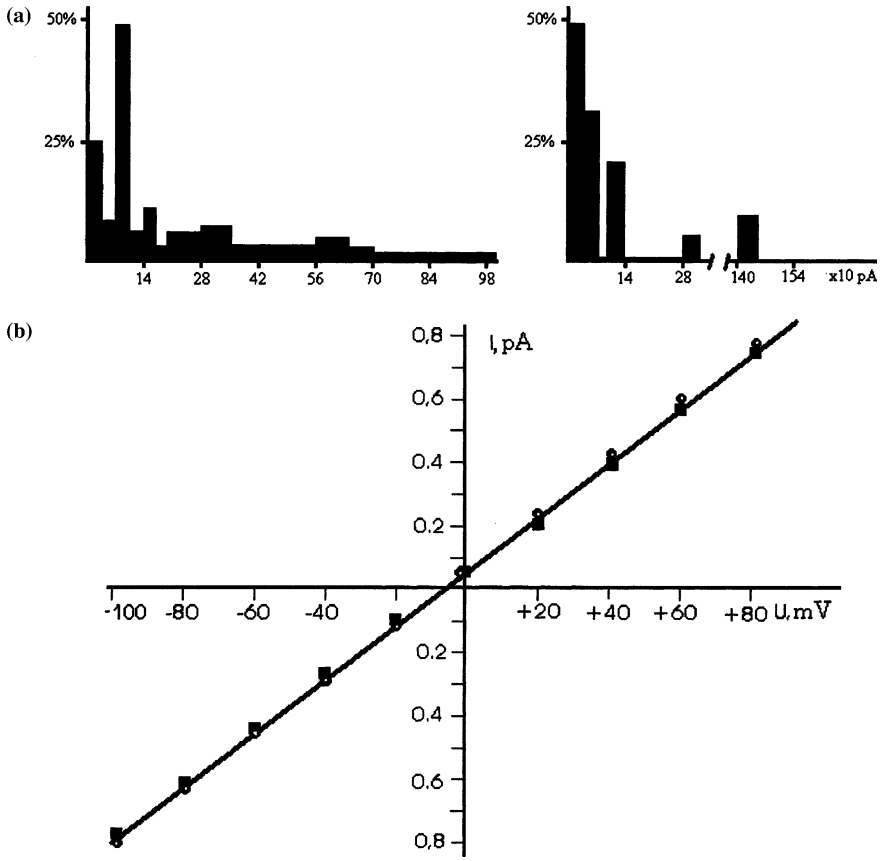

Figure 3. (a) Histograms of current amplitudes of single channels in S-45 BLM registered in different Ca^{2+} concentrations: 1 mM (left) and 5 mM (right). (b) Current-voltage characteristics of S-45 BLM at different Ca^{2+} concentrations: 1 mM (squares) and 5 mM (cycles).

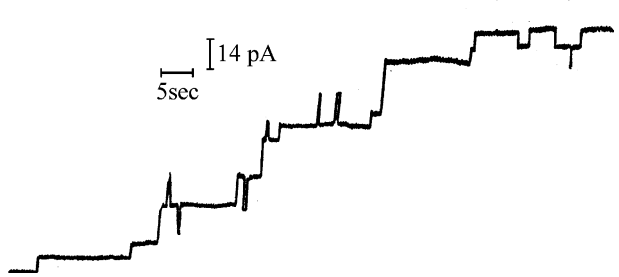

Figure 4. Recordings of Ca^{2+} currents through BLM composed from W-256 phospholipids. Bath solutions contained CaCl 4×10^{-3} mM, Tris-HCl 25 mM, pH 8.6, $U_{clamped} = +50$ mV.

changes were multiple to this magnitude. In the presence of Mg^{2+}, current fluctuations were registered being multiple to minimal discrete current changes that were 14 pA. Ca^{2+}/Mg^{2+} selectivity of BLM calculated from reversion potentials measured under biionic conditions was 4.3. Current–voltage characteristics of BLM were non-linear and showed dependence of conductance on voltage applied. Ca^{2+} and Mg^{2+} currents through BLM were insensitive to ionol being applied at a concentration of 4×10^{-5} M and were suppressed by pronase at concentrations of 15–20 mg/ml. The channels, obviously, do not relate to lipid peroxidation products. Thin layer chromatography of W-256 total phospholipids demonstrated the presence of unidentified ninhydrin- and biuret-positive component (~1% (w/w) phospholipids). Brought together, the data obtained give evidence of the protein nature of the channel-forming structures.

Diacetyl-DB18C6 (“trans”-isomer) when added two-sidedly to bath solutions at a concentration of 2×10^{-4} M suppressed completely the Ca^{2+} channels currents through W-256 composed BLM.

Intracellular location of Ca^{2+} channels in S-45 cells

With the purpose of finding out the intracellular location of Ca^{2+} channels, we obtained phospholipid fractions from nuclei, mitochondria, “light mitochondria” and microsomes. No Ca^{2+} channels similar to those above described were observed in BLM formed from phospholipids of nuclear, mitochondrial, and “light mitochondrial” fractions. But microsomal fraction formed BLM were found to contain Ca^{2+} channels with current amplitude being multiple to 90 pA. Thus, it is obvious that the structures responsible for realization of the channel effect are located in plasma membranes. Although further more accurate definition of the channels topography was not made, their location in the plasma membrane of S-45 cells seems to be very likely.

Our next step was to analyze the phospholipid composition of the obtained fractions using thin layer chromatography. Qualitatively, phospholipids from every separated fraction were identical, with the exception of the particularities, which are characteristic to a given type of organelle. However, chromatographic analyses of the microsomal fraction showed the presence of unknown component on the start. This component gave a positive reaction with ninhydrin, which indicates the presence of amino-groups. Thus, there is all likelihood that this component has a protein nature. An unidentified spot similar to this is present as well on the chromatogram of a phospholipid fraction obtained from the whole tumor mass. It is very possible that the channel properties of BLM from the tumor phospholipids are related to the presence of this unidentified component.

Diacyl-derivatives of dibenzo-18-crown-6 in oncology experiments

The Ca^{2+} channels found in S-45 and W-256 phospholipids fractions can account for the phenomenon of the high permeability of the tumor cells plasma membranes. In this connection, influence of diacetyl-DB186, which was shown to be a Ca^{2+} complexon [18], on a grafted S-45 tumor was assayed. It was of interest as well to find out changes, if any, in the S-45 growth kinetics caused by administration of divaleryl-DB18C6, the compound of the same homology row but possessing Ca^{2+} ionophore properties [19]. In addition, another DB18C6 diacyl-derivative – dinanoyl-DB18C6 was investigated.

In I experimental series (see Table 1, groups 1–4) a comparing study of influence of intratumorally applied crown ethers liposomal preparations upon the S-45 tumor’s growth were conducted. As seen from Table 1, diacetyl-DB186 effectivelly reduced the rate of the tumor growth, whereas divaleryl-DB18C6 being applied in the same doses had a growth-stimulating effect. No effect was found caused by dinanoyl-DB18C6.

An intratumoral application of diacetyl-DB186 liposomal preparations in later periods (series II) also resulted in an inhibition of the tumor growth rate. By 35th day 2-fold decrease in animals’ deaths in experimental group as compared to the control group, was observed. However, when applied intraperitonially in even higher doses (series III), the preparation was completely ineffective.

As seen from Table 1, effectiveness of the tumor growth inhibition caused by diacetyl-DB186 was markedly dependent on terms when the crown ether was administered. Thus, when first applied on the next day after the tumor grafting, the compound caused as much as 86% inhibition of the tumor growth. If applied in later terms (not earlier than on 10th day) it accounted for inhibition extent being not more than 28%. The growth inhibition rate failed to accelerate by increasing total dose of the compound. Seemingly, the mechanism of the anticancer action of diacetyl-DB186 does not consist in killing tumor cells, but inhibition of transformed cells fusion may take place, which does not lower notably their vitality. In this case, the experimentally observed inhibition rates will be in direct dependence on the tumor mass by the moment of the preparation administration and therefore on time between the tumor grafting and the administration.

Divaleril-DB18C6 when applied in the same doses and regimes had a growth-stimulating effect on the tumor. This fact confirms a connection between enhanced intracellular Ca^{2+} concentrations and cell fusion.

Interestingly, dinanoil-DB18C6 which is known as neither possessing significant ionophore properties nor being a blocker of S-45 Ca^{2+} in *in vivo* experiments, had no effect on tumor growth.

The anticancer effect of intratumoral application of diacetyl-DB186 liposomal preparations on W-256 is expressed to a much lesser extent (see Table 2, groups 1–2). No inhibition of the tumor growth is observed and a prolongation of mean life times values are insignificant. The most expressive are changes in the death-rate dynamics within experimental group in comparison with control animals (the differences of the time passed between the deaths of the first animal and the last one).

Analyzing possible mechanisms of the observed effects of diacetyl-DB186 and divaleril-DB18C6, one should, probably, rule out the traditional set for chemotherapy of cancer diseases: alkylation, metabolism competition, inhibition of protein synthesis and so on. The slightest differences in the structures of diacetyl-DB186 and divaleril-DB18C6 caused directly opposite biological effects. It suggested that interactions between the inhibitor (or stimulator) and the cell target which controls proliferation, occurs with high affinity. As diacyl-derivatives DB186 are hydrophobic, the hypothetical target must be localized in the plasma membrane of tumor cells. Similarity or rather identity of chemical properties of the three DB186 diacyl-derivatives makes unlikely any explanations other than direct influence of the compounds on Ca^{2+} permeability of tumor cells, which is a condition of their enhanced proliferation activity.

The inhibition of proliferation by blocking Ca^{2+} channels demonstrated in our experiments is a new, earlier undescribed cytostatic mechanism of biological activity of crown ethers.

Conclusion

Thus, it has been established that in bilayers made from S-45 phospholipids, two types of Ca^{2+} channels are present having current amplitudes being multiple to 35 pA and 90 pA. With alkalization of bath solutions, one channel type (multiple to 90 pA) disappeared. Bilayers from W-256 phospholipids were found to contain Ca^{2+} channels with amplitudes being multiple to 7 pA.

Investigation of properties of BLM made from nuclear, mitochondrial and microsomal phospholipids showed that Ca^{2+} channels are located in plasma membranes of cancer cells.

Thin layer chromatography of S-45 and W-256 liposomal phospholipid fractions showed the presence in them of unidentified component containing aminogroups.

Correlations between membrane activities of the investigated DB186 diacyl-derivatives and their action on S-45 and W-256 in *in vivo* experiments were found. A blocker of Ca^{2+} channels in tumor derived BLM – diacetyl-DB186 caused inhibition of the tumor growth, Ca^{2+} ionophore divaleril-DB18C6 stimulated the tumor growth and dinanoil-DB18C6 had no effect on the growth rate of cancer cells.

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