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INHIBITION OF MELANOGENESIS BY BMY-28565, A NOVEL COMPOUND DEPRESSING TYROSINASE ACTIVITY IN B16 MELANOMA CELLS

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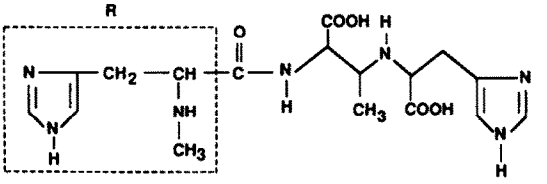
Abstract—The mechanism of a novel melanin synthesis inhibitor, BMY-28565, was studied using mouse B16 melanoma cells. This compound was active in depressing the intracellular accumulation of melanin with an IC_{50} of 5 μM. At dose levels causing no cytotoxicity, the melanolytic effect of this compound was correlated strongly with depression of the enzymatic activity of tyrosinase (monophenol oxygenase, EC 1.14.18.1), the key enzyme in the melanin synthesis pathway. Transcription of the tyrosinase gene was not inhibited by BMY-28565, as determined by RNA blotting analysis. BMY-28565 and three other active derivatives of this compound caused increased glycosylation of proteins in B16 melanoma cells, as assessed by radioactive mannose incorporation. It is, thus, suggested that the mechanism of inhibition of tyrosinase might be related to modifications of the sugar moiety of this enzyme or of a protein(s) that is essential for the expression of its enzymatic activity.

Melanins are widespread bathochromic aromatic polymers which are found both in animals and in plants. Neuromelanins are thought to be formed by the autoxidation of dopamine [1] whereas most of the melanins found in skin melanocytes, hair follicles, eyes and in pigmented melanomas are synthesized by a distinct biochemical pathway involving the progressive oxidation of tyrosine [2]. Tyrosinase (monophenol oxygenase, EC 1.14.18.1) is a membrane-bound glycoprotein that catalyses the first two steps of melanogenesis [3] and deficiency of this enzyme is known to be associated with albinism [4].

A few anti-melanogenic reagents, such as monobenzone and hydroquinone, are clinically useful. The former is used for permanent depigmentation in patients with generalized vitiligo who are not responsive to methoxsalen or trioxsalen photochemotherapy, and who wish to be one color [5–7]. The latter is used to lighten localized areas of darkened skin in patients with melasma, postinflammatory hyperpigmentation and severe freckling [7]. Their mechanism of action is due to selective cytotoxicity on skin melanocytes through the generation of reactive radical metabolites [7]. During the course of a screening for melanin biosynthesis inhibitors, we noticed that one compound (BMY-28565 = Feldamycin) [8, 9] isolated from *Streptomyces calvus*, strain N924-1, had a strong depigmenting activity on *S. bikiniensis* as well as mouse B16 melanoma cells [10]. In this communication, the depigmenting activity of this compound was investigated in terms of its mechanism of action. The data suggest that BMY-28565 constitutes the prototype of a new class of depigmenting agents acting through indirect modulation of the enzymatic activity of tyrosinase.



BMY-28565

Fig. 1. Chemical structure of BMY-28565. The chemical group indicated as "R" represents the part of the molecule which is structurally modified in BMY-28565 analogs (see also Table 1).

MATERIALS AND METHODS

Cell culture and drug treatment. BMY-28565 is originally a natural product obtained from the fermentation broth of *S. calvus*. BMY-28700, BMY-28769 and BMY-28733 are semisynthetic structural analogs (see Fig. 1 for their respective chemical structures) and they were synthesized by methods described elsewhere [9]. F10, a clone of B16 melanoma (obtained from Dr R. Giavazzi, Istituto di Ricerche Farmacologiche "Mario Negri Bergamo", Italy) was cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum. The semi-confluent B16 cells (2.5×10^4 cells) were plated in a dish (3.5 cm in diameter) with 2 mL culture medium and after 3 hr, the compound to be tested was added.

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The control and drug-treated cells were harvested and used for subsequent analyses.

Measurement of melanin content and tyrosinase assay. Cells were lysed in phosphate-buffered saline 1% NP-40 solution (4×10^6 cells/mL) with a Potter–Elvehjem Teflon/glass homogenizer. An aliquot of the homogenate was used for the measurement of the intracellular melanin content, according to Siegrist and Eberle [11], using a Beckman DU 65 spectrophotometer equipped with a 100-μL quartz cuvette. The remaining portion of the homogenate was centrifuged at 2000 *g* for 5 min and the resultant supernatant was used for tyrosinase assay. The measurement of tyrosinase activity is based on tyrosine hydroxylation, using L-[3,5-^{3}H]tyrosine (Amersham International, Amersham, U.K.) as a substrate according to Townsend *et al.* [12] with minor modifications [12]. The assay was performed using 25 μL of cell homogenate, 80 nmol L-tyrosine, 40 nmol L-DOPA and 25,000 dpm of L-[3,5-^{3}H]-tyrosine. Preparation of the reagents, reaction buffers, the elimination of unreacted tyrosine by charcoal and Dowex column were exactly as described previously [12]. The reaction was performed at 37° for 1 hr instead of 3 hr, due to the high content of tyrosinase in B16 melanoma cells. The assay was carried out in conditions of linearity in respect to proteins and time of incubation. One mU of tyrosinase is defined as the activity of enzyme that catalyses the oxidation of 1 nmol of tyrosine in 1 min. Therefore, the tyrosine hydroxylase activity (mU) is calculated as:

$$\frac{80.0 \text{ nmol} \times (\text{sample dpm} - \text{blank dpm})}{(\text{total dpm} - \text{blank dpm}) \times 0.5 \times 60 \text{ min}.}$$

In the formula shown above, the factor 0.5 is introduced because there are two atoms of tritium in L-[3,5]tyrosine but only the tritium in the 3 position is lost during the conversion of tyrosine to DOPA.

Protein concentrations were determined by the Bradford method [13] with a Bio-Rad kit using bovine serum albumin (Fraction V, the Sigma Chemical Co., St Louis, MO, U.S.A.) as a standard. Concentrations of BMY-28565 inhibiting 50% of intracellular melanin content and tyrosinase activity (IC_{50}) were calculated using the non-linear fitting computer program "ALLFIT" [14].

RNA blotting analysis. Total RNA was prepared according to the guanidiumthiocyanate/cesium chloride method [15] from B16 melanoma cells grown for 4 days in the absence or in the presence of BMY-28565. RNA samples were electrophoresed on a 1.2% agarose gel containing formaldehyde [15] and transferred to a Zeta probe nylon membrane (Bio-Rad, Richmond, CA, U.S.A.). The RNA blot was probed with a 1530 bp *Eco*RI-*Pst*I fragment of the mouse tyrosinase cDNA isolated from B16 melanoma cells [16]. Labeling of the probe was carried out according to Feinberg and Vogelstein [17]. Hybridization and subsequent washings of the membrane were performed according to the instructions of the manufacturer. The membrane was dried and exposed to a Kodak-Xomat X-ray film with two intensifying screens (Dupont Cronex, Dupont De Nemours, F.R.G.) at −70°.

Radioactive mannose incorporation. Cells were plated and treated with the appropriate compound as described above. After 3 days of incubation, medium was removed and replaced with fresh medium alone or with the test compound and [^{3}H]-mannose (2 μCi/mL) (Amersham). Cells were further incubated for the required amount of time, washed twice with Dulbecco's phosphate-buffered saline (Gibco, Grand Island, NY, U.S.A.) and harvested by trypsinization. After counting cells, proteins were precipitated with 10% trichloroacetic acid (TCA) and washed successively with 70, 90 and 100% ethanol. The washed pellet was dissolved in Soluene (Packard, Downers Grove, IL, U.S.A.), and the radioactivity was measured in a liquid scintillation counter (Beckman Instruments, Palo Alto, CA, U.S.A).

RESULTS

The chemical structure of BMY-28565 is shown in Fig. 1. This compound was found to inhibit melanin synthesis in *S. bikiniensis* with an IC_{50} of 1.29 μg/mL [9]. As shown in Fig. 2A, concentrations of BMY-28565 up to 100 μM did not affect the growth of cells. However, higher concentrations of this compound (200 and 300 μM) were cytostatic, reducing the number of cells to 75 and 47% relative to control cells, respectively. The viability (approximately 95–98%), as assessed by Trypan blue exclusion and the morphology as assessed by light microscopy were not influenced by the treatment with BMY-28565 at concentrations of 100 μM. Therefore, the following experiments were carried out at a concentration of 100 μM, at which the drug depressed tyrosinase activity as well as melanogenesis maximally without affecting the viability or the growth rate of the cells. As shown in Fig. 2B, addition of BMY-28565 to the culture medium of B16 melanoma cells for 4 days inhibited melanogenesis in a dose-dependent manner and this decrease in the intracellular melanin content was consistent with the depression of tyrosinase activity. The approximate IC_{50} extrapolated from these data was 12.0 ± 2.0 μM for the effect on melanin and 5.0 ± 1.2 μM for the inhibition of tyrosinase enzymatic activity.

The time course of the effect of BMY-28565 on melanogenesis and on tyrosinase activity is presented in Fig. 2C. After 2 days of incubation with the compound, the intracellular melanin content (and secreted melanin as well, data not shown) started to decrease in parallel with tyrosinase activity and reached less than 20% of the values of non-treated cells after 4 days. The onset of tyrosinase inhibition and consequently the inhibition of melanin accumulation was slow and developed gradually, starting from the second day of incubation.

BMY-28565 itself was unlikely to act simply as a direct inhibitor of the enzyme because addition of the drug to the assay system did not decrease tyrosinase activity significantly. In fact, homogenates of B16 melanoma cells preincubated at 37° for 1 hr in the absence of the drug showed a tyrosinase enzymatic activity of 8.0 ± 1.7 mU/mg of protein (mean ± SD, N = 6). The same homogenates

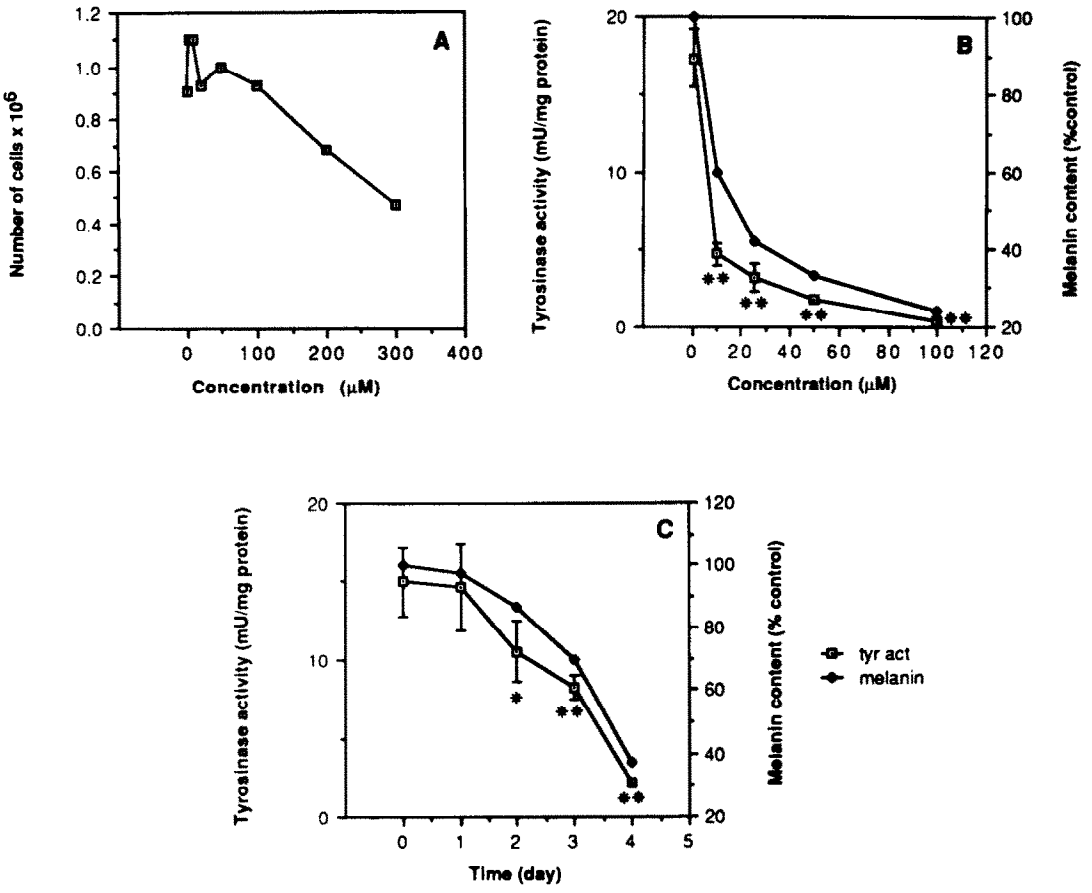


Fig. 2. (A) Effect of various concentrations of BMY-28565 on B16 melanoma cell number. After treatment for 4 days with the indicated concentrations of BMY-28565, B16 melanoma cells were harvested by trypsinization, stained with Trypan blue and counted in a Burker's chamber. Results are expressed as means of the counts in three different fields (SD is less than 10% and is not indicated) obtained from a pool of three different culture wells. (B) Effect of various concentrations of BMY-28565 on tyrosinase activity and melanin content of B16 melanoma cells. After treatment for 4 days with the indicated concentrations of BMY-28565, B16 melanoma cells were harvested by trypsinization and extracts were prepared for the measurement of tyrosinase enzymatic activity and intracellular melanin content. For tyrosinase activity, results are expressed as means ± SE of three separate culture dishes. For melanin content, the value of a pool of three dishes is indicated. (C) Time course of the effect of BMY-28565 on tyrosinase activity and melanin content in B16 melanoma cells. After treatment with 100 μM of BMY-28565 for the indicated amount of time, B16 melanoma cells were harvested by trypsinization and extracts were prepared for the measurment of tyrosinase enzymatic activity and intracellular melanin content. Results are expressed as means ± SE of three separate culture dishes.
Statistically significant (** $P < 0.01$, * $P < 0.05$) relative to control according to Dunnett's test.

preincubated in the presence of 10 and 100 μM BMY-28565 had enzymatic activities of 8.1 ± 10 and 7.6 ± 1.2 mU/mg of protein (means ± SD, N = 6), respectively. This did not rule out the possibility that the compound was acting through an active metabolite(s). To address this point, tyrosinase activity was determined in control cell extracts after addition of various amounts of B16 melanoma cell extract treated with BMY-28565 for 4 days. Even after addition of a 4-fold excess (on a cell basis) of BMY-28565 treated cell extracts, tyrosinase activity was not inhibited. In fact, values of 14.3 ± 1.4 mU/mg of protein in the control samples (means ± SD of three replicates) and 13.3 ± 0.3 mU/mg of protein in the samples fortified with BMY-28565-treated cell extracts (mean ± SD of three replicates) were determined. These data suggest that a diffusible metabolic product of this compound was not responsible for the decrease in enzymatic activity.

To understand whether the depression of tyrosinase activity was due to the regulation of gene expression at the transcriptional level, RNA blotting analysis was performed with total RNA prepared from cells treated with BMY-28565 for 4 days and the level of tyrosinase-specific message was compared to that of control cells. As shown in Fig. 3, the cDNA coding for tyrosinase recognized an approximately 2000 nucleotide-long message in addition to other higher

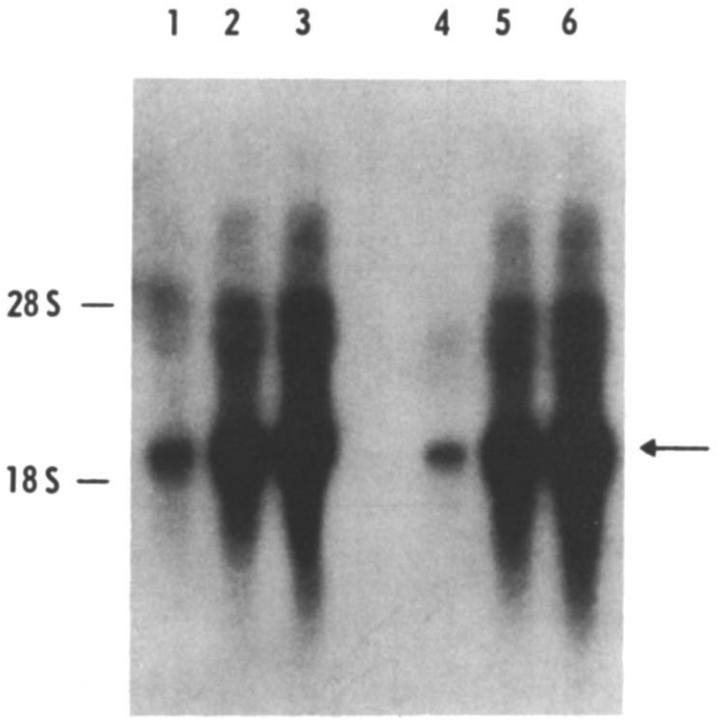


Fig. 3. Effect of BMY-28565 on mouse tyrosinase mRNA level. ^{32}P-labelled mouse tyrosinase cDNA probe was hybridized to total RNA from control and BMY-28565 treated B16 melanoma cells. Lanes 1–3: 5, 10, 20 μg of RNA from control cells, respectively. Lanes 4–6: 5, 10, 20 μg on RNA from BMY-28565-treated cells, respectively. The arrow on the right side of the figure indicates the position of approximately 2000 bases tyrosinase mRNA that constitute the most abundant transcript.

molecular weight messages in RNA samples derived from non-treated and BMY-28565-treated cells, as expected from our previous experiments [16]. However, there was no difference in the amount of tyrosinase-specific messages between the two samples. Therefore, it was concluded that the depression of tyrosinase activity and melanogenesis was not due to a decreased steady state level of tyrosinase message by blockade of tyrosinase gene expression.

Since tyrosinase is known to be a highly glycosylated protein and this post-translational modification is essential for its enzymatic activity *in vivo* [18], the effect of BMY-28565 on the incorporation of ^{3}H-labeled mannose into TCA-precipitable proteins in B16 melanoma cells was studied. As shown in Fig. 4, there was an obvious difference in terms of the incorporation of radioactivity in BMY-28565-treated cells relative to non-treated cells. The incorporated radioactivity of the xenobitic-treated cells was higher than those of control cells by 5.9- and 4.1-fold after incubation with [^{3}H]mannose for 1 and 3 hr, respectively. This difference in [^{3}H]mannose incorporation between control and BMY-28565-treated cells was maintained even after 6 hr. It is to be noticed that similar results were obtained when the mannose incorporation was normalized for the amount of proteins instead of the number of cells (data not shown).

To know whether or not the inhibition of tyrosinase activity by BMY-28565 was related to its ability to increase the level of glycosylation of proteins, [^{3}H]-mannose incorporation was examined in parallel with tyrosinase activity after treatment with different concentrations of BMY-28565. As shown in Table 1, the incorporated radioactivity in the xenobiotic-treated cells was gradually lowered with decreasing amounts of BMY-28565, showing a good inverse correlation between tyrosinase activity and the glycosylation of proteins.

To support the idea that the melanolytic activity of BMY-28565 was inversely correlated to the glycosylation of cellular proteins, similar experiments were performed with three other synthetic derivaties of BMY-28565 whose chemical structures are presented in Table 1. All the compounds depressed tyrosinase activity and melanogenesis albeit with different potencies (data not shown). Table 1 shows the results obtained with each compound at the concentrations that reduce tyrosinase activity to its minimal level without interfering with cell viability. For BMY-28700 and BMY-28733, the same experiments were performed using the same concentration as that used for the most potent compound, BMY-28769. It is obvious that increased glycosylation of cellular proteins and inhibition of tyrosinase are two inversely correlated parameters for each member of this class of compounds. The

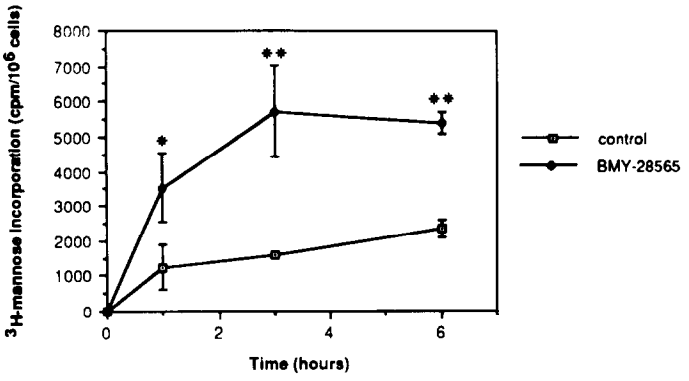
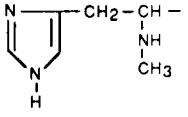
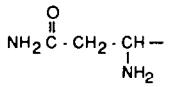
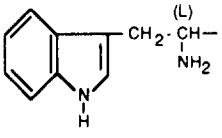
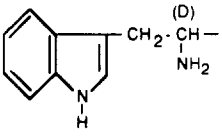


Fig. 4. Time course of the incorporation of [^{3}H]mannose in B16 melanoma cells before and after treatment with BMY-28565. After incubation of the cells in the absence or in the presence of 100 μM of BMY-28565 for 4 days, fresh medium containing the radiolabelled mannose was added and the incubation continued for the indicated amount of time. Mannose incorporation was determined by liquid scintillation counting of TCA-precipitable proteins. Results are expressed as means ± SE of three separate culture dishes.

Statistically significant (** $P < 0.01$, * $P < 0.05$) relative to control according to Dunnett's test.

Table 1. Effect of BMY-28565 and its congeners on tyrosinase activity, melanin content and the incorporation of mannose into proteins

R Group	Compound	Concn (μM)	Tyrosinase activity* (mU/mg protein)	(%)	[^{3}H]Mannose incorporation† (cpm/10^6 cells)	(%)
	Control	—	15.10	100	4554 ± 253	100
	BMY-28565	100	0.62	4.1	12,270 ± 355§	269
		50	1.92	12.7	9839 ± 3606‡	216
		25	2.54	16.8	7968 ± 978§	174
		10	5.18	34.3	5969 ± 600	131
	BMY-28700	50	0.37	2.5	13,509 ± 1646§	296
		25	3.48	23.0	8030 ± 1823§	176
	BMY-28769	25	0.94	6.2	15,166 ± 917§	333
	BMY-28733	400	1.36	9.0	10,442 ± 2888‡	229
		25	5.43	36.0	5251 ± 1446	115

* Results are obtained by determination of tyrosinase activity in a pool of three culture dishes. One milliunit of tyrosinase is defined as the activity of the enzyme that catalyses the oxidation of 1 nmol of tyrosine in 1 min.

† Results are expressed as means ± SE of three separate culture dishes.

‡ Statistically significant ($P < 0.05$) relative to control according to Dunnett's test.

§ Statistically significant ($P < 0.01$) relative to control according to Dunnett's test.

relationship between these two parameters ($r = 0.92$) is evident in Fig. 5, where the data appearing in Table 1 are represented graphically.

DISCUSSION

Some of the well known depigmenting agents include derivatives of hydroquinone and cathecol. Topical application of these compounds seems to cause selective cytotoxicity to skin melanocytes through the generation of reactive free radicals during the oxidation of these substances by the enzyme tyrosinase [7]. These two classes of compounds, however, are not active in all the clinical

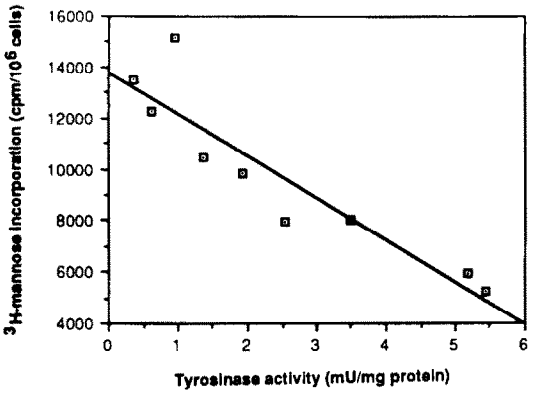


Fig. 5. Correlation between tyrosinase activity inhibition and the increase of glycosylation in proteins. The experimental values presented in this graph were obtained from Table 1. The correlation coefficient between the two parameters taken into consideration is 0.92 which was obtained by linear fitting of the data using the Cricket Graph software (Apple Computers, Cupertino, CA, U.S.A.).

situations where demelanizing treatment is required [7]. A search for melanin synthesis inhibitors with different mechanisms of action is thus called for.

In this report, we describe in detail the possible mechanism of action of the prototype of a novel class of depigmenting compounds, BMY-28565, on B16 mouse melanoma cells. BMY-28565 dramatically depressed tyrosinase activity and intracellular melanin content in the micromolar range at concentrations that were not cytotoxic to the cells. Optical microscopy demonstrated that the morphological appearance of B16 melanoma cells was not influenced by treatment with BMY-28565 (data not shown). Electron microscopy, however, showed a significant decrease in mature melanosomes again without significantly altering their morphology.

This compound is not a direct inhibitor of tyrosinase since it did not decrease the activity of the enzyme in cellular extracts. Moreover, it did not act through the down regulation of tyrosinase gene expression because the steady state level of tyrosinase mRNAs was not influenced. The data obtained suggest that this compound inhibits tyrosinase activity through post-translational modification of the enzyme itself or of other modulatory protein(s). This inhibition, in fact, is inversely correlated with the ability of BMY-28565 to cause an increase in the glycosylation of cellular proteins or glycoproteins. Moreover, this inverse correlation was observed for other compounds with similar chemical structure showing different depigmenting potencies. If protein glycosylation is indeed directly linked to tyrosinase inhibition, it would be interesting to know whether this enzyme is a target protein, in this respect. It is tempting to speculate that quantitative and perhaps qualitative modifications of the glycosylation pattern of tyrosinase or other melanosome proteins induced by BMY-28565 and its structural analogs might influence the activity of this enzyme by altering its export or localization to the melanosome itself [19].

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