ORIGINAL ARTICLE

Novel murine tumour models depend on strain and route of inoculation[1]

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[1]Dedicated to the memory of Professor Feng Liu, PhD, 1955–2014, University of North Carolina at Chapel Hill.
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SUMMARY

This study describes variations in tumour growth patterns which occur when changes in the routes of inoculation and mouse strain are used to introduce tumours into established murine model systems that are known to vary in location and aggression. Intraperitoneal, subcutaneous, intravenous and hydrodynamic inoculations of B16F10 cells were compared among CD-1, C57BL/6 and Balb/c mice. Most surprisingly, allogeneic tumour growth in Balb/c mice after intravenous and hydrodynamic inoculation of B16F10 cells was faster than tumour growth in the syngeneic C57BL/6 mice. These and other variations in the tumour growth patterns described here can help provide the researcher with more experimental control when planning to use the optimal tumour model for any particular study.

Introduction

In vivo testing of cancer treatment is a necessary step in drug development, and employing an effective and relevant tumour model is essential for obtaining convincing results and determining accurate conclusions. For decades, murine models have been the most facile and efficient of the tumour models (Leenders *et al.* 2008). Tumours have been generated *in vivo* through carcinogenic (Imaoka *et al.* 2009; Ramos *et al.* 2012) or viral induction (Lewis *et al.* 2001), through genetic engineering to increase susceptibility to spontaneous tumour formation (Heyer *et al.* 2010; Politi & Pao 2011), and, most commonly, by inoculating mice with cancer cells that have been grown *in vitro* (Tsutsumi *et al.* 2009; Oliva *et al.* 2012).

Intraperitoneal (i.p.), subcutaneous (s.c.), intravenous (i.v.) and hydrodynamic (h.d.) (Liu *et al.* 1999; Zhang *et al.* 1999) injections are all viable routes for inoculation, and each produces a distinct pattern of tumour growth. Hydrodynamic injection is carried out by quickly pushing 1.6 ml (equal to the total blood volume of the mouse) of cells/solution through the tail vein (Liu *et al.* 1999;

Zhang *et al.* 1999). Quickly injecting this large volume distends and allows efficient delivery to tissues, especially the liver. Thus, while the site of injection for both i.v. and h.d. inoculation is the tail vein, the sites of tumour growth are quite distinct (Kang *et al.* 2009; Li *et al.* 2011). In addition, another factor which may affect the fine tuning of murine tumour model growth patterns are differences between the susceptibility between growth patterns which can occur between the mouse strains themselves. For a given route of inoculation, CD-1, C57BL/6 (C57), and Balb/c mice can generate significant differences in tumour growth rates and patterns, but there are few reports where this question specifically has been studied in detail. This report describes how differences in the strain or route of B16F10 murine melanoma inoculation can generate new and distinct tumour models for use in tumour characterization or therapeutic experiments.

Materials and methods

B16F10 murine melanoma cells were used for this study. Cells were maintained in DMEM with penicillin/streptomycin and 10% foetal bovine serum. Female CD-1, C57 and Balb/c mice were purchased from the University of North Carolina's in-house breeding facility.

CD-1, C57 and Balb/c mice were ordered at the same time and were all aged 6 weeks at the time of inoculation. Four different inoculation routes were studied ($n = 5$ for each mouse strain and inoculation route, for a total of 12 different groups). All mice were inoculated on the same day with the same number, passage and population of cells. For i.v. injection, all three strains of mice were inoculated with 2×10^5 B16F10 cells in 200 µl PBS through the tail vein. For h.d. injection, all three strains of mice were inoculated with 2×10^5 B16F10 cells suspended in 1.6 ml of PBS by injecting the entire volume through the tail vein within a few seconds. For s.c. injection, all three strains of mice were inoculated with 2×10^5 B16F10 cells in 100 µl PBS. For i.p. injection, all three strains of mice were inoculated with 2×10^5 B16F10 cells in 200 µl PBS.

All mice were sacrificed 14 days after inoculation to allow quantitative comparisons between strains and routes of inoculation. Organs were fixed in 4% paraformaldehyde, and relevant organs were embedded in paraffin. These organs were sectioned and stained with H&E to visualize regions of tumour growth.

s.c. and i.p. tumour growth was quantified by dissecting and weighing the tumours. Lung tumour growth was measured by counting tumour nodules on each lung. Statistical analysis was performed using Student's *t*-test and Holm–Sidak test to determine the statistical significance.

Ethical approval statement

All work performed on animals was in accordance with and permitted by the University of North Carolina Institutional Animal Care and Use Committee, protocol #14-045.

Results and discussion

Tumour growth rates were compared among CD-1, C57 and Balb/c mice 14 days after inoculation with 2×10^5 B16F10 cells. Five mice from each of the three mouse strains were inoculated via one of four different routes: intravenous (i.v.), hydrodynamic (h.d.), subcutaneous (s.c.) and intraperitoneal (i.p.). When the mice were inoculated i.v., all three strains of mice developed tumours specifically in their lungs, and the rates of tumour growth differed depending on mouse strain. Figure 1 shows that the C57 mice, the syngeneic hosts for B16F10 cells, rapidly developed tumours exclusively in their lungs. This result was expected, as this strain and cell line has long been used as a melanoma lung metastasis model (Lin *et al.* 1998; Peer & Margalit 2004). Unexpectedly, the tumour growth rate in the lungs of Balb/c mice after i.v. inoculation was significantly faster than that in C57 mice. This result was surprising because B16F10 inoculation in Balb/c mice should constitute a less robust allograft model when compared to the syngraft model in C57 mice. In contrast, the outbred and more heterozygous
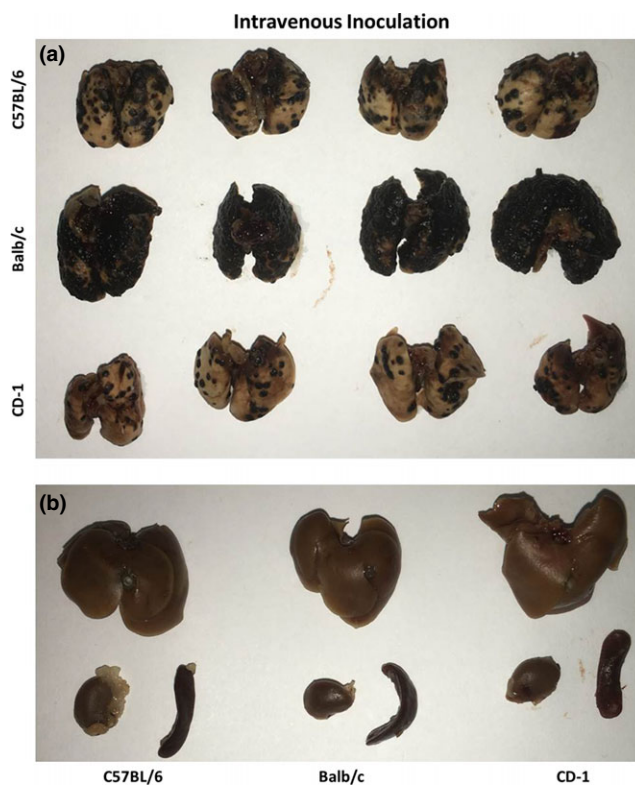

Figure 1 Tumour growth after i.v. inoculation in CD-1, C57 and Balb/c mice. Fourteen days after i.v. inoculation of 2×10^5 B16F10 cells in 200 µl PBS, mice were sacrificed and their organs were fixed in 4% paraformaldehyde. (a) Tumour growth in lungs. Tumour growth in Balb/c lungs was much faster than that in C57 or CD-1 lungs. (b) Representative liver, kidney and spleen from each strain showing lack of tumour growth outside of the lung.
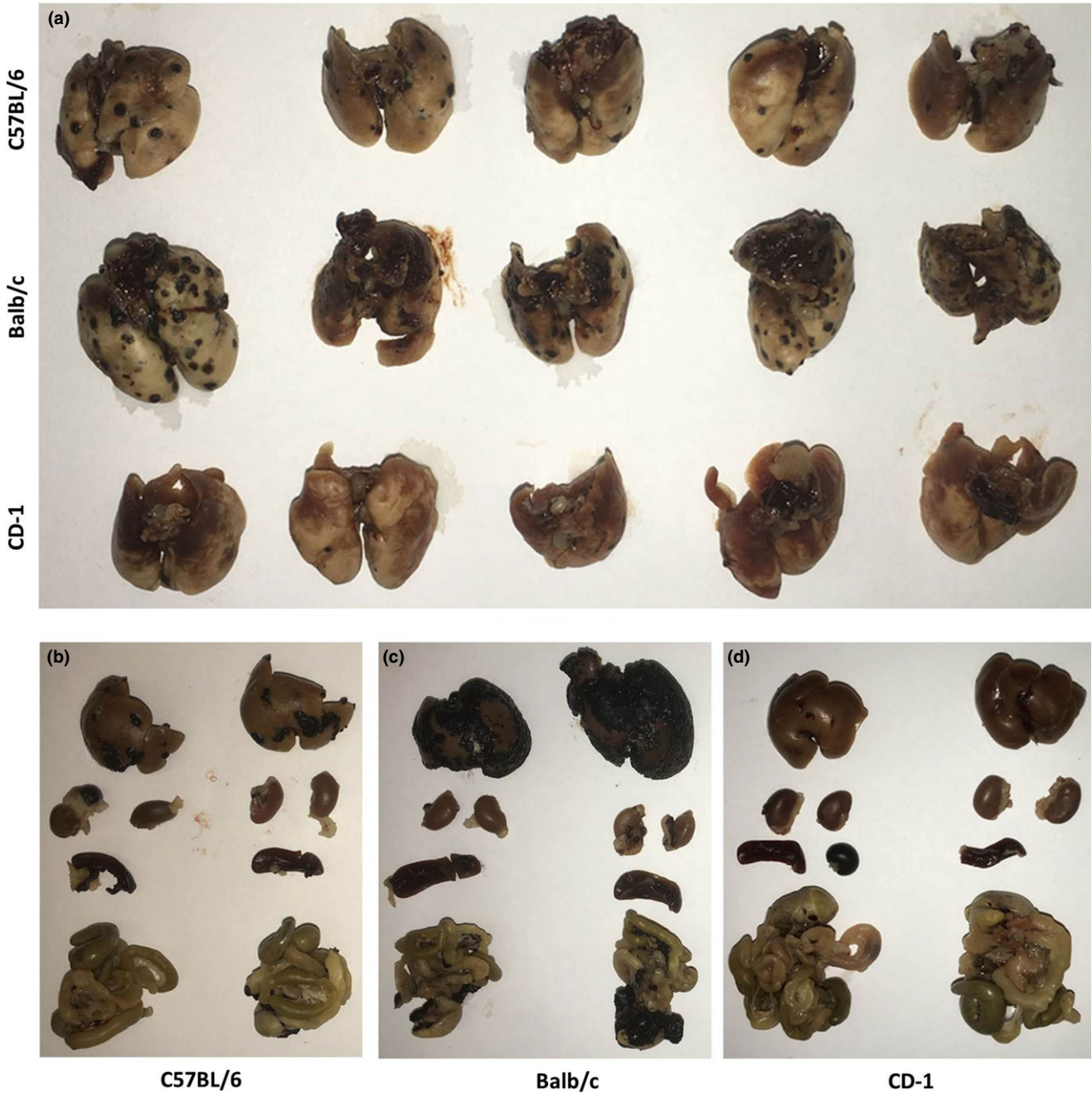
Hydrodynamic Inoculation

Figure 2 Tumour growth after h.d. inoculation in CD-1, C57 and Balb/c mice. Fourteen days after inoculation of 2×10^5 B16F10 cells in 1.6 ml PBS, mice were sacrificed and their organs were fixed in 4% paraformaldehyde. (a) Tumour growth in lungs. Tumour growth in Balb/c lungs was again faster than that in C57 or CD-1 lungs, but lung tumour growth was overall slower than the lung tumour growth after i.v. inoculation. (b,c,d) Representative liver, kidney, spleen and intestines from C57, Balb/c and CD-1 mice, respectively, showing broad tumour growth outside of the lung. Again, Balb/c mice showed faster tumour growth in liver than did C57 or CD-1 mice.

CD-1 strain of mice developed lung tumours significantly more slowly than C57 mice.

Strain- and route-dependent differences in tumour growth were also observed after h.d. inoculation (Figure 2). The large injection volume forced the injected tumour cells into several organs, especially the liver, generating tumours in the liver, lung, kidney, spleen and peritoneum. An overview of tumour growth patterns can be found in Table 1. The

tumour growth rate in the lungs after h.d. inoculation was significantly slower than its rate after intravenous inoculation (Figure 3), most probably because the force of h.d. injection is sufficient to push the tumour cells through the lung capillaries and into downstream organs. Again, tumour growth in Balb/c mice was faster than that in C57 mice, although tumours were present in the liver, lung, kidney, spleen and peritoneum in all five mice per group. The tumour growth pattern after h.d. inoculation in both C57 and Balb/c mice was consistent with the literature describing growth in C57 mice (Kang *et al.* 2009; Li *et al.* 2011). Tumours in CD-1 mice grew more slowly in the lung, but did not show many signs of growth in other organs after h.d. inoculation. The histological findings illustrated in Figure 4 show differences in lung and liver tumour burden after i.v. and h.d. inoculation respectively.

Table 1 Overview of tumour growth patterns across all tested strains and routes of inoculation

	Mice with tumours present in this organ					
	Liver	Spleen	Kidney	Lung	Peritoneum	s.c.
Hydrodynamic						
C57BL/6	5/5	5/5	5/5	5/5	4/5	0/5
Balb/c	5/5	5/5	5/5	5/5	5/5	0/5
CD 1	0/5	0/5	1/5	5/5	1/5	0/5
Intravenous						
C57BL/6	0/4	0/4	0/4	4/4	0/4	0/4
Balb/c	0/4	0/4	0/4	4/4	0/4	0/4
CD 1	0/4	0/4	0/4	4/4	0/4	0/4
Intraperitoneal						
C57BL/6	5/5	5/5	5/5	0/5	5/5	0/5
Balb/c	5/5	5/5	5/5	0/5	5/5	0/5
CD 1	3/5	3/5	3/5	0/5	3/5	0/5
Subcutaneous						
C57BL/6	0/5	0/5	0/5	0/5	0/5	5/5
Balb/c	0/5	0/5	0/5	0/5	0/5	5/5
CD 1	0/5	0/5	0/5	0/5	0/5	5/5

Other routes of B16F10 inoculation were performed to investigate any other strain-dependent tumour growth differences. Intraperitoneal tumour growth after i.p. injection was very fast in all three mouse strains, filling the peritoneal cavity and surrounding the intestines, spleen and kidneys, although the take rate in CD-1 mice was only three of the five inoculated mice. There was no statistical difference between tumour growth rates among all three strains, and at the conclusion of the study, no tumours had yet metastasized to the lungs (data not shown). Subcutaneous tumour growth after s.c. injection was slowest in CD-1 mice and significantly faster in C57 mice (Figure 5). Subcutaneous tumours in Balb/c mice grew more slowly than those in C57 mice, but not significantly so.

The differences in tumour growth patterns shown here provide some interesting, albeit unexpected, results. The syngeneic nature of B16F10 inoculation in C57 mice is evidenced in its rapid tumour growth along all inoculation routes, but what is most interesting is the even faster tumour growth in Balb/c mice after i.v. and h.d. inoculation, even though the introduction of B16F10 cells generates an allogeneic model in which tumour growth should be suppressed by the host's immune system. The outbred CD-1 mice did not show this increased tumour growth; their tumours grow more slowly than the tumours of both C57 and Balb/c mice.

The differences in the observed rate of tumour growth among CD-1, C57 and Balb/c mice have generated many questions. It is simple enough to explain how the syngeneic C57 host may not mount a robust immune response against the injected B16F10 cells, resulting in a fast, widespread tumour growth among several routes of inoculation. Immune surveillance against B16F10 cells may also play a role in the slower tumour growth in the outbred CD-1 mice. The most puzzling is the consistently faster tumour growth in Balb/c mice after i.v. and h.d. inoculation, both of which use injection via the tail vein as the route of inoculation. This unexpected result may have occurred at least in part because of inadequate rejection of the allogeneic cells by the Balb/c host.
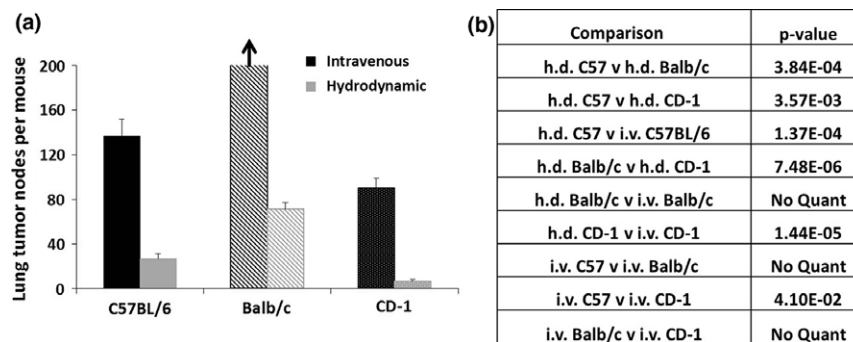

Figure 3 (a) Quantification of tumour nodes on lungs after i.v. or h.d. inoculation. Tumour nodes per mouse were counted and averaged. (b) Relevant *P*-values were calculated using a Student's *t*-test, and statistical significance was confirmed for all comparisons using the Holm–Sidak corrections. Because the tumour growth in Balb/c mice that received i.v. inoculation covered nearly the entire lung, tumour nodes were unable to be quantified, but the overall tumour burden in these mice was obviously much higher than in all other groups. *P*-values were not calculated for comparisons between Balb/c i.v. and other groups.
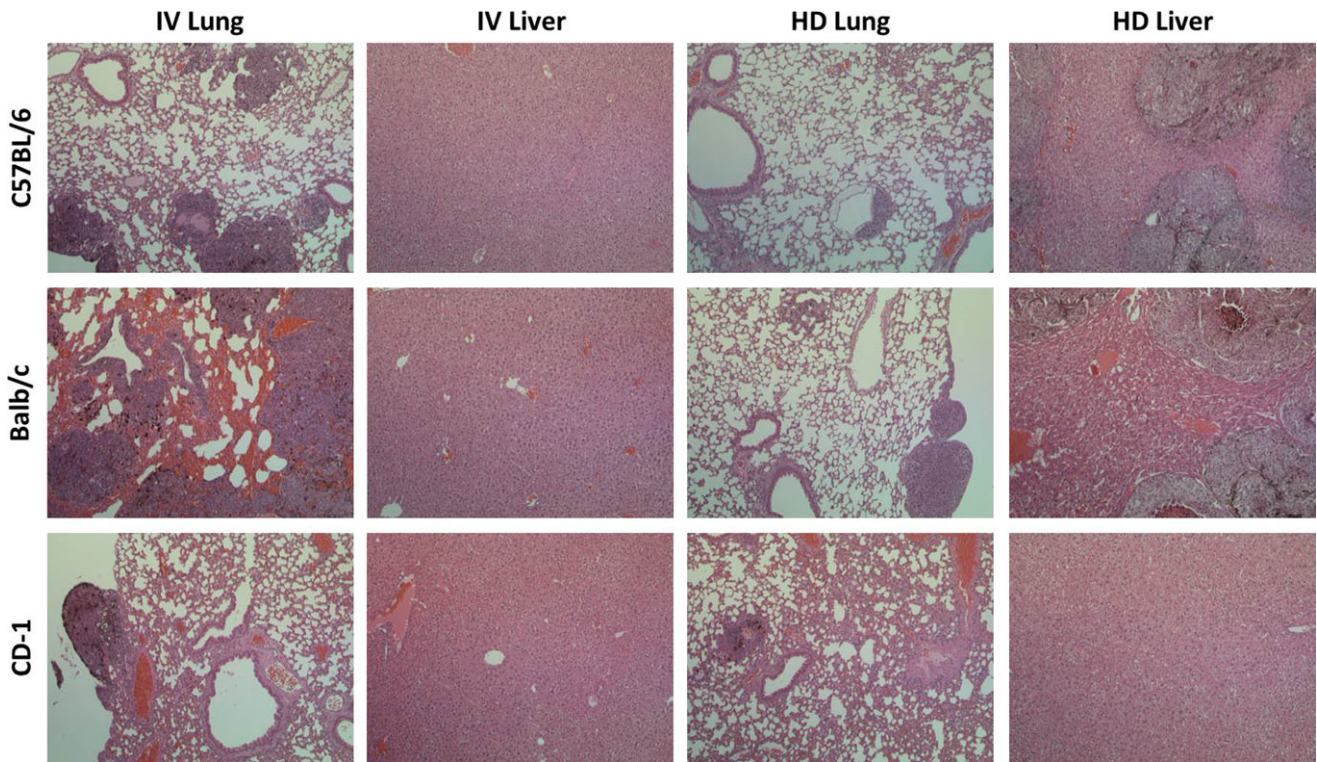
Figure 4 H&E-stained sections of lung and liver in CD-1, C57 and Balb/c mice. Tumour burden in Balb/c i.v. lungs is high, and the normal epithelium is surrounded with blood. No livers show tumour burden after i.v. inoculation, but there is a large burden in C57 and Balb/c tumours after h.d. inoculation.

Figure 5 Tumour growth after s.c. inoculation in CD-1, C57 and Balb/c mice. Fourteen days after s.c. inoculation of 2×10^5 B16F10 cells in 100 µl PBS, mice were sacrificed, and tumours were harvested and weighed. (a) Relative s.c. tumour sizes in all three strains; (b) Average tumour weights; **$P = 0.01$.

The cells may have overwhelmed the immune system in the Balb/c mice or induced a immune tolerance providing a niche for the tumour cells to grow quickly. Recent literature has shown that B16F10 cells produce anti-inflammatory and immunosuppressive microvesicles containing phosphatidylserine and that these microvesicles contribute to increased tumour growth in Balb/c mice (Luize *et al.* 2009). While Balb/c and C57 mice were both used in this study, tumour growth in the different strains was not compared directly. Thus the nature of the immunological differences between the routes of innoculation, combined with mouse strain, should be taken into account when mice are used as animal models in the field of cancer therapy.

Conclusions

The differences in tumour growth among the different strains and inoculation routes implies that they constitute a series of independent B16F10 tumour models. Intravenous inoculation into C57 mice generates the expected fast-growing lung metastasis model, while h.d. inoculation in C57 mice produces a model in which metastasis has spread throughout the body. Inoculations in Balb/c mice produce aggressive, allogeneic tumour models that might be used to study how immune contributions affect tumour growth, while the CD-1 models provide less aggressive tumour growth that could be used to replicate slow-growing tumours. The differences in

growth speed will allow researchers to select a more or less aggressive tumour model that will best fit for their studies.

Identifying the variations in tumour models which could be generated by modifying mouse strain and route of inoculation might have many practical applications. The models described here provide some indication of the range of possible selection, but only compares the growth of one tumour line in a combination of three mouse strains and four routes of inoculation. If this effort is expanded to include other mouse strains and tumour lines, this could generate an extremely useful source to aid scientists in choosing the most effective model for their research.

Conflict of interest

No conflict of interest is involved in this research work.

Funding source

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