Evaluation the Potential of Novel Antitumor Candidates on Ehrlich Ascites Tumor-Bearing Mice

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ABSTRACT
In our previous work fused pyrimidine and thiopyrimidine nucleoside analogs (2, 3b, 3c, 4a and 4c) as well as sulfated oligosaccharides (Malto SO₄, Raffinose SO₄, Stachyose SO₄, Chondroitin-6-sulfate, Maltohexaose SO₄) were prepared. The objective of this study was to elucidate the antitumor activity of prepared compounds against Erlich ascites carcinoma cells (EAC) bearing mice through monitoring the tumor volume and life span of the mice using ascites tumor and solid tumor models. The results revealed that all the tested compounds when supplemented at 1/10 of their LD₅₀ (median lethal dose), showing antitumor potential and caused increased in the life span of mice. Otherwise, the antitumor potential in simultaneous treatment groups was higher than the groups in which treatment was started 10 days post tumor inoculation. Compound 3C and Malto SO₄ showed highest activity on reduction tumor volume from 5.30±0.60 CC in control mice to 2.20±0.19 and 2.00±0.18 CC respectively, while the positive drug, doxorubicin treated group revealed reduction of tumor volume from 5.30±0.60 to 1.10±0.14 CC. Otherwise 3C and Malto SO₄ also showed the most highest survival rate (39.00±2.70 and 40.00±3.40 days, respectively) with the increase of life span 77% and 82% respectively compared to control, while doxorubicin showed increased in survival rate by 86% (41.00±4.30 days) as compared to control. It is obviously from the present study that the tested compounds especially 3c and Malto SO₄ possessed antitumor activity and prolong the life span of mice bearing tumor.

Keywords: Antitumor, thiopyrimidines, sulfated oligosaccharides, Erich ascites carcinoma.

INTRODUCTION
There is a need for chemotherapeutic agents for treatment of neoplastic diseases that are safe for therapeutic use and that exhibit selective toxicity with respect to the pathological condition. Furthermore, there is a need for chemotherapeutic agents with modified or improved profiles of activity. The search therefore continues to develop new antitumor agents has been extensively studied. It is well known that, pyrimidine and fused heterocyclic pyrimidine derivatives have attracted a great deal of interest in particular 4-hydrizinopyrimidine derivatives, which were tested for their medicinal, bactericidal and fungicidal activity. Pyrimidine and heterocyclic annulated pyrimidine derivatives attracted great interest due to the wide variety of interesting biological activities observed for these compounds, such as anticancer, antiviral, anti-HIV-1 activity, anti-inflammatory and antimicrobial activity. Sulfated oligosaccharides, such as heparan, heparan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate and dermatan sulfate, are important ingredients of extracellular matrix. Recently, many sulfated oligosaccharides have been extracted from bacteria, plants and animals for studying their effects as anti-tumor agents. Furthermore, some experimental studies suggested that the anti-thrombotic activity play an important role in the antitumor effects of sulfated polysaccharides. Sulfated oligosaccharides could suppress the proliferation and metastasis of tumor cells by the inhibition of tissue factor, thrombin, thrombus formation and platelet aggregation. Sulfated oligosaccharides could suppress the proliferation and metastasis of tumor cells by inhibiting heparanase and directly bind to growth factors to inhibit the growth of tumors.

Owing to the above facts, the aim of the present work is to study the antitumor effect of previously synthesized thiopyrimidine acyclic nucleosides and thioglycoside analogs as well as sulfated oligosaccharides (Malto SO₄, Raffinose SO₄, Stachyose SO₄, Chondroitin-6-sulfate, Maltohexaose SO₄) against Erlich ascites carcinoma cells (EAC) bearing mice through monitoring the tumor volume and life span of the mice.

MATERIALS AND METHODS
Previously prepared fused pyrimidine and thiopyrimidine nucleoside analogs (2, 3b, 3c, 4a and 4c) as well as
sulfated oligosaccharides (Maltose \(SO_4\), Raffinose \(SO_4\), Stachyose \(SO_4\), Chondroitin-6-sulfate, Maltohexaose \(SO_4\)) were used. All chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA).

Animal

Male Swiss albino mice (body weight 20±2 g) were purchased from animal breeding center of National Research Centre, Egypt. They were kept for a week under environmentally controlled conditions (constant temperature 25-27 °C, with 12 h light/dark cycle) for one week prior to starting the experiments, and they were provided with water and commercial diets. All experimental procedures were carried out in accordance with the protocol was approved by the institutional animal care and ethics guidelines of National Research Centre, Egypt.

Cell line

Ehrlich’s ascites carcinoma (EAC) cell line was obtained from National Cancer Institute, Cairo University, Egypt. The cells were maintained by intraperitoneal inoculation of 1x10⁶ viable cells in mice.

Assay of acute toxicity

The acute toxicity of the tested compounds was determined in vivo according to Prieur et al. 15; Ghosh 16. Briefly, adult Swiss albino mice fasted for 12 h were randomly divided into groups of 10 per group. Each group was separately administrated once intraperitoneal (i.p.) in a volume of 1 ml/kg b.w. gradually increasing doses (0-500 µg/kg b.w.) for the fused pyrimidine and thiopyrimidine nucleoside analogs and (0-1500 µg/kg b.w.) for sulfated oligosaccharides. Control animals received the vehicle alone. The fasted mice in both test and control groups were then provided with food and water immediately after the administration. Mortality of the animals was observed up to one month post-treatment. \(LD_{50}\) (the median lethal doses) of each compound was determined (the dose resulted in 50% mortality of the animals).

Antitumor activity

The antitumor activity of the tested compounds was determined using ascites tumor and solid tumor models.

### RESULTS

**Acute toxicity of compounds**

As shown in table 1, the median lethal doses (\(LD_{50}\), the dose resulted in 50% mortality of the animals) of each compound was determined. Fused pyrimidine and thiopyrimidine nucleoside analogs compounds 2, 3b, 3c, 4a and 4c showed marked acute activity. The concentrations required by compounds 2, 3b, 3c, 4a and 4c for 50% mortality of the animals were found to be 132, 108, 80, 120 and 160 µg/kg body weight, respectively.

**Sulfated oligosaccharides (Maltose \(SO_4\), Raffinose \(SO_4\), Stachyose \(SO_4\), Chondroitin-6-sulfate, Maltohexaose \(SO_4\))**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>(LD_{50}) µg/Kg b.w.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>132.00±15.50</td>
</tr>
<tr>
<td>3b</td>
<td>108.00±12.75</td>
</tr>
<tr>
<td>3c</td>
<td>80.00±10.00</td>
</tr>
<tr>
<td>4a</td>
<td>120.00±13.70</td>
</tr>
<tr>
<td>4c</td>
<td>160.00±17.50</td>
</tr>
<tr>
<td>Maltose (SO_4)</td>
<td>600.00±65.75</td>
</tr>
<tr>
<td>Raffinose (SO_4)</td>
<td>1006.00±115.00</td>
</tr>
<tr>
<td>Stachyose (SO_4)</td>
<td>800.00±85.00</td>
</tr>
<tr>
<td>Chondroitin-6-sulfate</td>
<td>1210.00±125.00</td>
</tr>
<tr>
<td>Maltohexaose (SO_4)</td>
<td>680.00±70.00</td>
</tr>
</tbody>
</table>

Values are mean ± SE

**Ascites tumor model**

Animals were divided into groups of 10 animals in each group. All the animals were injected i.p. with 1 x 10⁶ viable EAC cells in PBS (aspirated from 15 day old EAC ascites tumor in mice). After 24 h of tumor inoculation the tested compounds were administered i.p. at dose of 1/10 of their \(LD_{50}\) values and continued for 10 consecutive days. The group administrated with vehicle alone (DMSO) was maintained as control. Doxorubicin (2 mg/kg body weight, i.p., for 10 days) was used as the standard reference drug. The mortality rate was noted in each group and the percent increase in life span (ILS) was calculated as described by Alhuwalia et al. 17; Joy et al. 18.

**Effect of tested compounds when administered simultaneously with tumor inoculation.**

Viable EAC cells (1 x 10⁶) in 0.1 ml PBS were transplanted subcutaneous into the right groin of mice. Tested compounds were administered i.p., at 1/10 of their \(LD_{50}\) values 24 h post tumor implantation and extended for 10 consecutive days. The control group was treated with vehicle (DMSO) and the standard reference group was treated with doxorubicin (2 mg/kg body weight, i.p., 10 days). The tumor development on animals in each group was determined by measuring the diameter of tumor growth in two perpendicular planes using vernier calipers on every fifth day. The tumor volume was calculated by measuring the radii of the tumor at two different planes as mentioned by Ma et al. 19, Mary et al. 20.

**Effect of tested compounds when administered after tumor development.**

Antitumor activity of the compounds was tested after tumor development in mice. Solid tumor development in mice was induced as described earlier. After 13 days of tumor transplantation animals were divided into groups of 10 animals each and were administrated i.p. at 1/10 of their \(LD_{50}\) values for 10 consecutive days. The group treated with vehicle was maintained as control and the standard reference group was treated with doxorubicin (2 mg/kg body weight, i.p., 10 days). Tumor diameter was measured using a vernier caliper on every fifth day and volume was calculated.

**Statistical analysis**

Values are recorded as mean ± S.E. The data were analyzed by Student’s t-test; differences below the 0.5 level (\(P<0.05\)) was considered as statistically significant.
SO₄) showed marked acute activity. The concentrations required by Maltose SO₄, Raffinose SO₄, Stachyose SO₄, Chondroitin-6-sulfate, Maltohexaose SO₄ for 50% mortality of the animals were 600, 1006, 800, 1210 and 680 µg/kg body weight, respectively.

Antitumor activity

Antitumor activity of the tested compounds was determined using ascites tumor and solid tumor models.

Ascites tumor model

As shown in Table 2. In the ascites tumor model the administration of prepared compounds (2, 3b, 3c, 4a and 4c) significantly increase (*P<0.05) the life span of the animals as compared with control group (animals in the EAC injected alone) except compound 4e; which revealed insignificant change. There is a gradual increase in life span using the tested compounds from compound 4c, 2, 4a, 3b to compound 3c. The increases in life span were 9, 32, 45, 64, and 77% respectively. The standard reference drug (doxorubicin 2 mg/kg body weight) exhibited 86% increase life span of the animals. The animals in the EAC control group were dead after 22 days. It is clear from the data that compound 3c was the best compound exerting a significant anticancer activity compared with doxorubicin.

On the other hand, the administration of sulfated oligosaccharide compounds (Maltose SO₄, Raffinose SO₄, Stachyose SO₄, Chondroitin-6-sulfate, Maltohexaose SO₄) significantly increase (*P<0.05) the life span of the animals as compared with control group (animals in the EAC injected alone). There is a gradual increase in life span using the prepared compounds from compound Chondroitin-6-sulfate, Raffinose SO₄, Stachyose SO₄, Maltohexaose SO₄ to compound Maltose SO₄ follows the order Maltose SO₄ > Maltohexaose SO₄ > Stachyose SO₄ > Raffinose SO₄ > Chondroitin-6-sulfate. Maltose SO₄ was the best compound exerting a significant anticancer activity. The increases in life span were 82, 41, 54, 27, and 73% respectively in Maltose SO₄, Raffinose SO₄, Stachyose SO₄, Chondroitin-6-sulfate, Maltohexaose SO₄. Doxorubicin exhibited 86% increase life span of the animals. All the animals in the EAC injected alone group were dead after 22 days (table 2).

Effect of the tested compounds when administered simultaneously with tumor inoculation

All the tested compounds of fused pyrimidine and thiopyrimidine nucleoside analogs or sulfated oligosaccharide possessed significant antitumor activity against solid tumor models. i.p. administration of the of fused pyrimidine and thiopyrimidine nucleoside analogs in simultaneously treated groups showed significant antitumor activities against solid tumor and reduce the tumor volume (Figure 1). Among the tested compounds, 3c show the highest antitumor activity. The tumor volume of animals without treatment on the 30th day after tumor inoculation was found to be 5.30 CC. The tumor volume reduced to 2.20 CC when treated with compound 3c, and to 2.60 CC when treated with compound 3b. While reduced to 4.70, 3.60 and 3.20 CC when treated separately with compound 4c, 2 and 4a respectively.

Table 2: Effect of treatment with fused pyrimidine and thiopyrimidine nucleoside analogs compounds as well as sulfated oligosaccharides on the survival of ascites tumor harboring mice inoculated with EAC cell line.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Survival time (days)</th>
<th>% increase in life span of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.00±1.80</td>
<td>0</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>41.00±4.30*</td>
<td>86</td>
</tr>
<tr>
<td>2</td>
<td>29.00±2.50*</td>
<td>32</td>
</tr>
<tr>
<td>3b</td>
<td>36.00±2.70*</td>
<td>64</td>
</tr>
<tr>
<td>3c</td>
<td>39.00±2.70*</td>
<td>77</td>
</tr>
<tr>
<td>4a</td>
<td>32.00±1.90*</td>
<td>45</td>
</tr>
<tr>
<td>4c</td>
<td>24.00±2.30</td>
<td>9</td>
</tr>
<tr>
<td>Maltose SO₄</td>
<td>40.00±3.40*</td>
<td>82</td>
</tr>
<tr>
<td>Raffinose SO₄</td>
<td>31.00±2.50*</td>
<td>41</td>
</tr>
<tr>
<td>Stachyose SO₄</td>
<td>34.00±3.00*</td>
<td>54</td>
</tr>
<tr>
<td>Chondroitin-6-sulfate</td>
<td>28.00±3.20*</td>
<td>27</td>
</tr>
<tr>
<td>Maltohexaose SO₄</td>
<td>38.00±3.70*</td>
<td>72</td>
</tr>
</tbody>
</table>

Values are mean ± SE.; n = 10 mice; *P<0.05 significant with respect to control.

On the other hand, i.p. administration of the sulfated oligosaccharide in simultaneously treated groups showed significant antitumor activities against solid tumor and reduce the tumor volume (Figure 2). Among the compounds, Maltose SO₄ showed the highest antitumor. The tumor volume of animals without treatment on the 30th day after tumor inoculation was found to be 5.30 CC. The tumor volume reduced to 2.00 CC when treated with Maltose SO₄, and to 2.50 CC when treated with Maltohexaose SO₄. While reduced to 3.30, 2.90 and 4.00 CC when treated separately with Raffinose SO₄, Stachyose SO₄, Chondroitin-6-sulfate respectively.

Effect of tested compounds when administered after tumor development

The fused pyrimidine and thiopyrimidine nucleoside analogs or sulfated oligosaccharides were also highly effective against development solid tumor. The treatment with the fused pyrimidine and thiopyrimidine nucleoside analogs for 10 consecutive days after tumor development showed reduction in the volume tumor (Figure 3). The tumor volume of animals without treatment on the 30th day after tumor inoculation was found to be 6.00 CC. The tumor volume reduced to 2.90 CC when treated with compound 3c, to 3.25 CC when treated with compound 3b, and to 3.50 CC when treated with compound 4a. While reduced to 4.00 and 5.27CC when treated separately with compounds 2 and 4c respectively. From the forgoing results we noticed that administration of fused pyrimidine and thiopyrimidine nucleoside analogs (2, 3b, 3c, 4a and 4c) shows more antitumor activity in simultaneous treated groups than the groups in which treatment was started 10 days after tumor inoculation. The sulfated oligosaccharide compounds were also highly effective against development solid tumor. The treatment with the compounds for 10 consecutive days after tumor development showed reduction in the volume tumor (Figure 4). The tumor volume of animals without
treatment on the 30th day after tumor inoculation was found to be 6.00 CC. The tumor volume reduced to 2.70 CC when treated with Maltose SO₄, to 3.00 CC when treated with Maltohexaose SO₄, and to 3.50 CC when treated with Stachyose SO₄. While reduced to 3.80 and 4.80 CC when treated separately with Raffinose SO₄ and Chondroitin-6-sulfate respectively. From the forgoing results we noticed that administration of sulfated oligosaccharide compounds (Maltose SO₄, Raffinose SO₄, Stachyose SO₄, Chondroitin-6-sulfate, Maltohexaose SO₄) shows more antitumor activity in simultaneous treated groups than the groups in which treatment was started 10 days after tumor inoculation.

DISCUSSION
Cytotoxicity is one of the chemotherapeutic targets of antitumor activity 21. Most of the clinically used antitumor agents possess significant cytotoxic activity in cell culture systems. In our previous work, the in vitro cytotoxic activity study against cancer cell lines revealed that the fused pyrimidine and thiopyrimidine nucleoside analogs were potent cytotoxic agents in the order 3c > 3b > 4a > 2 > 4c 13. Otherwise, sulfated oligosaccharides has shown that the growth inhibitory potency against cell lines follows the order Maltose SO₄ > Maltohexaose SO₄ > Stachyose SO₄ > Raffinose SO₄ > Chondroitin-6-sulfate 14. The higher cytotoxic activity of both tested groups against cell lines partially explains its significant antitumor activity against ascites and solid tumors bearing mice.

In the present study the fused pyrimidine and thiopyrimidine nucleoside analogs as well as sulfated oligosaccharide were examined for their in vivo antitumor action against Erlich ascites carcinoma bearing mice.
through monitoring the tumor volume and life span of the mice. All the investigated compounds exhibit a relevant antitumor activity and showing potency near doxorubicin cytotoxic activity specially compound 3c and Maltose SO₄. The preliminary evaluation of in vivo antitumor activity of tested compounds, in ascetic and solid Ehrlich tumor bearing mice showed noticeable activity near that of doxorubicin in increasing the life span of treated animals; furthermore, in case of solid tumor-bearing mice, the data suggest that the treatment with these compounds resulted in a significant tumor mass reduction in comparison with control animals.

In EAC ascites tumor bearing mice, a regular rapid increase in ascetic tumor volume was observed. Ascetic fluid is the direct nutritional source for tumor cells and increase in ascetic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells. this was completely noticed in the control groups in comparison to the treated groups. So it may be concluded that these synthesized compounds by decreasing the nutritional fluid volume and arresting the tumor growth increases the life span of EAC-bearing mice. Otherwise, the tested compounds significantly reduced the solid tumour development in mice. The tumour reduction was higher in animals treated simultaneously than after tumor development.

Antitumour activity of these compounds is through regulation of free radicals generation with affecting on the proliferation, differentiation and apoptosis of cancer cells. Interestingly the carcinogenesis involves mainly...
three steps namely initiation, promotion and progression. The implication of free radicals in different steps of carcinogenesis is well documented [23,24]. In our earlier studies we found that fused pyrimidine and thiopyrimidine nucleoside analogs as well as sulfated oligosaccharide were possess hepatoprotective effect [13,25]. The free radical hypothesis supported the fact that the regulation of free radicals production can effectively inhibit carcinogenesis.

In conclusion, the present study reveals that the investigated compounds exhibit a relevant antitumor activity comparable to the activity of commonly used anticancer drug, doxorubicin especially compound 3e and Maltose SOs. They may be considered future drug candidates for cancer therapy.

ACKNOWLEDGEMENT
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REFERENCES