# *In vitro* and *in vivo* effects of polyhaemoglobin–tyrosinase on murine B16F10 melanoma

Binglan Yu and Thomas Ming Swi Chang

Melanoma is an increasingly common fatal skin cancer. Many groups are carrying out research on potential treatments for melanoma. One of these approaches has shown that lowering tyrosine can inhibit the growth of melanoma in cell cultures and of B16BL6 melanoma in mice. However, humans cannot tolerate tyrosine-restricted diets for lowering tyrosine because of nausea, vomiting and weight loss. We report here our preparation and characterization of a novel soluble polyhaemoglobin–tyrosinase complex. This preparation prevents native tyrosinase from having adverse effects and from rapid removal after injection. The preparation inhibited murine B16F10 melanoma cell growth in culture and delayed its growth in a mice model. Intravenous injection of the preparation lowers the systemic tyrosine level without causing adverse effects such as vomiting and weight loss in mice. It is therefore possible that this complex could be useful in the treatment of human melanoma. *Melanoma Res* 14:197–202 © 2004 Lippincott Williams & Wilkins.

## Introduction

Melanoma, a fatal skin cancer, is a common tumour that accounts for 10% of all malignancies. Melanoma is most commonly found on the skin, but 10% arise in the eye [1]. At least 20% of people diagnosed with melanoma progress to advanced disease and die within 5 years of diagnosis [2]. At present, despite the use of adjuvant therapy, chemotherapy, immunotherapy and radiation therapy, the median survival of patients with metastatic melanoma is about 6 months [3].

Tyrosine is important in the metabolic cycle of melanoma [4–6], and malignant melanomas require higher concentrations of tyrosine for growth. Research, especially that of Meadows' group, has shown that lowering systemic tyrosine using a tyrosine- and phenylalanine-restricted diet can inhibit the growth of melanoma *in vitro* and *in vivo* [7–9]. However, low tyrosine diets are not well tolerated by humans, resulting in weight loss and other adverse effects [10]. Furthermore, a restricted diet can only lower the systemic tyrosine level to about 67% of normal levels [11].

We therefore investigated a new approach of chemically cross-linking tyrosinase with excess haemoglobin to form a soluble polyhaemoglobin–tyrosinase (PolyHb–tyrosinase) complex. In this way, after intravenous injection, tyrosinase is covered and protected by the PolyHb from causing adverse effects and from rapid removal from the circulation. PolyHb by itself has already been shown to be safe and effective as a red blood cell substitute in the final stages of a phase III clinical trial, with up to 10l infused to replace lost blood in trauma surgery [12–15]. We have also successfully prepared PolyHb cross-linked together with superoxide dismutase and catalase [16,17].

In the present study, we investigated the preparation, structural and functional properties and the *in vitro* effect on the growth of the B16F10 melanoma cell line of this novel PolyHb–tyrosinase. This was followed by animal studies in rats and in mice, including a B16F10 melanoma-bearing mice model.

## Materials and methods
### Materials
Purified bovine haemoglobin was purchased from Biopure Corporation (Boston, Massachusetts, USA). Glutaraldehyde (25%) was obtained from Polysciences (Warrington, Pennsylvania, USA). L-Lysine monohydrochloride (SigmaUltra > 99%), L-tyrosine [98% thin layer chromatogra (TLC)] and tyrosinase from mushroom (EC 1.14.18.1, 3000 units/mg stated activity) were purchased from Sigma-Aldrich (Ontario, Canada). All other reagents were of analytical grade.

## Preparation of PolyHb and PolyHb–tyrosinase

Reaction mixtures were prepared containing haemoglobin (10 g/dl) and tyrosinase (6000 U/ml) in 0.1 M potassium phosphate buffer, pH 7.6. In the PolyHb mixtures, an equivalent volume of buffer replaced the enzyme. Prior to the start of cross-linking, 1.3 M lysine was added at a molar ratio of 7:1 lysine/haemoglobin. The cross-linking reaction was started with the addition of glutaraldehyde (5%) at molar ratio of 16:1 glutaraldehyde/haemoglobin. Glutaraldehyde was added in four equal aliquots over a 15 min period. After 3.5–48 h at 4°C under aerobic conditions with constant stirring, the reaction was stopped with 2.0 M lysine at a molar ratio of 200:1 lysine/haemoglobin. Solutions were dialysed in physiological saline solution and passed through a sterile 0.45 μM filter. Aliquots (500 μl) of the 16:1 cross-linked preparation were concentrated using 100 kDa microconcentrators (Amicon, Beverly, Massachusetts, USA). Samples were centrifuged at 2500 g for 55 min at 23°C and the retentate was collected. The haemoglobin concentration was determined using cyanomethaemoglobin at 540 nm.

## Determination of tyrosinase activity

Tyrosinase activity was assessed by measuring the formation of enzymatic products at 300 nm [18]. The absorbance at 300 nm was followed continuously for 8–14 min using a Perkin Elmer Lambda 4B spectrophotometer (Norwalk, Connecticut, USA), and changes in optical density per minute were used to analyse the activity of the enzyme.

## Effects of intravenous injection of PolyHb–tyrosinase in rats

Fasted male Sprague-Dawley rats (245–260 g) were obtained from Charles River Canada (St Constant, Quebec, Canada). Animals were anaesthetized with an intraperitoneal injection of 65 mg/kg pentobarbitone (Somnotol, Decton Dickinson, New Jersey, USA). Body temperature was maintained by a warming blanket. Incisions were carefully performed in one hindlimb below the inguinal ligament, and the femoral vessels were carefully isolated. Polyethylene cannulas (PE-10 and PE-50, Clay Adams, Becton Dickinson, Sparks, Maryland, USA) were inserted and secured distal to the superficial epigastric branches in the femoral artery and vein. Proper vessel access was tested with a small volume injection of heparinized saline (50 IU/ml). Blood samples were taken at the beginning of the experiment, then PolyHb–tyrosinase was injected through the femoral vein. The femoral artery cannula was connected to the venous cannula for blood to circulate thoroughly for a short interval. Blood samples were then taken from the femoral artery at different time intervals. The plasma in each blood sample was separated from the blood and placed in a 1.5 ml plastic tube and stored at −80°C until analysed. The tyrosine concentration in the plasma was measured by a fluorometric method using a Perkin Elmer Luminescence Spectrometer LS50B [19].

## Tumour cells and culture conditions

B16-F10 murine melanoma cells were obtained from American Type Tissue Collection (Manassas, Virginia, USA). The tumour cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum. Cells were passaged every 2–3 days. For the experiment, melanoma cells were cultured in complete DMEM until they became 30–40% confluent, when one of the following was added (0.57 ml sample per 10 ml medium): (i) saline solution (0.9 g/dl NaCl); (ii) free tyrosinase solution; (iii) PolyHb solution; or (iv) PolyHb–tyrosinase solution. The cell viability was then determined by trypan blue exclusion for up to 4 days [20–22]. Tumour cells were routinely monitored by phase microscopy. Cell counts were obtained daily with a haemacytometer.

## Intravenous injection of PolyHb–tyrosinase in normal mice

BD2F1 female mice (C57BL/6 × DBA/2F1) aged 57–63 days were purchased from Charles River Canada. Mice were kept with a 12 h light/dark schedule and fed conventional food and water *ad lib*. All animals were housed and cared for according to the animal care regulations of McGill University. All mice were acclimatized for at least 7 days prior to use in the experiment. Two groups of mice (five mice per group) were studied. An intravenous injection of 0.1 ml saline (control group) or 0.1 ml PolyHb–tyrosinase solution (test group) was administered every day, and blood was taken after each injection in both groups.

## B16F10 melanoma-bearing mice model

B16F10 melanoma cells prepared at a concentration of $1 \times 10^6$ cells in 0.1 ml of Hanks' balanced salt solution were injected subcutaneously into the shaved lateral flank of each mouse. The size of the primary tumour was measured every 2 days using callipers, and the tumour volume was calculated using the formula $V = (A \times B^2)/2$, where $V$ is the volume (mm³), $A$ is the long diameter (mm) and $B$ is the short diameter (mm) [23]. When the tumours reached an average size of 125 mm³, three groups of mice were treated as follows: (i) the sham control group received no intravenous injections; (ii) the saline control group received daily intravenous injections of 0.1 ml saline; and (iii) the test group received daily intravenous injections of 0.1 ml of PolyHb–tyrosinase solution. The endpoint of the study was when the tumour burden in any group of animals reached 10% of the body weight, based on the regulations of the ethics committee of the Faculty of Medicine Animal Care Committee of McGill University.

## Data analysis

Statistical analysis was performed using the Student's *t*-test within analysis of variance and was considered to be significant at $P < 0.05$.

## Results

### Molecular weight distribution of PolyHb–tyrosinase and analysis of tyrosinase activity

We first optimized the cross-linking of tyrosinase to haemoglobin to form PolyHb–tyrosinase with regard to the degree of polymerization, molecular weight distribution and enzyme activity. We then prepared the PolyHb–tyrosinase complex with a cross-linking time of 3.5–48 h for use in the animal studies. The longer cross-linking time did not decrease enzyme activity significantly [24]. We also found that increasing the cross-linking time from 3.5 h to 24 h resulted in an increasing percentage of the complex in the larger molecular weight fraction (over 400 kDa) [24]. However, there was no significant difference in the percentage in the larger molecular weight fraction (over 400 kDa) between a cross-linking time of 24 h and 48 h [24]. Thus, for the present study we used a maximal cross-linking time of 24 h, giving 74% with a molecular weight over 400 kDa for the preparation of the complex for the following experiments [24].
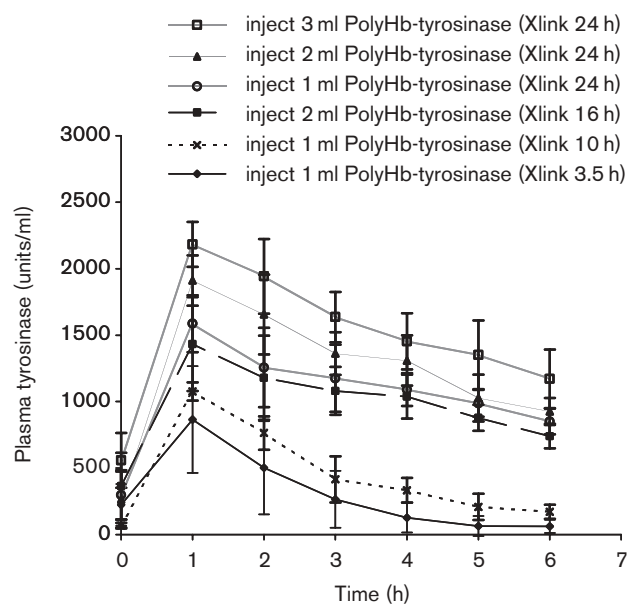
### Intravenous injection of PolyHb–tyrosinase

Rats were used for this initial study since it is not possible to obtain the large total volumes of blood samples needed for analysing the plasma tyrosinase activity and retention time of the enzyme in mice. After the injection of 1 ml/250 g body weight of PolyHb–tyrosinase with a cross-linking time of 3.5 h, tyrosinase activity reached $1303 \pm 411$ U/ml in the first hour, then decreased rapidly to $61 \pm 49$ U/ml by 6 h (Fig. 1). Use of PolyHb–tyrosinase with a cross-linking time of 24 h more than doubled the tyrosinase activity in the plasma, and the retention time in the circulation was markedly improved (Fig. 1). Increasing the volume from 1 ml to 2–3 ml/250 g body weight only increased the tyrosinase activity and retention time slightly. We therefore decided to use a 24 h cross-linked complex of 1 ml/250 g body weight for the subsequent studies in mice.

### Effects of PolyHb–tyrosinase on the growth of cultured melanoma cells

B16F10 melanoma cells were cultured in DMEM and treated with saline, free tyrosinase, PolyHb or PolyHb–tyrosinase. Figure 2 shows that PolyHb by itself did not have any effect on the growth of B16F10 cells when compared with saline. The PolyHb–tyrosinase complex was not significantly different from free tyrosinase in inhibiting the growth of the melanoma cells *in vitro* (Fig. 2).

Fig. 1



Tyrosinase activity after intravenous injection of PolyHb–tyrosinase (6000 units/ml) prepared by cross-linking for 3.5–24 h.

Fig. 2
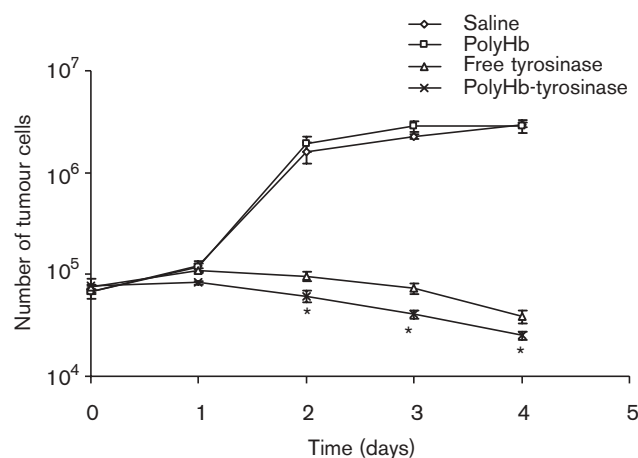


*In vitro* growth curves of B16F10 cell lines after the addition of saline, PolyHb, free tyrosinase or PolyHb–tyrosinase to the medium. Results shown are the mean ± SD of three separate experiments. *$P < 0.05$ compared with saline and PolyHb.

### Intravenous injection of PolyHb–tyrosinase in normal mice

Daily intravenous injections of PolyHb–tyrosinase in the test group reduced plasma tyrosine levels rapidly to $0.19 \pm 0.09$ mg/dl on day 2 compared with $1.44 \pm 0.16$ mg/dl in the control group (Fig. 3a). Since one of the adverse effects of the use of a tyrosine-restricted diet is nausea,
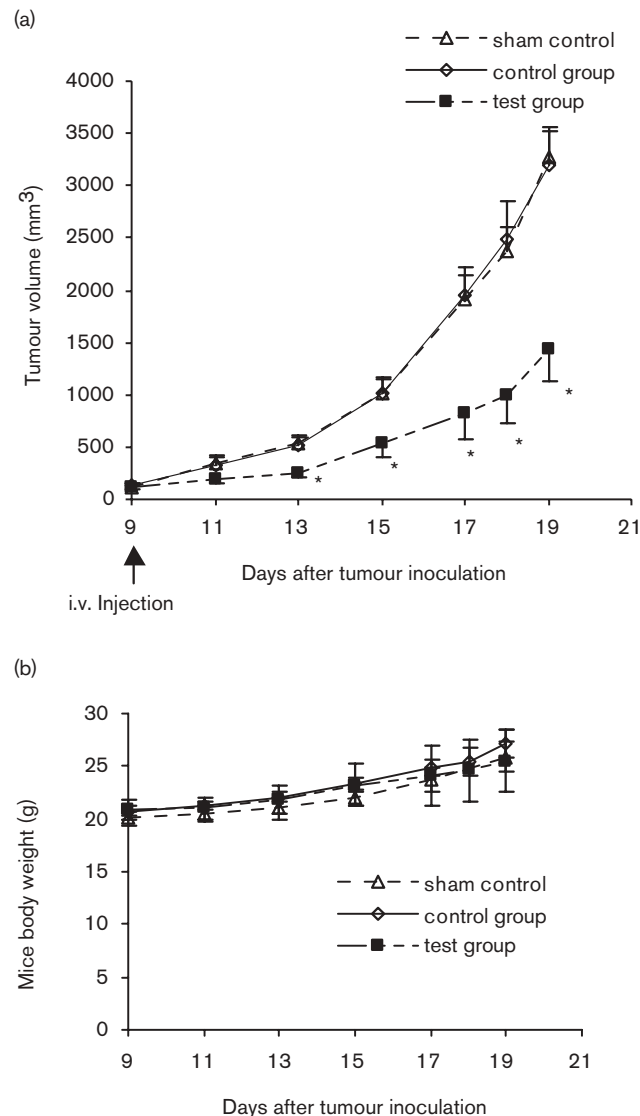
**Fig. 3**

(a)



(b)
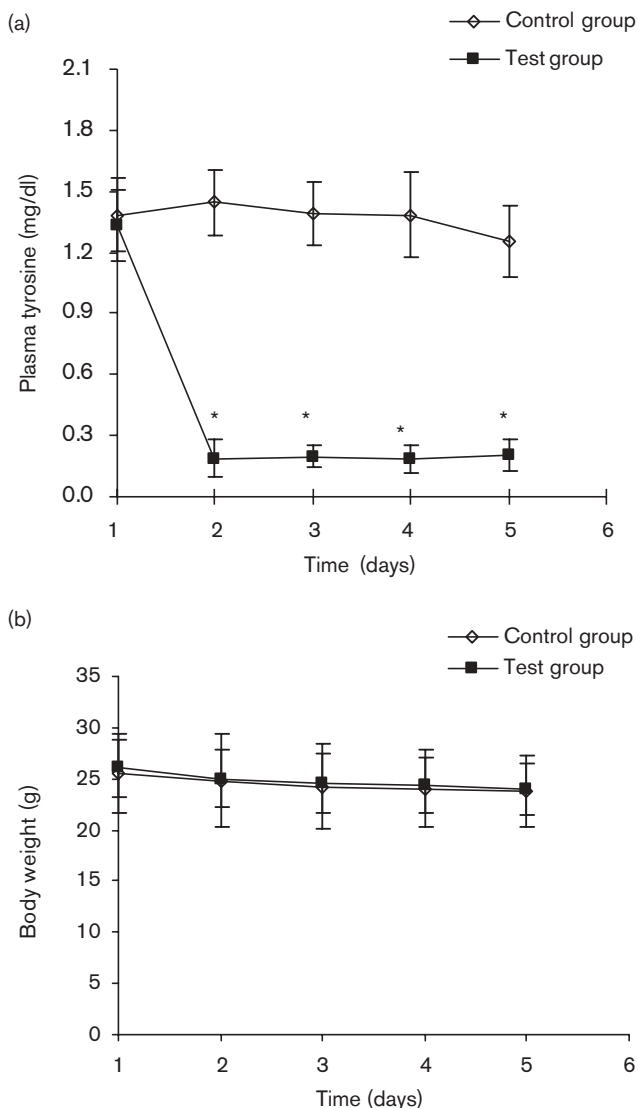


Effects of intravenous injection of PolyHb–tyrosinase into normal mice. (a) Plasma tyrosine concentration after daily intravenous injection of 0.1 ml saline (control group, $n=5$) or 0.1 ml PolyHb–tyrosinase (test group, $n=5$). *$P < 0.05$ compared with control group. (b) Body weight.

**Fig. 4**

(a)



(b)



Effects of daily intravenous injections of PolyHb–tyrosinase in mice. (a) Tumour growth of B16F10 melanoma in mice receiving no intravenous injection (sham control), 0.1 ml intravenous saline daily (saline control) or 0.l ml intravenous PolyHb–tyrosinase daily (test group). *$P < 0.05$ compared with sham control and control group. Endpoint of study was when the tumour burden in any one group reached 10% of the normal body weight. All values are the mean $\pm$ SEM. (b) Body weight after tumour implantation.

vomiting and weight loss, we also followed these effects in the mice. Daily measurements of body weight showed no difference between the control group and the test group (Fig. 3b). Furthermore, no vomiting was observed in these mice.

### Effect of intravenous injection of PolyHb–tyrosinase in melanoma mice model

B16F10 cells were inoculated subcutaneously into mice and when the tumour volume reached an average of 125 mm³, on day 9, the sham control group received no intravenous injections, the saline control group received

daily intravenous injections of 0.1 ml saline, and the test group received daily intravenous injections of 0.l ml of PolyHb–tyrosinase.

There was no significant difference in tumour size between the control group and the sham control group (Fig. 4a). However, 4 days after the daily intravenous injections of PolyHb–tyrosinase, the tumour volume was significantly lower than that in the control

group. After 6 days of daily intravenous injections of PolyHb–tyrosinase, the tumour volume in the test group was only $53.49 \pm 13.91\%$ of that in the control group. Nineteen days after the inoculation of the B16F10 melanoma cells, the tumour volume of the control has reached the maximum of 10% of the body weight allowed by the Animal Care Committee and the study was terminated. At this time, the tumour size in the test group was only $45.28 \pm 10.09\%$ of that in the control group (Fig. 4a). Our results suggest that PolyHb–tyrosinase retards the growth of B16F10 melanoma in mice.

We also followed the body weight of these three groups of mice. There was no significant difference in weight gain (Fig. 4b). The growth of tumours in the tumour-bearing mice contributed to the gain in weight (Fig. 4b) compared with that observed for the normal mice (Fig. 3b).

## Discussion

The incidence of melanoma, a fatal skin cancer that is the fifth most common cancer in North America, is increasing dramatically, doubling every 10 years [25]. Despite extensive research, at present only adjuvant therapies are available [1,26,27]. These include immunotherapy, chemotherapy, autologous bone marrow transplantation, biochemotherapy and chemoimmunotherapy. Meadows' group has carried out important studies showing that lowering the systemic tyrosine can inhibit the growth of melanoma in mice [7–9,28–31]. Unfortunately, a tyrosine-restricted diet is not well tolerated in humans and results in weight loss, nausea and vomiting in patients who are already severely ill from their melanoma. Thus it has not been possible to carry out meaningful clinical trials. We have reported here a possible biotechnological solution to this problem based on intravenous PolyHb–tyrosinase. Unlike free tyrosinase [28–33], the tyrosinase in PolyHb–tyrosinase is covered and protected from being exposed to the body by PolyHb (with a haemoglobin:tyrosinase molar ratio of 100:2), which prevents the tyrosinase from having any adverse effects. PolyHb–tyrosinase decreases the systemic tyrosine levels in mice to 13% of the original level without the adverse effects of nausea, vomiting or weight loss, whereas a tyrosine-restricted diet lowers the tyrosine levels only to 67% of the original level but has the adverse effects of nausea, vomiting or weight loss. *In vitro*, PolyHb–tyrosinase inhibited the growth of B16F10 cells. When injected intravenously into B16F10 melanoma-bearing mice, PolyHb–tyrosinase also delayed the growth of the melanoma when compared with the control group. These results should prompt further studies to optimize this approach and to investigate its combination with other methods for treating malignant melanoma.

## References

1 Borden EC (editor). *Melanoma: Biologically Targeted Therapeutics*. Totowa: Humana Press; 2002.
2 Beahers OH, Henson DE, Hutter RVP, Kennedy BJ. Malignant melanoma of the skin (excluding eyelid). In: *American Joint Committee on Cancer Manual for Staging of Cancer*, 4th edition. Philadelphia: JB Lippincott; 1992, pp. 143–148.
3 Black J. Malignant melanoma: an update on treatments. *Plast Surg Nurs* 1999; **19**:143–147.
4 Jimbow K, Iwashina T, Alena F, Yamada K, Pankovich J, Umemura T. Exploitation of pigment biosynthesis pathway as a selective chemotherapeutic approach for malignant melanoma. *J Invest Dermatol* 1993; **100**:231S–238S.
5 Letellier S, Garnier JP, Spy J, Stoitchkov K, Le Bricon T, Baccard M, *et al.* Development of metastases in malignant melanoma is associated with an increase in the plasma. *Melanoma Res* 1999; **9**:389–394.
6 Potterf BS, Hearing VJ. Tyrosine transport into melanosomes is increased following stimulation of melanocyte differentiation. *Biochem Biophys Res Commun* 1998; **248**:795–800.
7 Fu YM, Yu ZX, Ferrans VJ, Meadows GG. Tyrosine and phenylalanine restriction induces $G_0/G_1$ cell cycle arrest in murine melanoma *in vitro* and *in vivo*. *Nutr Cancer* 1997; **29**:104–113.
8 Pelayo BA, Fu YM, Meadows GG. Inhibition of B16BL6 melanoma invasion by tyrosine and phenylalanine deprivation is associated with decreased secretion of plasminogen activators and increased plasminogen activator inhibitors. *Clin Exp Metastasis* 1999; **17**:841–848.
9 Pelayo BA, Fu YM, Meadows GG. Decreased tissue plasminogen activator and increased plasminogen activator inhibitors and increased activator protein-1 and specific promoter-1 are associated with inhibition of invasion in human A375 melanoma deprived of tyrosine and phenylalanine. *Int J Oncol* 2001; **18**:877–883.
10 Lorincz AB, Kuttner RE, Brandt MB. Tumor response to phenylalanine-tyrosine limited diets. *J Am Diet Assoc* 1969; **54**:198–205.
11 Meadows GG, Pierson HF, Abdallah RM, Desai PR. Dietary influence of tyrosine and phenylalanine on the response of B16 melanoma to carbidopa-levodopa methyl ester chemotherapy. *Cancer Res* 1982; **42**:3056–3063.
12 Chang TMS. Red blood cell substitutes. *Best Pract Res Clin Haematol* 2000; **13**:651–668.
13 Chang TMS (editor). *Blood Substitutes: Principles, Methods, Products and Clinical Trials*, vols I and II. Basel: Karger; 1997, 1998.
14 Squires JE. Artificial blood. *Science* 2002; **295**:1002–1005.
15 Gould SA. The life-sustaining capacity of human polymerized hemoglobin when red cells might be unavailable. *J Am Coll Surg* 2002; **195**:445–452.
16 Chang TMS. Future generations of red blood cell substitutes. *J Intern Med* 2003; **253**:527–535.
17 D'Agnillo F, Chang TMS. Polyhemoglobin-superoxide dismutase-catalase as a blood substitute with antioxidant properties. *Nat Biotechnol* 1998; **16**:667–671.
18 Duckworth HW, Coleman JE. Physiochemical and kinetic properties of mushroom tyrosinase. *J Biol Chem* 1970; **245**:1613–1625.
19 Fisher MJ, Pogson CI. The determination of flux through phenylalanine hydroxylase and homogentisate oxidase in isolated hepatocytes. *Biosci Rep* 1984; **4**:835–841.
20 Rodriguez-Ayerbe C, Smith-Zubiaga I. Effect of serum withdrawal on the proliferation of B16F10 melanoma cells. *Cell Biol Int* 2000; **24**:279–283.
21 Yoon SS, Fidler IJ, Beltran PJ, Bucana CD, Wang YF, Fan D. Intratumoral heterogeneity for an epigenetic modulation of *mdr-1* expression in murine melanoma. *Melanoma Res* 1997; **7**:275–287.
22 Kayaga J, Souberbielle BE, Sheikh N, Morrow WJ, Scott-Taylor T, Vile R, *et al.* Anti-tumor activity against B16-F10 melanoma with a GM-CSF secreting allogeneic tumor cell vaccine. *Gene Ther* 1999; **6**:1475–1481.
23 Barthelmes HU, Niederberger E, Roth T, Schulte K, Tang WC, Boege F, *et al.* Lycobetaine acts as a selective topoisomerase IIβ poison and inhibits the growth of human tumor cells. *Br J Cancer* 2001; **85**:1585–1591.
24 Yu B, Chang TMS. *In vitro* and *in vivo* enzyme studies of polyhemoglobin–tyrosinase. *Biotechnol Bioeng* 2004; in press.
25 Katsambas A, Nicolaidou E. Cutaneous malignant melanoma and sun exposure. *Arch Dermatol* 1996; **132**:444–450.
26 Caraceni A, Gangeri L, Martini C, Belli F, Brunelli C, Baldini M, *et al.* Neurotoxicity of interferon-α in melanoma therapy. *Cancer* 1998; **83**:482–489.
27 Johnson TM, Yahanda AM, Chang AE, Fader DJ, Sondak VK. Advances in melanoma therapy. *J Am Acad Dermatol* 1998; **38**:731–741.

28 Elstad CA, Meadows GG, Aslakson CJ, Starkey JR. Evidence for nutrient modulation of tumor phenotype: impact of tyrosine and phenylalanine restriction. *Adv Exp Med Biol* 1994; **354**:171–183.

29 Uhlenkott CE, Huijzer JC, Cardeiro DJ, Elstad CA, Meadows GG. Attachment, invasive, chemotaxis, and proteinase expression of B16-BL6 melanoma cells exhibiting a low metastatic phenotype after exposure to dietary restriction of tyrosine and phenylalanine. *Clin Exp Metastasis* 1996; **14**:125–137.

30 Fu YM, Yu ZX, Pelayo BA, Ferrans VJ, Meadows GG. Focal adhesion kinase-dependent apoptosis of melanoma induced by tyrosine and phenylalanine deficiency. *Cancer Res* 1999; **59**:758–765.

31 Elstad CA, Thrall BD, Raha G, Meadows GG. Tyrosine and phenylalanine restriction sensitizes adriamycin-resistant P388 leukemia cells to adriamycin. *Nutr Cancer* 1996; **25**:47–60.

32 Jimbow K. Development of targeted chemoradiotherapy for malignant melanoma by exploitation of metabolic pathway. *J Med Sci* 1998; **73**:105–110.

33 Gili A, Thomas PD, Ota M, Jimbow K. Comparison of *in vitro* cytotoxicity of *N*-acetyl and *N*-propionyl derivatives of phenolic thioether amines in melanoma and neuroblastoma cells and the relationship to tyrosinase and tyrosine hydroxylase enzyme activity. *Melanoma Res* 2000; **10**:9–15.