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PHOTOLYSIS OF 2-AZIDOFLUORENE IN SITU AS A PROBE IN CHEMICAL CARCINOGENESIS: BYPASS OF REQUIREMENT FOR METABOLIC ACTIVATION

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SUMMARY: 2-Azidofluorene was synthesized to serve as a specific photoaffinity label in chemical carcinogenesis studies. The drug exhibited little toxicity, and was non-transforming in the C3H 10T½ CL8 cells. When photolysed at 360 nm in water to generate N-hydroxy-2-aminofluorene, then added to the cells, the drug again exhibited little toxicity but was very weakly transforming. When it was irradiated inside the cells cytotoxicity was enhanced greatly. Both Types II and III transformed foci were observed in transformation experiments and were comparable to those obtained with N-acetoxy-2-acetylaminofluorene. Irradiation alone was neither toxic nor transforming. These results suggest that photoaffinity labeling is a promising tool in studying chemical carcinogenesis by bypassing metabolic activation.

The biological effects of a large number of chemical carcinogens depend, in part, on their metabolic activation and detoxification in target tissues (1). The active metabolites are electrophiles which cause initiation following interaction with nucleophilic centers of critical cellular molecules (1). The precise reactive forms for many carcinogens are not known and this has contributed to the complexity of understanding the molecular basis of chemical carcinogenesis. With benzo(a)pyrene, for example, a large number of metabolites are generated, and until recently, it was not clear which of these metabolites is/are responsible for the mutagenic and/or carcinogenic activities of the drug (2).

To avoid the problem of metabolic activation we have used photoaffinity labeling as a probe for studying carcinogenesis in mammalian cell culture. We have thus synthesized 2-azidofluorene (2-AzF)**, an analog of the established carcinogens 2-aminofluorene (2-AF) and its N-acetyl derivative (2-AAF). Both 2-AF

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**Abbreviations used: 2-AzF, 2-azidofluorene; 2-AF, 2-aminofluorene; NAAAF, N-acetoxy-2-acetylaminofluorene; TPA, 12-0-tetradecanoylphorbol-13-acetate; PE, plating efficiency; 10T½, C3H 10t½ CL8 cells.

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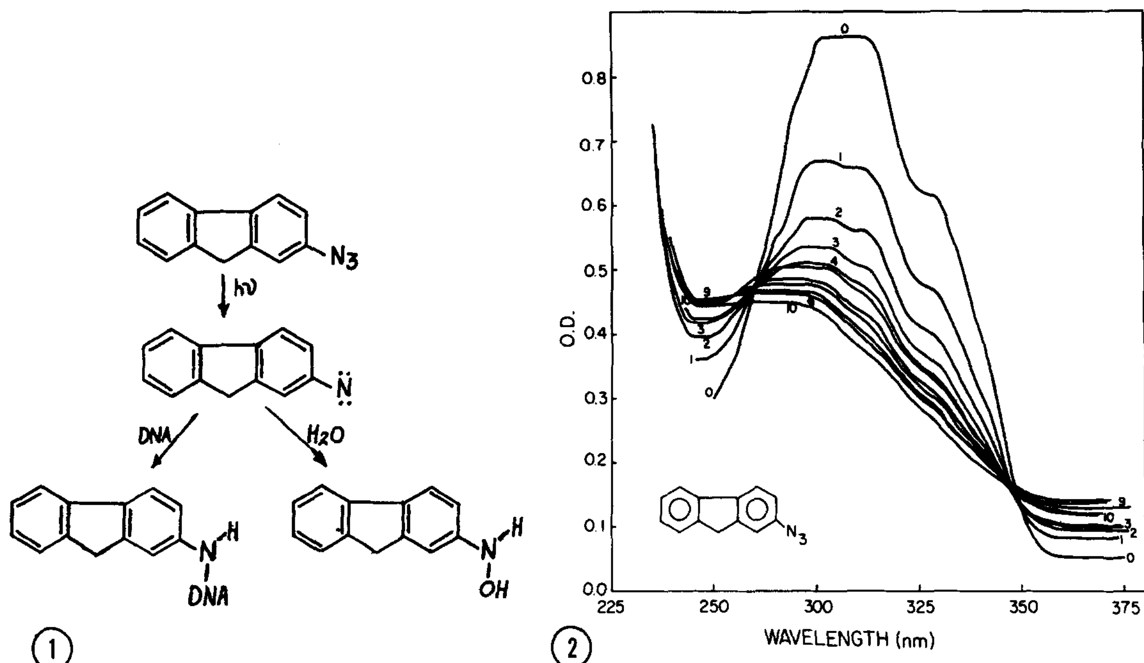


Fig. 1 The interaction of 2-azidofluorene with DNA and water following photolysis at 360 nm.

Fig. 2 Changes in uv spectrum of 2-azidofluorene as a function of irradiation time in water: 10.6 mg of 2-AzF were dissolved in 10 ml dimethylsulfoxide of which 50 μl were dissolved in 50 ml of water. The spectrum was read at various times of irradiation (0-10 min) in a 10 cm cuvette in a Cary 15 spectrophotometer.

and 2-AAF are oxidized to their respective N-hydroxy metabolites and then converted to one of several esters that are believed to be ultimate carcinogens (1). To bypass these processes which are lacking in many in vitro systems (3), 2-AzF is allowed to interact with cellular macromolecules and then photolysed; a short-lived nitrene is generated which covalently attaches the drug to a neighboring group. The interaction of the nitrene with water, on the other hand, leads to an N-hydroxy derivative (Fig. 1). In this communication, we report the utilization of photoaffinity labeling in in vitro carcinogenesis studies. The transformation of the C3H 10T½ CL8 (10T½) mouse embryo fibroblasts, which are sensitive to post-confluence inhibition of cell division (4,5), by 2-AzF when photolyzed in situ at 360 nm is described.

MATERIALS AND METHODS

Synthesis of 2-Azidofluorene: 2-AzF was synthesized from 2-AF (Aldrich Chemical) through a diazonium intermediate as described recently (3).

Cell Culture: Conditions and Transformation Assays: The 10T½ cells were grown in Eagle's basal medium with Earle's salts supplemented with 10% heat inactivated fetal calf serum (Grand Island Biological Co.) in the presence of penicillin and streptomycin (4). Cytotoxicity was determined by the plating efficiency (PE) method as described by Reznikoff et al (5). Percent survival was calculated as the ratio of PE of treated dishes relative to PE of controls. Transformation experiments were carried out by the Reznikoff et al (5) procedure as modified by Bertram (6). Transformed foci of Types II and III were scored six weeks after treatment. The transformation frequency was calculated as the number of transformed foci per 60 mm dish, adjusted for survival.

Irradiation by Near-Ultraviolet Light: Irradiation of 2-AzF in the presence and absence of the cells was carried out using light box fitted wth filtered black ray lamps having maximum output at 360 nm (Buchler Instruments). The lamp was kept 4 cm away from the dishes. The uv light was filtered through the polystyrene covers which absorb greater than 99% of the radiation below 300 nm, essentially allowing light in the 360 nm through. The effect of 12-0-tetradecanoylphorbol-13-acetate (TPA) on irradiated cultures were carried out as described by Mondal and Heideberger (8) adding TPA (0.1 μg/ml) twice weekly.

RESULTS

Irradiation of 2-AzF in Water: The usefulness of 2-AzF as a photoaffinity probe required that it be photosensitive. Its photolysis at 360 nm as function of time of irradiation was measured spectrophotometrically, and as shown in Fig. 2, changes in the spectrum were observed due to the formation of a new species of the probe and the disappearance of the azide. Maximal changes in the uv/vis spectrum were obtained when the photolysis was carried out for 8-10 minutes.

The Cytotoxic Effects of Pre-incubation and Irradiation Times of the Azide on the 10T½ cells: 2-AzF (25 μM) was incubated with the 10T½ cells for 5, 10, or 15 min prior to photolysis for 15 min. After 24 hr the medium was changed and the cytotoxicity was determined 7 days later. As shown in Fig. 3a, the azide reaches its targets within 5 min. To ensure maximum uptake, the cells were incubated with the drug for 10 min prior to photolysis in the remaining experiments. When the cells were incubated with the drug for 10 min, then irradiated for different periods of time, maximal cytotoxicity was induced after 15 min of irradiation (Fig. 3b). Irradiation of the cells in the absence of the drug was virtually nontoxic. The production of substantial toxicity required that the drug be photolyzed *in situ*.

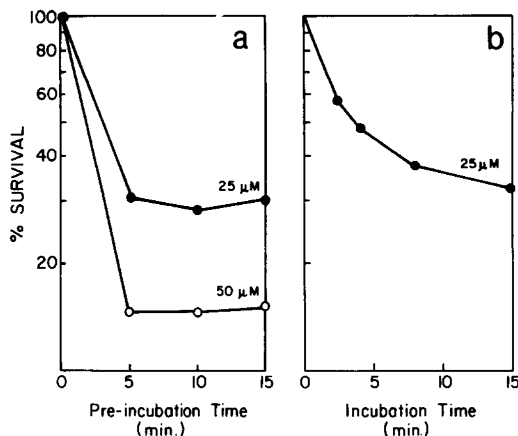
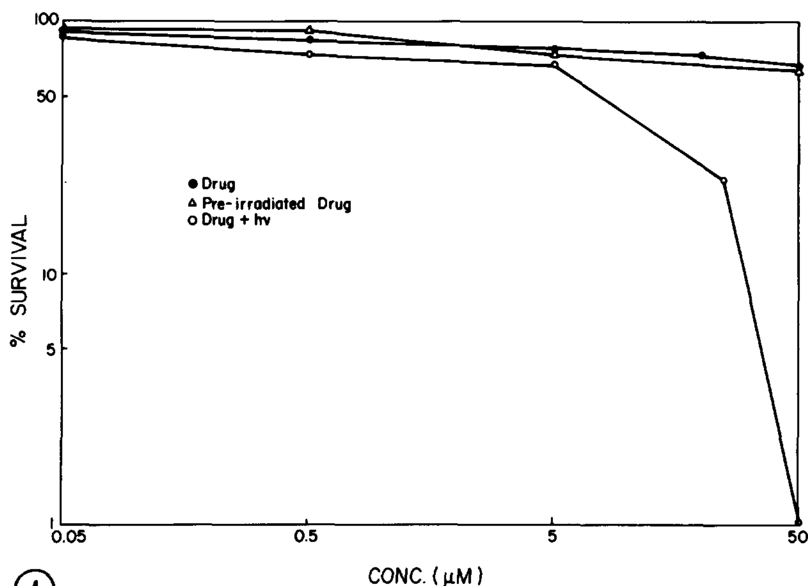


Fig. 3 a. The effect of incubation time of 2-AzF (25 µM) prior to irradiation for 15 min on the cytoxicity against the 10T½ cells.

b. The effect of irradiation time of the 10T½ cells in the presence of 2-AzF (25 µM) on cytotoxicity. The drug was added to the cells in complete fresh medium 10 min before irradiation at 350 nm.

Incubation of cells in the dark either with 50 µM 2-AzF or with 2-AzF irradiated prior to mixing resulted in cell survival of 75%. However when the cells were incubated with the drug for 10 min and then were irradiated cell survival was lowered to 11%. The cytotoxicity profile of the drug plus irradiation was similar to that with N-acetoxy-2-acetylaminofluorene (NAAAF), a postulated ultimate carcinogenic form of 2-acetylaminofluorene in the absence of radiation. These results are presented in Fig. 4 and also in Table I.

Cell Transformation: The data on transformation of 10T½ cells under different conditions are summerized in Table I. The acetone control, uv light or 2-AzF in the dark were not transforming at any concentration. In addition, uv light at 360 nm does not seem to cause initiation, since when followed by TPA treatment no transformation was obtained (7). The pre-irradiated drug, principally N-hydroxy-2-aminofluorene, is weakly transforming, the transformation frequency being less than 10% of that of the drug irradiated in the cells. Also no Type III transformed foci were obtained with the pre-irradiated compound. The drug plus uv light caused significant transformation and both Types II and III foci were ob-



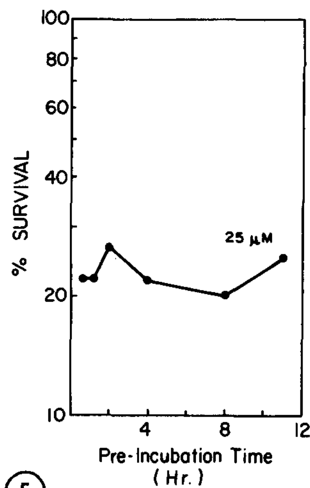


Fig. 4 The cytotoxic effect of 2-AzF on the 10T½ cells in the presence and absence of uv irradiation. The effect pre-irradiated drug is also given. The cells (200/60 mm dish) were seeded 24 h before treatment.

Fig. 5 The stability of the azido group of 2-AzF in the 10T½ cells: 20,000 cells were seeded in 60 mm dishes 24 hr before the addition of the drug (25 μM) in complete fresh medium. The cells were incubated for selected intervals prior to irradiation at 360 nm. Percent survival was calculated as the ratio of the number of cells in the treated dishes relative to the number of cells in appropriate controls incubated for the given period of time and then irradiated for 15 min.

served. The oncogenicity of NAAAF is also reported, and is similar to that of 2-AzF plus light treatment. Type II transformed foci are considered moderately malignant while Type III are highly malignant. Both give rise to fibrosarcomas when inoculated subcutaneously into irradiated C3H mice (5).

Stability of the Azido Group in the 10T½ Cells: 2-AzF (25 μM) was incubated with 20,000 cells in 60 mm dishes in complete medium for selected intervals over a 12 hr period, and then irradiated as described above. The percent survival was determined for each incubation period. As shown in Fig. 5, the cell survival remained essentially unchanged over a number of hours. These results suggest that even if 2-AzF is metabolized by the cells, the azido group is relatively stable under the conditions of the experiment.

TABLE I

Transformation of C3H 10T½ CL8 Cells by 2-Azidofluorene (2-AzF) and N-Acetoxy-2-Acetylaminofluorene (NAAAF)

Treatment	Cytotoxicity % Survival	Transformation Frequency† Type II	Type III	Total
Control	100	0	0	0
hυ (360 nm)	95	0	0	0
hυ (360 nm)+TPA(0.1μg/ml)	95	0	0	0
2-AzF				
5μM	76	0	0	0
25μM	72	0	0	0
Pre-Irradiated 2-AzF				
5μM	78	0.3	0	0.3
25μM	75	0.1	0	0.1
2-AzF + hυ				
5μM	68	1.5	0.4	1.9
25μM	24	2.7	0.8	3.5
NAAAF				
4μM	70	1.7	1	2.7
7μM	50	1.8	1.2	3

†No. of Transformed Foci/Dish, Adjusted for % Survival

DISCUSSION

In this report, we have shown that photoaffinity labeling may be used as specific probes in cell transformation studies. Similar probes have been applied in various areas of molecular biology to study estrogen receptors (8,9), enzyme binding sites (10), mutagenesis (11-13) and DNA repair (12).

Since the azido moiety is relatively stable in the cells in the dark (Fig. 5), photoaffinity probes have the advantage of bypassing metabolic activation and detoxification processes. Another advantage is that with photoaffinity labeling, we can control the timing of the insult since the covalent attachment to the critical cellular targets occurs only when the drug and the cells are irradiated. These advantages will enable us to address ourselves critically to specific problems in chemical carcinogenesis.

The effects of near uv irradiation on the cells are not fully known, but the short interval of exposure apparently does not lead to initiation since post treatment with TPA did not lead to transformation. In comparison, Mondal and

Heidelberger (7) demonstrated that the irradiation of the 10T½ cells in the uv region causes initiation, and that TPA promotes the formation of visible transformed foci. In order to minimize side effects of near uv irradiation on the cells, we used filtered black ray lamp having a maximum output at 360 nm with little emission below 310 nm. In addition, the cells were irradiated through a polystyrene filter which absorbs radiation below 300 nm. Nevertheless, we are currently exploring other probes which can be photolyzed at longer wavelengths and/or require a much shorter time for photolysis.

We believe that photoaffinity labeling is useful in studies in mammalian cell culture as has been proven in bacteria and yeast (11-13).

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