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Tyrosine transport in a human melanoma cell line as a basis for selective transport of cytotoxic analogues

James M. PANKOVICH and Kowichi JIMBOW*
Division of Dermatology and Cutaneous Sciences, 260G Heritage Medical Research Building, University of Alberta, Edmonton, Alberta T6G 2S2, Canada

Tyrosine is an essential amino acid for the initial step of melanin synthesis, yet little is known concerning its transport in melanocytes. As an important first step in the development of new anti-melanoma agents based upon chemical and pharmacological modifications of melanin synthesis, the present study characterized the transport mechanism of tyrosine *in vitro* using the human melanoma cell line SK-MEL 23. Several tyrosine transport systems may be involved in melanocytes: systems L and T, which transport neutral amino acids with branched or aromatic side chains, and systems A and ASC, which transport neutral amino acids with smaller side chains. In order to determine which system or combination of systems is involved in tyrosine transport in melanoma cells, studies of kinetics, Na^+-dependence and competitive inhibition were undertaken. The K_m and $V_{max.}$ for the Na^+-independent transport system were found to be 0.164 ± 0.016 mM and 21.6 ± 1.1 nmol/min per mg of protein respectively. This transport was preferentially inhibited by the system L specific analogue, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid, the system T substrate tryptophan, and the sulphur homologue of tyrosine, 4-*S*-cysteinylphenol. Sequential addition of these inhibitors at increasing concentrations indicated that they inhibit the same transporter. Our results suggest that tyrosine transport in SK-MEL 23 melanoma cells is similar to system L transport previously characterized in other cell types. This one transport system appears to supply all the tyrosine required for both cell growth and melanin synthesis. The transport system may be subject to manipulation by melanogenic stimulating factors, making the transport of cytotoxic tyrosine analogues an important area for further study.

INTRODUCTION

Most attempts to develop chemotherapeutic agents specific to malignant melanoma have involved exploitation of intermediates of melanin synthesis or agents which are assumed to enhance melanin pigmentation, or both. Tyrosine is a natural precursor for the production of the melanin pigments eumelanin and pheomelanin, which occurs in the presence of tyrosinase. Since tyrosinase activity and melanin synthesis are generally increased in neoplastic counterparts, it is expected that the transport of tyrosine into melanoma cells might be greatly elevated [1].

Neutral amino acids, e.g. alanine, leucine, tyrosine and tryptophan, are transported by amino acid transporters such as system L, system T, system A and system ASC [2,3]. These transport systems are characterized by several biological properties, including (*a*) affinity for specific substrates, (*b*) dependence on Na^+, and (*c*) inhibition of transport by specific inhibitors [4].

System L is the leucine-preferring Na^+-independent transporter that is responsible for transport of branched-chain and aromatic amino acids. It has a specific inhibitor, the non-metabolizable leucine analogue BCH (2-aminobicyclo[2.2.1]heptane-2-carboxylic acid). Tryptophan is the preferred substrate for system T transport, a Na^+-independent transport system similar to system L [5,6]. System A is most effective in transporting amino acids with short, polar or linear side chains in a Na^+-dependent manner. The non-metabolizable analogues AIB (2-aminoisobutyric acid) and MeAIB (2-methylaminoisobutyric acid) are both transported through this system. Another Na^+-dependent transport system, designated system ASC, preferentially transports alanine, serine and cysteine. It can be differentiated from system A because of its inability to transport *N*-methylated substrates such as MeAIB [2].

Various approaches have been utilized to take advantage of the unique metabolic pathway of melanin synthesis in the treatment of melanoma [7–9]. Tyrosine and dopa analogues have been developed, assuming that they can act as substrates for tyrosinase to produce potentially cytotoxic quinones [7,10–13]. These analogues may be transported into melanoma cells via amino acid transport similar to that of their parent compounds. In our previous study [10], a sulphur homologue of tyrosine, 4-*S*-cysteinylphenol (4-S-CP) (Fig. 1), was synthesized and tested for its incorporation *in vivo*. 4-S-[^{3}H]CP was injected into mice bearing subcutaneous melanoma tumours and, by whole-body autoradiography, shown to be selectively incorporated into these tumours. It is, however, still unclear how and why tyrosine and the tyrosine homologue 4-S-CP are selectively transported in melanoma cells. The present study characterizes the transport of tyrosine *in vitro* for the human melanotic melanoma cell line SK-MEL 23.

MATERIALS AND METHODS

Materials

Minimal essential medium (MEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco (Grand

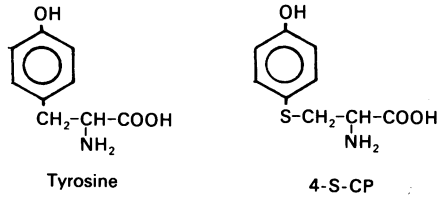

Fig. 1. Structures of tyrosine and its sulphur homologue 4-S-CP

Abbreviations used: 4-S-CP, 4-*S*-cysteinylphenol; MEM, minimal essential medium; FBS, fetal bovine serum; BCH, 2-aminobicyclo[2.2.1]-heptane-2-carboxylic acid; MeAIB, 2-methylaminoisobutyric acid; AIB, 2-aminoisobutyric acid; MSH, melanocyte-stimulating hormone.

* To whom correspondence should be addressed.

Island, NY, U.S.A.). 24-well plates and T-150 flasks (Corning, New York, NY, U.S.A.), were used for culturing the cells. L-[*ring*-3,5-^{3}H]tyrosine (46.7 Ci/mmol) was obtained from NEN (Mississauga, Ontario, Canada) and stored at 4 °C. BCH was obtained from Calbiochem (San Diego, CA, U.S.A.), and the remainder of the inhibitors were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The synthesis of 4-S-CP was reported previously [14].

Cell culture

SK-MEL 23 human melanotic melanoma cells were kindly supplied by Dr. A. N. Houghton of the Memorial Sloan-Kettering Cancer Centre, New York. This cell line was derived from a metastatic lesion of human melanoma and has been characterized as both having of high tyrosinase level and being highly pigmented [15]. These cells were incubated at 37 °C in an atmosphere of 5% CO_2/95% humidity using MEM supplemented with 10% FBS, 100 units of penicillin/ml and 100 μg of streptomycin/ml. After 2–3 days of culture in 150 cm^2 flasks, the cells were collected by trypsin treatment and transferred to 24-well plates, where the assay procedure was performed.

Measurement of tyrosine uptake

Tyrosine uptake was measured using subconfluent cultured cells grown in 24-well plates by techniques similar to those described by Richelson [16]. Briefly, the cells were seeded at a density of 4×10^4 per well and allowed to grow for 2–3 days prior to the procedure being performed. To prepare the cells for the assay procedure, they were washed three times with a transport buffer (pH 7.4) consisting of 140 mM-NaCl, 5 mM-KCl, 5.6 mM-glucose, 0.9 mM-$CaCl_2$, 1.0 mM-$MgCl_2$ and 25 mM-Hepes. The cells were preincubated in this transport buffer for 30 min prior to the initiation of the transport assay. They were then incubated at room temperature for specific periods of time in the uptake medium, made by supplementing the transport buffer with L-[*ring*-3,5-^{3}H]tyrosine and, when appropriate, with unlabelled inhibitors. Transport was terminated by aspiration of the uptake medium and rapidly washing three times with ice-cold transport buffer. NaOH (0.2 M, 1 ml) was added to each well to dissolve the cells. A sample was taken to determine radioactivity by liquid scintillation counting and another was assayed for protein content by the Bradford dye-binding method, using BSA as the standard [17].

RESULTS

Time course of tyrosine uptake

SK-MEL 23 cells were washed and preincubated in the amino acid-free transport buffer for 30 min before initiation of the transport assay. The time course of tyrosine uptake was measured by incubating the cells for up to 60 s in a NaCl or KCl uptake medium containing 0.1 mM-tyrosine (Fig. 2). There was no difference between the time courses with the two uptake media. Therefore all subsequent experiments used the NaCl medium and an incubation time of 20 s to approximate initial rates of transport.

Kinetics of tyrosine transport

The kinetics of tyrosine transport in the SK-MEL 23 cell line was studied at concentrations ranging from 0.05 to 0.8 mM (Fig. 3*a*). Based on the Eadie–Hofstee plot of the data in the presence of Na^+, the K_m was calculated to be 0.164 ± 0.016 mM and the $V_{max.}$ to be 21.6 ± 1.1 nmol/min per mg of protein (Fig. 3*b*).

Inhibition analysis

Inhibition analysis was performed using a tyrosine concentration of 0.1 mM and an inhibitor concentration of 1.0 mM in the uptake media. Of the 13 compounds tested as transport inhibi-

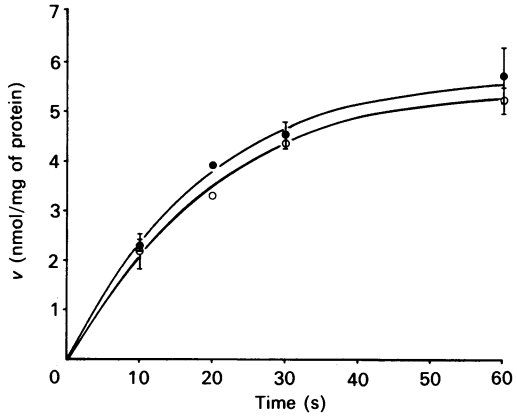

Fig. 2. Time course of tyrosine transport in SK-MEL 23 melanoma cells *in vitro*

The time course of tyrosine transport was measured for up to 60 s at a tyrosine concentration of 0.1 mM in either NaCl (●) or KCl (○) uptake medium. All subsequent experiments used a 20 s incubation to approximate initial rates of transport.

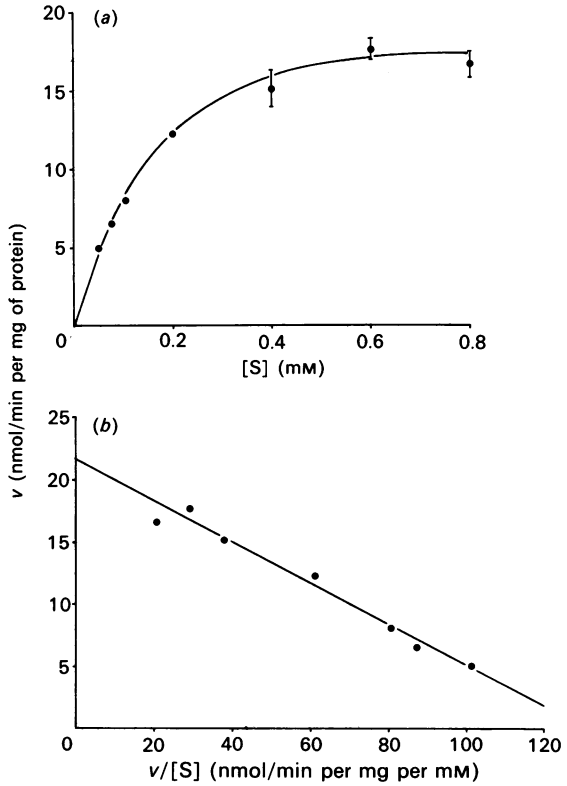

Fig. 3. Concentration-dependence of tyrosine uptake by SK-MEL 23 melanoma cells

(*a*) A tyrosine concentration range of 0.05–0.8 mM was used. (*b*) Eadie–Hofstee plot of the tyrosine transport data used in the calculation of K_m and $V_{max.}$ values. Tyrosine concentrations ranged from 0.05 to 0.8 mM and transport was measured for 20 s in NaCl uptake medium. Liquid scintillation was used to determine the [^{3}H]tyrosine uptake, and protein content was determined using the Bradford dye-binding assay.

Table 1. Inhibition of tyrosine transport into malignant melanoma cells *in vitro* by amino acids and amino acid analogues

Tyrosine transport was measured with 0.1 mM-tyrosine in the presence of 1.0 mM inhibitors. K^+ was used as a replacement for Na^+ in the KCl medium experiments. Values are means ± S.E.M. of triplicate determinations.

	Inhibition (%)	
Inhibitor	NaCl medium	KCl medium
Tryptophan	78.1±1.0	69.2±1.0
Phenylalanine	77.1±1.2	76.1±3.1
Leucine	66.6±0.8	55.0±2.6
BCH	63.0±0.8	62.3±1.4
4-S-CP	61.2±2.8	63.0±2.4
Cysteine	28.2±0.9	0
Alanine	18.6±1.1	14.1±1.4
Cystine	17.7±1.2	27.9±1.6
Arginine	14.2±3.6	0.9±0.9
Serine	13.5±0.6	3.5±1.9
Glutamine	13.3±2.5	3.4±1.7
MeAIB	9.8±1.9	0
AIB	5.8±0.6	0

Table 2. Comparison of tyrosine transport into melanoma cells *in vitro* in the presence or absence of Na^+ in the uptake medium

K^+, in the form of KCl, was used as the replacement for Na^+. Cells were incubated for 20 s in transport buffer supplemented with either 0.1 or 0.6 mM-tyrosine. The cells were washed three times with ice-cold buffer then lysed using 0.2 M-NaOH and samples were taken for determination of [^{3}H]tyrosine by liquid scintillation counting and protein content by the Bradford dye-binding assay. Values are means ± S.E.M. of at least triplicate determinations.

	Tyrosine uptake (nmol/mg of protein)	
[Tyrosine] (mM)	NaCl medium	KCl medium
0.1	2.49±0.12	2.87±0.09
0.6	6.23±0.81	7.02±0.35

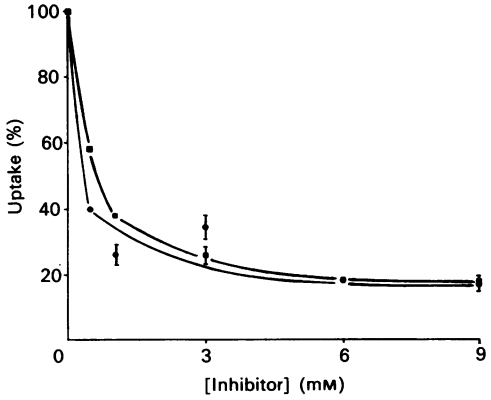

Fig. 4. Effects of inhibitors on tyrosine uptake by SK-MEL 23 melanoma cells

Tyrosine transport was measured using the NaCl uptake medium supplemented with 0.1 mM-tyrosine and 0–9 mM-BCH (■) or -tryptophan (●) as inhibitor.

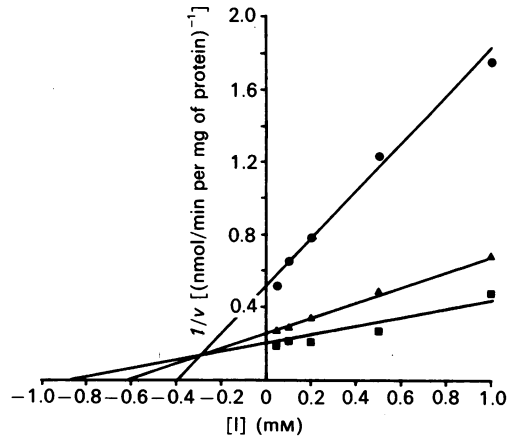

Fig. 5. Dixon plot of inhibition of tyrosine uptake by 4-S-CP

Tyrosine concentrations of 0.05 mM (●), 0.1 mM (▲) and 0.2 mM (■) were used with 4-S-CP concentrations of 0.05–1.0 mM to determine the effect of the addition of this compound on tyrosine transport. The K_i of this competitive inhibition was determined to be 0.288 mM.

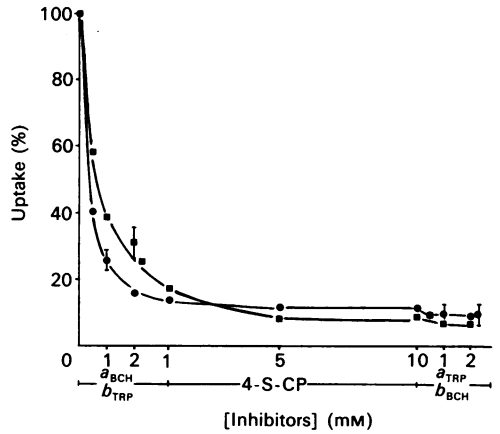

Fig. 6. Effect of BCH, tryptophan and 4-S-CP on tyrosine transport in SK-MEL 23 cells

SK-MEL 23 cells were incubated with 0.1 mM-tyrosine and (*a*) increasing concentrations of BCH, 2.25 mM-BCH plus increasing concentrations of 4-S-CP, or 2.25 mM-BCH plus 10 mM-4-S-CP plus increasing concentrations of tryptophan (TRP) added simultaneously (■, BCH/4-S-CP/tryptophan). (*b*) Effects of the same compounds on tyrosine transport, but with a reversed order of BCH and tryptophan addition (●, tryptophan/4-S-CP/BCH).

tors, the most significant inhibition came from the aromatic amino acids tryptophan and phenylalanine, and from the branched-side-chain amino acid leucine (Table 1). The leucine analogue BCH, a specific inhibitor of system L transport, and the sulphur homologue of tyrosine, 4-S-CP, were also very effective inhibitors of tyrosine transport. The remaining compounds had little or no effect on the transport of tyrosine. These experiments were repeated using K^+, in the form of KCl, as a replacement for Na^+ to produce a Na^+-free buffer. There was little difference in the inhibition observed in the absence of Na^+.

Na^+-dependence of transport

Table 2 directly compares the transport of tyrosine at two substrate concentrations in NaCl and KCl uptake media. As with the time course and inhibition assays, there was no significant difference between transport in the NaCl medium and transport in the KCl medium.

Further inhibitor analysis

Of the effective inhibitors tested, tryptophan and BCH were chosen for further analysis because each has been associated with specific amino acid transport systems in other cell types [2,6]. The tyrosine homologue 4-S-CP was also further analysed because of its close structural similarity to tyrosine and its relatively high inhibition of tyrosine transport.

When tryptophan or BCH was added to 0.1 mM-tyrosine uptake medium at concentrations ranging from 0 to 9.0 mM, both compounds showed the same effect with increasing inhibitor concentrations (Fig. 4). Using a Dixon plot, the K_i for the competitive inhibition of 4-S-CP with tyrosine transport was found to be 0.288 mM (Fig. 5). All three of these inhibitors were then sequentially added at increasing concentrations and the inhibition of tyrosine transport was measured (Fig. 6). Sequential addition of the tyrosine transport inhibitors BCH, tryptophan and 4-S-CP would allow the identification of a transport system that may be masked by the presence of a higher-capacity transporter. The response to the increasing concentrations of the inhibitors was similar even when the sequence of addition of the BCH and tryptophan was reversed.

DISCUSSION

Tyrosine has a unique function in melanocytes and melanoma cells. In addition to its involvement in general protein synthesis, tyrosine is required for the production of melanin pigments. If excess tyrosine is added to the medium of cultured melanocytes or melanoma cells, the products of tyrosine oxidation through the interaction with tyrosinase are potentially toxic [18,19]. Tyrosine and dopa have been suggested to act as bioregulators of the melanogenic apparatus in a similar way as melanocyte-stimulating hormone (MSH) [20–22]. The actual nature of this regulation has yet to be determined, but a hormone-like function for tyrosine has been postulated [20].

The transport of tyrosine and other aromatic amino acids into various cell types, including mouse blastocysts [23], neuroblastoma [24] and melanoma [25], and red blood cells of humans [26,27] and sheep [28], has been studied, but the transport into human melanoma cells has yet to be elucidated. The kinetic data from the present study indicate that tyrosine transport in SK-MEL 23 human melanoma cells has a K_m of 0.164 ± 0.016 mM and a $V_{max.}$ of 21.6 ± 1.1 nmol/min per mg of protein. In the three studies where the effects of NaCl and KCl medium were compared, there appeared to be little change in the transport characteristics. Therefore this transport can be considered to be Na^+-independent.

The Na^+-independence and poor inhibition by AIB and MeAIB rule out the involvement of the Na^+-dependent systems A or ASC in tyrosine transport. Both system L and system T transport aromatic amino acids and are known to have overlapping specificities, with some cell types having only one system [2]. Inhibition by BCH is a characteristic feature of system L transport because of its selectivity for only this transporter. The addition of BCH caused substantial inhibition of tyrosine transport in the melanoma cells. Inhibition by tryptophan has characteristics similar to those with BCH (Table 1, Figs. 4 and 6).

All of our present findings, i.e. substrate specificity, Na^+-dependence, inhibition analysis and kinetics, indicate that the transport of tyrosine into the human melanotic melanoma cell line SK-MEL 23, is similar to that previously described as system L. Tyrosine transport in B16 mouse melanoma cells has been studied and similarly found to be via a single mechanism, system L [25,29]. The difference in methodologies between these studies prevents a direct comparison of all results, but it is apparent that tyrosine transport into these melanin-producing cells is an essential step in the melanogenesis pathway.

Analogues of amino acids have been used as chemotherapeutic agents [10,11,13], with the transport of these analogues into various cell types being implicated, at least partially, in their efficacy. One example is the L-phenylalanine mustard melphalan, used clinically in the treatment of multiple myeloma [30] and malignant melanoma [31]. Melphalan transport has been characterized in normal human lymphoblasts and bone marrow cells, cells of human breast and ovarian cancer [30,32], and in mouse leukaemia cells [33]. Its effectiveness as a cytotoxic agent has been associated with the transport via amino acid transporters, particularly system L and system ASC [30,32].

The sulphur homologue of tyrosine, 4-S-CP, has been synthesized in an effort to utilize the melanogenic pathway in the treatment of melanoma [14]. 4-S-CP was previously shown to be a substrate of tyrosinase and to preferentially accumulate in mouse melanoma tumours, as well as increasing the survival time of tumour-bearing mice *in vivo* [10,14]. In the present study, 4-S-CP was shown to be a competitive inhibitor of tyrosine transport, with a K_i of 0.288 mM, slightly higher than the K_m for tyrosine transport. This would indicate a possible involvement of the tyrosine system in the previously observed selective accumulation and cytotoxicity of 4-S-CP in melanoma cells.

Manipulation of the cells' biochemical needs may allow for the increasing effectiveness of this homologue as a chemotherapeutic agent. It is expected that an enhancement or activation of melanogenesis through administration of MSH, which increases the utilization of tyrosine or dopa for melanin production, may allow greater accumulation of 4-S-CP in melanoma cells.

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REFERENCES

1. Jimbow, K., Fitzpatrick, T. B. & Quevedo, W. C., Jr. (1986) in Biology of the Integument (Matoltsy, A. G., ed.), vol. 2, pp. 278–292, Springer-Verlag, Berlin
2. Shotwell, M. A. & Oxender, D. L. (1983) Trends Biochem. Sci. **8**, 314–316
3. Oxender, D. L., Lee, M., Moore, P. A. & Cecchini, G. (1977) J. Biol. Chem. **252**, 2675–2679
4. Christensen, H. N. (1985) J. Membr. Biol. **84**, 97–103
5. Vadgama, J. V. & Christensen, H. N. (1985) J. Biol. Chem. **260**, 2912–2921
6. Rosenberg, R., Young, J. D. & Ellory, J. C. (1980) Biochim. Biophys. Acta **598**, 375–384
7. Wick, M. M. (1980) J. Invest. Dermatol. **74**, 63–65
8. Thody, A. J., Oliver, I. & Sherbet, G. V. (1988) Eur. J. Cancer Clin. Oncol. **24**, 1879–1884
9. Graham, D. G., Tiffany, S. M. & Vogel, F. S. (1978) J. Invest. Dermatol. **70**, 113–116
10. Jimbow, K., Miura, T., Ito, S. & Ishikawa, K. (1989) Pigm. Cell Res. **2**, 34–39
11. Ito, Y. & Jimbow, K. (1987) Cancer Res. **47**, 3278–3284
12. Ito, S., Kato, T., Ishikawa, K., Kasuga, T. & Jimbow, K. (1987) Biochem. Pharmacol. **36**, 2007–2011
13. Alena, F., Jimbow, K. & Ito, S. (1990) Cancer Res. **50**, 3743–3747
14. Miura, S., Ueda, T., Jimbow, K., Ito, S. & Fujita, K. (1987) Arch. Dermatol. Res. **279**, 219–225
15. Houghton, A. N., Real, F. X., Davis, L. J., Condon-Cardo, C. & Old, L. J. (1987) J. Exp. Med. **164**, 812–829
16. Richelson, E. (1973) Anal. Biochem. **52**, 563–573
17. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
18. Pawlek, J. M. (1976) J. Invest. Dermatol. **66**, 201–209
19. Pawlek, J. M. & Lerner, A. B. (1978) Nature (London) **276**, 627–628
20. Slominski, A. & Paus, R. (1990) J. Theor. Biol. **143**, 123–128

21. Slominski, A., Moellmann, G., Kuklinska, E., Bomirski, A. & Pawelek, J. (1988) J. Cell Sci. **89**, 287–296
22. Slominski, A. (1989) Life Sci. **45**, 1799–1803
23. Van Winkle, L. J., Christensen, H. N. & Campione, A. L. (1985) J. Biol. Chem. **260**, 12119–12123
24. Richelson, E. (1974) J. Biol. Chem. **249**, 6218–6224
25. Saga, K. & Shimojo, T. (1982) J. Biochem. (Tokyo) **92**, 343–355
26. Young, J. D., Wolowyk, M. W., Jones, S. M. & Ellory, J. C. (1983) Biochem. J. **216**, 349–357
27. Hoare, D. G. (1972) J. Physiol. (London) **221**, 311–329
28. Young, J. D., Jones, S. M. & Ellory, J. C. (1980) Proc. R. Soc. London B **209**, 355–375
29. Jara, J. R., Martinez-Liarte, J. H., Solano, F. & Penafiel, R. (1990) J. Cell Sci. **97**, 479–485
30. Begleiter, A., Lam, H. P., Grover, J., Froese, E. & Goldenberg, G. J. (1979) Cancer Res. **39**, 353–359
31. Souhaami, R. & Peters, W. (1986) Clin. Haematol. **15**, 219–234
32. Dufour, M., Panasci, L. C., St. Germain, J. & Boulet, L. (1985) Cancer Chemother. Pharmacol. **15**, 125–131
33. Vistica, D. T. (1979) Biochim. Biophys. Acta **550**, 309–317

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