Drug Effects in Melanoma: Tumor-Specific Interactions of Proflavine and Ethidiumbromide

B.-R. BALDA AND G. D. BIRKMAYER

Departments of Dermatology and Cell Biology of the University of Munich, Munich, Germany

INTRODUCTION

Drugs which act as metabolic inhibitors are of increasing importance for bio-chemical–pharmacological research. This is comparable to the development of radionuclids some years ago, but it is necessary in this case that they act specifically. From experiments with such compounds information can be obtained on the number and kind of metabolic pathways as well as on the mechanisms of structural relations at the molecular level. In other words, the value of these inhibitors for therapeutic research has two aspects: (1) they are *per se* potential therapeutics simply by their characteristic inhibitory effect, and (2) the elucidation of metabolic disturbances leads to an understanding of the pathogenesis and sometimes to the etiology of a given disease. This could be the basis for further therapeutic experiments.

To find a useful therapy for human malignant melanoma we studied the effects of proflavine and ethidiumbromide on the hamster melanoma A Mel 3 of Fortner as a model.

MATERIALS AND METHODS

All methods used here were described in detail previously (1–3). Radioactive-labeled chemicals were obtained from Radiochemical Centre, Amersham, England, all other chemicals from Serva, Heidelberg, Germany. Proflavine was purchased from Fluka AG, Buchs/SG, Switzerland.

The hamster melanoma A Mel 3 of Fortner was propagated serially by sc inoculation of 1.5–3.0×10^6 cells into female golden Syrian hamsters weighing 80–100 g.

Subcellular particles were obtained after tumor homogenization in 0.25 M sucrose containing 10 mM Tris/HCl and 2 mM EDTA at pH 8.3 followed by differential centrifugation. To obtain the "purified" virus fraction the microsomal fraction was layered on a discontinuous sucrose gradient (50%/35%/20%) and centrifuged to equilibrium.

Incubation mixture for RNA-directed DNA synthesis contained in micromoles: 8 of Tris/HCl (pH 8.3), 1 of $MgCl_2$, 6 of KCl, 3 of dithiothreitol, 0.2 each of the

unlabeled deoxyribonucleoside triphosphates (dATP, dGTP, dCTP), and 0.01 of dTTP. 25 μliters of [³H]dTTP (2 mCi/ml) were added and when indicated ½ O.D. unit poly dT:rA. Particle protein was not more then 150 μg, suspended in 10 nM Tris/HCl (pH 8.3), 150 mM NaCl, and 2 mM EDTA. The final concentration of Nonidet P 40 was 0.08% in the final volume of 125 μliters. After 30 min at 37°C the reactions were stopped by addition of 10% perchloric acid containing 50 mM sodium pyrophosphate, the precipitates washed in ethanol and counted, using PBD scintillator.

For nucleic acids extraction in a volume of 1.0 ml the samples were incubated with 1% pronase in Tris/HCl (pH 8.3) and thereafter extracted three times by phenol/m-cresol/8-hydroxyquinoline/water 500:70:0.5:55 (w/w) and 0.5% final concentration of sodium dodecylsulfate. The aqueous phase was washed four times with ether and the nucleic acids were precipitated by addition of 2 vol of absolute ethanol, recrystallized, and lyophilized. The dried nucleic acids were redissolved in 10 mM Tris/HCl (pH 8.0), 20 mM NaCl, and 2 mM EDTA and further characterized by isopycnic centrifugation in a linear gradient formed from 15 to 30% glycerol. Concentrations of nucleic acids were calculated from their absorbance ratio at 260 and 280 nm.

The subcellular fractions were examined by electron microscopy after fixation in 1% OsO₄ in 0.1 M cacodylate buffer (pH 7.2) and postfixation in 0.5% aqueous uranyl acetate, for 1 hr each, dehydration in ethanol and embedding in Epon 812. Thin sections were cut on a LKB ultrotome III with a diamond knife, mounted on copper grids covered with collodium and carbon films, stained with magnesium uranyl acetate and lead citrate and examined in a Siemens Elmiskop IA or 101 operated at 80 kV with a 50 μm objective aperture. The *in vivo* experiments are described in the text.

RESULTS

1. *Survival Time*

Tumor-bearing animals receiving 2.4 mg proflavine dissolved in drinking water each day (from the time of sc inoculation with tumor cells until they died) survive about twice as long as untreated ones (Table 1). Depending on dosage and time schedule, the survival time can be enlarged up to 10 wk after sc injection of the drug.

2. *RNA-Directed DNA Synthesis*

Subcellular particles of the hamster melanoma exhibit an RNA-directed DNA polymerase activity (3, 4). The isolated RNA–DNA hybrid is indeed strong evi-

TABLE 1

EFFECT OF PROFLAVINE ON THE SURVIVAL TIME (A MEL 3)[a]

	Survival time	
	Days after tumor inoculation	Factor
Control (untreated)	~14	—
Proflavine treated	~27	×1.9

[a] All animals were inoculated sc by 1.0–2.6 × 10⁶ melanoma cells. Proflavine was given orally in a dosage of 2.4 mg per day and animal.

dence that this enzymic activity is dependent on high molecular weight RNA (4). The extent to which radioactivity was incorporated into polynucleotide, expressed as specific radioactivity, correlated with the source of the enzyme. For example, the microsomal fraction gave unsatisfactory results because of high levels of non virus-associated RNase and DNase activities. Virus particles are rare in this fraction, indicated by electron microscopical controls. Therefore we used a fraction
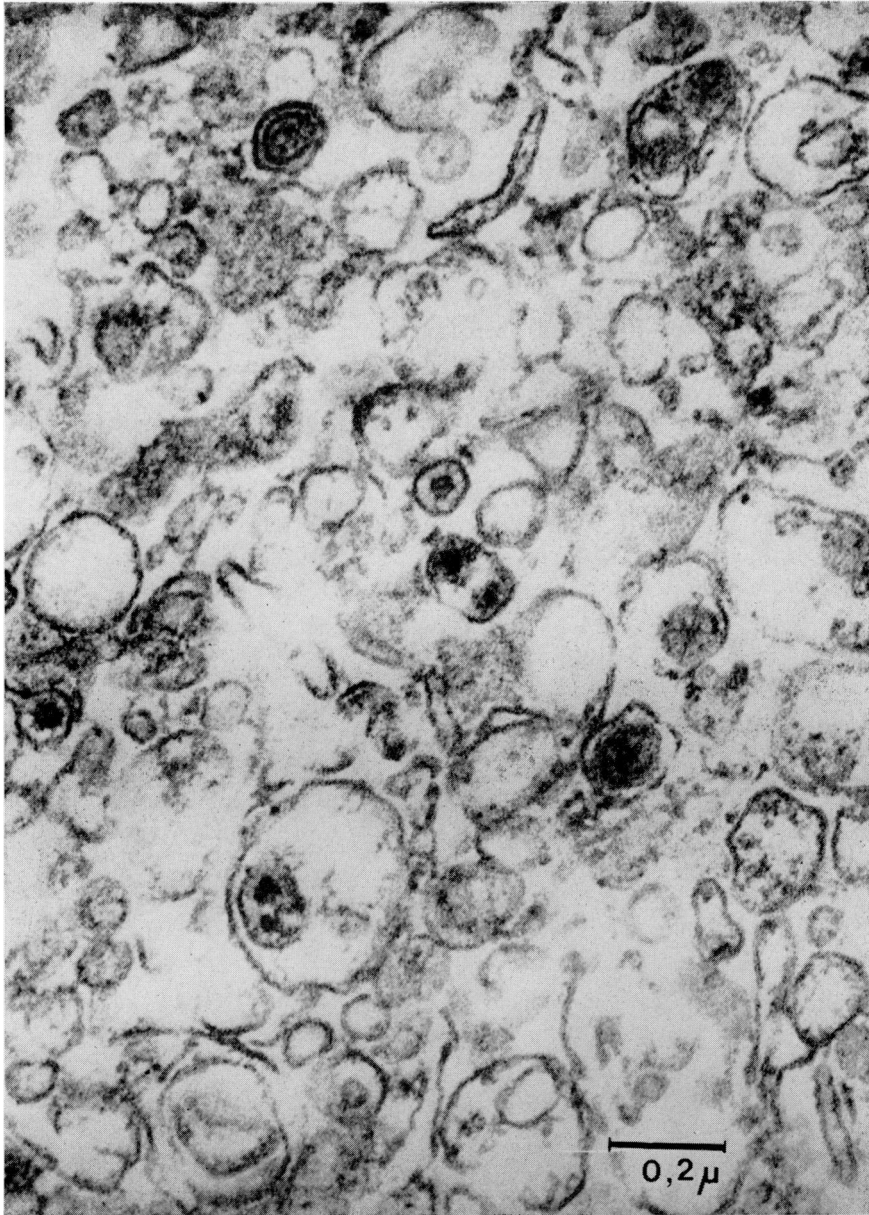

FIG. 1. Electron micrograph of the "purified" virus fraction as obtained from density gradient centrifugation in sucrose (50/35/20%). Virus particles are recognizable by the electron-dense nucleoid. ×100,000.

TABLE 2

EFFECT OF PROFLAVINE ON THE RNA-DIRECTED DNA SYNTHESIS (A MEL 3)[a]

	cpm/mg protein	% Inhibition
Standard assay (+poly dT:rA)	4250	—
+Proflavine (20 µg/ml)	2950	30.0
+Proflavine (40 µg/ml)	1380	68.0
+Proflavine (80 µg/ml)	570	86.5
+Proflavine (100 µg/ml)	158	96.2

[a] For details see Material and Methods.

TABLE 3

EFFECT OF ETHIDIUMBROMIDE ON THE RNA-DIRECTED DNA SYNTHESIS (A MEL 3)[a]

	cpm/mg protein	% Inhibition
Standard assay (without poly dT:rA)	650	—
+Ethidiumbromide (20 µg/ml)	550	15.0
+Ethidiumbromide (40 µg/ml)	324	50.0
+Ethidiumbromide (80 µg/ml)	272	58.0
Standard assay (+poly dT:rA)	4250	—
+Ethidiumbromide (20 µg/ml)	2150	50.0
+Ethidiumbromide (40 µg/ml)	1270	70.0
+Ethidiumbromide (80 µg/ml)	425	90.0

[a] For details see Material and Methods.

enriched with viruses, which could be obtained after an additional discontinuous density-gradient centrifugation step (4). The electron micrograph of this fraction (Fig. 1) shows an appreciable number of virus particles besides numerous reaggregated membranes.

Another important consideration is the requirement of the nonionic detergent Nonidet P 40. The optimal assay concentration is about 0.1%.

Small amounts of proflavine (20 µg/ml) added to the incubation mixture inhibit remarkably the RNA-directed DNA synthesis. Depending on dosage, the reaction is almost completely inhibited (Table 2). Ethidiumbromide has the same, perhaps a little stronger, effect (Table 3). It is of interest that the inhibition is more evident after stimulating the enzyme assay by poly dT:rA (Table 3).

3. [³H]-*Uridine Incorporation into RNA*

[³H]-uridine (0.2 mCi/animal) administered sc is, after 2 hr, incorporated into RNA species, which could be isolated from the mitochondrial and microsomal fraction and also from the virus-enriched fraction. Density-gradient centrifugation of the total RNA from all these fractions shows the presence of a high molecular weight RNA (3). When ethidiumbromide in a dosage of 40 mg/kg body weight is injected sc the incorporation of the radioactive labeled nucleoside into RNA is markedly reduced. As shown in Fig. 2, the most markedly reduced RNA species are the 4 S and 70 S RNA.
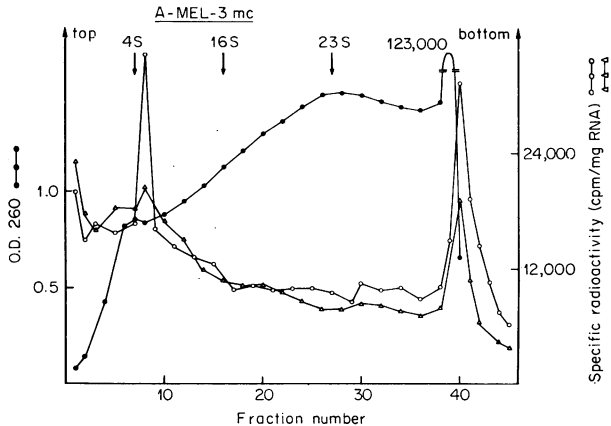
FIG. 2. Sedimentation profile and distribution of radioactivity of [³H]uridine 'labeled RNA from microsomes of melanoma. Closed circles: OD₂₆₀. Open circles: specific radioactivity in the absence of ethidiumbromide. Open triangles: specific radioactivity in the presence of ethidiumbromide. Ethidiumbromide was injected sc 2 hr prior to [³H]uridine in a dosage of 40 mg/kg body weight.

DISCUSSION

Using the acridine dye proflavine and the phenanthridine dye ethidiumbromide special features of the metabolism of the hamster melanoma A Mel 3 were analysed. Biochemical experiments were undertaken to clarify the twice-as-long survival time of proflavine-treated tumor-bearing animals in comparison to untreated ones.

As previously described, the *in vivo* incorporation of [³H]phenylalanine into mitochondrial and microsomal proteins of the melanoma is reduced by about 90% when proflavine is administered 60 min prior to phenylalanine (5). We had also found a tumor-characteristic and proflavine-sensitive protein synthesis (2). In principle these findings could be explained by the action of degradative enzymes in the melanoma, which become active by accidental derepression of some genes, but we have no support for it at the moments.

However, it is known that viruses can be detected in animal melanomas (6–8). Therefore, we checked the sensitivity of the tumor tissue against proflavine and ethidiumbromide. The two main characteristics of oncogenic RNA viruses are a RNA-directed DNA polymerase, the so-called reverse transcriptase (9), and a single-stranded high molecular weight or 70 S RNA (10). In addition to the previously described properties of this RNA-directed DNA synthesis (4), it is greatly of interest that this reaction could be strongly inhibited *in vivo* by proflavine and ethidiumbromide. These drugs also inhibited the *in vivo* synthesis of both high molecular weight and 4S RNA.

All these findings do not agree with the hitherto known proflavine and ethidiumbromide action on the molecular level. These two drugs are well known as intercalating agents for double-stranded circular DNA (11–15). More recently, Finkelstein and Weinstein and Weinstein and Finkelstein (16, 17) were able to show that tRNA species could also interact with proflavine.

From these results we may suggest the following more detailed mode of action of proflavine and ethidiumbromide at the molecular level in virus-transformed cells
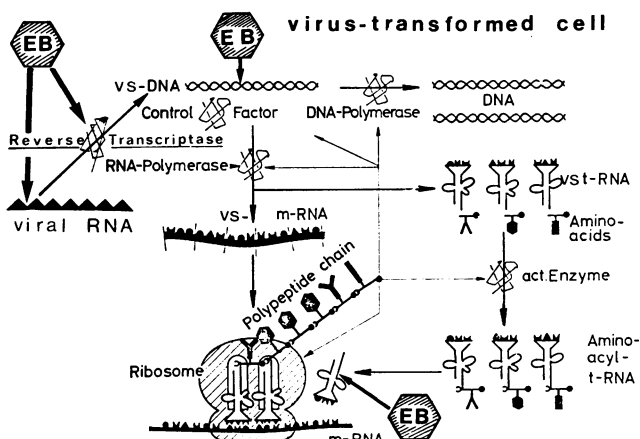
FIG. 3. The supposed action of proflavine and ethidiumbromide (both indicated by EB) at the molecular level in virus-transformed cells. For details see discussion.

(Fig. 3). First, the drugs act preferentially with double-stranded circular DNA (in normal cells mitochondrial DNA represents such a species). As found previously, the synthesis of mitochondrial DNA in the hamster melanoma is very strongly inhibited (1). Not clear at present is whether or not the drugs intercalate with such DNA sequences which contain viral sequences, called oncogens, indicated here as VS-DNA. Second, the dyes can interact with tRNA species. In this way regulatory functions (16) and the protein synthesis at the ribosomal level are blocked. It is supposed that most of 4 S RNA contains virus-coded species. Third, the 70 S RNA interacts with the dyes. Compared to other mammalian RNA species this implicates structural and base-sequence abnormalities of the 70 S RNA. Probably this single-stranded, high molecular weight RNA is at least partially double-stranded. Finally, the RNA-directed DNA polymerase probably interacts at least partially with the inhibitors, so that this enzyme may also be structurally peculiar.

In summarizing the presented results, it should be emphasized that the viral etiology of the hamster melanoma becomes clearer and the drugs used are potentially therapeutic for this tumor because of their specific action. For use in man they are at present too toxic, though slight change in the molecule may provide a useful drug. Preliminary results suggest a similar inhibitory effect on the RNA-directed DNA synthesis of these drugs in human melanoma (17). This is consistent with previously described observations of virus particles present in lymph node metastases of human melanomas detected by electron microscopy (18).

SUMMARY

Using the hamster melanoma A Mel 3 of Fortner as a model for the human malignant melanoma the effects of two dyes, proflavine and ethidiumbromide, were studied. Both drugs given *in vivo* prolong the survival time and inhibit the synthesis of 4 S and 70 S RNA. They also act as very strong inhibitors of the RNA-directed DNA synthesis *in vitro*. A specific action of these drugs on the oncorna-virus transformed melanoma cells is assumed. Preliminary results parallel these findings for the human melanoma also.

ACKNOWLEDGMENTS

REFERENCES

1. Balda, B.-R. and Birkmayer, G. D. Der Einfluss von Proflavin auf den DNS-Stoffwechsel des Hamstermelanoms. *Arch. Klin. Exp. Derm.* **239**, 176 (1970).

2. Birkmayer, G. D. and Balda, B.-R. Evidence for proflavine sensitive proteins in malignant hamster melanoma. *Hoppe Seyler Z. Physiol. Chem.* **352**, 780 (1971).

3. Birkmayer, G. D., Miller, F., and Balda, B.-R. Inhibition of high molecular weight RNA synthesis in a hamster melanoma by ethidiumbromide *in vivo. Hoppe Seyler Z. Physiol. Chem.* **353**, 1749 (1972).

4. Balda, B.-R. and Birkmayer, G. D. RNA-directed DNA synthesis in the hamster melanoma A Mel 3. *Arch. Derm. Forsch.* (in press).

5. Birkmayer, G. D. and Balda, B. R. Proflavine inhibition of protein synthesis in malignant hamster melanoma. *FEBS Lett.* **11**, 221 (1970).

6. Mishima, Y. Melanotic tumors. *In* "Ultrastructure of Normal and Abnormal Skin" (A. S. Zelickson, Ed.), p. 388. Lea & Febiger, Philadelphia, 1967.

7. Epstein, W. L. and Fukuyama, K. Light and electron microscopic studies of a transplantable melanoma associated with virus-like particles. *Cancer Res.* **30**, 1241 (1970).

8. Balda, B.-R., Wolff, H. H., Birkmayer, G. D., and BraunFalco, O. Virus-like particles in the hamster melanoma A Mel 3 of Fortner. *Naturwissenschaften* **57**, 548 (1970).

9. Temin, H. M. and Mizutani, M. S. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. *Nature (London)* **226**, 1211 (1970).

10. Robinson, W. S. and Baluda, M. The nucleic acid from avian myeloblastosis virus compared with the RNA from the Bryan strain of Rous sarcoma virus. *Proc. Nat. Acad. Sci. (Wash.)* **54**, 1686 (1965).

11. Lerman, L. S. Structural considerations in the intercalation of DNA and acridines. *J. Mol. Biol.* **3**, 18 (1961).

12. Lerman, L. S. The structure of the DNA–acridine complex. *Proc. Nat. Acad. Sci. (Wash.)* **49**, 94 (1963).

13. Dannenberg, H. and Sonnenbichler, J. Untersuchungen zur Wechselwirkung zwischen aromatischen Kohlenwasserstoffen und Aminen mit Desoxyribonucleinsäure. *Z. Krebsforsch.* **67**, 127 (1965).

14. Waring, M. J. Complex formation between ethidiumbromide and nucleic acids. *J. Mol. Biol.* **13**, 269 (1965).

15. Lepecq, J. P. and Paroletti, C. A fluorescent complex between ethidiumbromide and nucleic acids. Physical–chemical characterization. *J. Mol. Biol.* **27**, 87 (1967).

16. Gallo, R. C. and Pesta, S. Transfer RNA species in normal and leukemic human lymphoblasts. *J. Mol. Biol.* **52**, 195 (1970).

17. Balda, B.-R. and Birkmayer, G. D. in preparation.

18. Birkmayer, G. D., Balda, B.-R., Miller, F., and BraunFalco, O. Virus-like particles in metastases of human malignant melanoma. *Naturwissenschaften* **59**, 369 (1972).