Journal of Cellular Biochemistry 30:1–9 (1986)
Biochemical and Molecular Epidemiology of Cancer 33–41

Quantitative Relationship Between Initiation of Hepatocarcinogenesis and Induction of Altered Cell Islands

William K. Kaufmann, Susan A. MacKenzie, Richard J. Rahija, and David G. Kaufman

Department of Pathology and Lineberger Cancer Research Center, University of North Carolina, Chapel Hill, North Carolina 27514

We have quantified the initiation of hepatocytic neoplasms and the induction of altered cell islands in regenerating livers of rats given a single treatment with one of three carcinogens before or during the peak of DNA synthesis after partial hepatectomy. For up to 20 wk after treating livers during the peak of DNA synthesis with methyl(acetoxymethyl)nitrosamine (DMN-Ac), hepatocytic neoplasms were not seen. Thereafter, in rats fed the liver tumor promoter, phenobarbital, neoplasms emerged continuously so that by 60 wk after initiation, livers held an average of 5.5 neoplasms. Islands of cellular alteration, identified by their abnormal retention of glycogen on fasting, also appeared to emerge continuously between 20 and 60 wk after initiation. By 60 wk, promoted livers contained about 10,000 islands. In DMN-Ac-initiated, phenobarbital-promoted livers, neoplasms and islands maintained a constant numerical relationship over time with about 1,450 islands emerging for every neoplasm that emerged. This ratio of islands to neoplasms differed according to the type of carcinogen used to initiate hepatocarcinogenesis and depending on whether promotion with phenobarbital was included. In livers initiated with DMN-Ac but not promoted with phenobarbital, the ratio of islands to neoplasms was about 7,750:1. In livers initiated by treatment with $(\pm)$-7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene at the peak of DNA synthesis and then promoted with phenobarbital, the ratio of islands to neoplasms was 7,200:1. In livers exposed to gamma rays at the peak of DNA synthesis in regenerating livers and promoted, no neoplasms were seen in our sample although islands could be enumerated. Evaluation of another group of rats irradiated during the prereplicative phase of regeneration revealed two neoplasms in nine treated livers and a ratio of islands to neoplasms of greater than 12,000:1. Thus, when comparing livers treated once with carcinogen and then promoted, this ratio of islands to neoplasms differed considerably according to the carcinogen being tested. These results suggest that the induction of glycogen-retaining hepatocyte islands may not be a quantitative measure of the initiation of hepatocarcinogenesis.

Received April 17, 1985; revised and accepted September 5, 1985.

Key words: hepatocarcinogenesis, initiation, promotion, neoplasm, altered-cell island

The initiation phase of carcinogenesis is phenomenologically defined as that process whereby susceptible target cells are irreversibly altered by a subthreshold dose of carcinogen so that upon subsequent application of a noncarcinogenic stimulus (ie, promotion) neoplasms are produced [1,2]. However, suprathreshold doses of carcinogen or repetitive low doses typically induce benign and malignant neoplasms without the need for the promoting stimulus. Both the initiation of hepatocarcinogenesis by a subthreshold dose of carcinogen [3] and the induction of neoplasms directly by a suprathreshold dose of carcinogen [4,5] appear to require two elements [6]; (1) damage to DNA, and (2) proliferation of damaged cells. Seemingly, during the replication and division of hepatocytes with damaged DNA, irreversible alterations are produced that dispose these cells to neoplasia.

We are interested in identifying the factors that may influence the initiation of hepatocarcinogenesis. In a study of the relationship of the cell division cycle to the susceptibility of proliferating hepatocytes to initiation by the chemical carcinogen, DMN-Ac, we found the S phase to be a period of maximal sensitivity [3]. Hepatocytes in G_1 appeared to be significantly less sensitive, suggesting that prereplicative DNA repair reduces cellular risk of initiation by this chemical.

Recent studies suggest that initiation of hepatocarcinogenesis might be monitored in short-term assays that quantify hepatocytic islands of cellular alteration [7]. These islands are induced by hepatocarcinogens and display a variety of morphological, biological, and biochemical abnormalities (see [8,9] for reviews). To date, there have been few studies in which the yields of islands estimated by morphometric techniques are compared with the yields of hepatocytic neoplasms induced under the same conditions in the same livers. Here we describe the results of our preliminary efforts to quantify hepatocytic neoplasms and islands of cellular alteration in livers that were treated once with a hepatocarcinogen given before or during the peak of hepatocyte DNA synthesis after partial hepatectomy. We reasoned that if altered cell islands represent the clonal progeny of initiated hepatocytes, as some have suggested [8], then treatments with carcinogens that induce equivalent numbers of islands also should produce equivalent yields of neoplasms after promotion.

METHODS

Male F344 rats were obtained from Charles River Breeding Laboratories (Kingston, MA) at about 6 wk of age. They were acclimated for 1 wk and weighed 100 g at the time of treatment. Rats were treated with the chemical carcinogens during the peak of DNA synthesis 18–20 hr after a two-thirds partial hepatectomy [4]. DMN-Ac of greater than 98% purity was synthesized by Dr. G. Muschik, Program Resources, Inc., NCI-FCRF (Frederick, MD) by the method of Roller et al [10]. DMN-Ac was dissolved in phosphate-buffered saline, pH 5.5, immediately before use. (±)-7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) synthesized at Midwest Research Institute (Kansas City, MO) and of greater than 99% purity, was dissolved in anhydrous dimethylsulfoxide (silylation grade from Pierce Chemical Co., Rockford, IL). Immediately before administration, 25 μl of this solution was mixed with 0.5 ml of Steroid Suspending Vehicle (Armour Pharmaceutical Co., Kankakee, IL), and the mixture was then drawn into a syringe. Aqueous

solutions of carcinogen were administered directly into the hepatic portal vein (at 5 ml per kg body weight) as previously described [4]. For treatment with gamma radiation, rats were anesthetized with brevital and placed in a restraining apparatus with 3/8 inch of lead shielding the upper and lower thirds of the body. The middle third of the rat containing the regenerating liver remnant was not shielded. Irradiation from above and below was performed with a Gammacell 40 Cesium 137 source at a dose rate of 1.4 GY per min. For the dose-response study, irradiation was performed at 18 hr after partial hepatectomy. Another group of rats was irradiated at 4 hr after partial hepatectomy during the prereplicative phase of liver regeneration [4]. Three wk after treatment with carcinogen, rats were fed a Purina chow diet containing 0.05% phenobarbital. Some DMN-Ac-treated rats were fed the Purina chow diet alone.

At intervals after treatment, groups of rats were sacrificed for analysis of hepatocytic neoplasms and altered cell islands. One week before sacrifice, phenobarbital was removed from the diet. Rats were fasted for 24 hr before sacrifice to deplete livers of glycogen. After removal of the liver, a 1-cm^2 slice (2-mm thick) of the right lateral lobe was frozen on solid CO_2, and 8-μm-thick sections were cut on a cryostat. Sections were stained with periodic acid and the Schiff reagent (PAS) and counterstained with hematoxylin. The diameters of glycogen-storage islands seen in transections were measured with an eyepiece micrometer. The numbers of islands per liver were estimated according to the stereologic method of Pugh et al [11] as described in [12]. The remaining liver was fixed in formalin, then cut at 1–2 mm intervals to identify neoplasms. All gross identifications were confirmed by subsequent histologic analysis as previously described in detail [4].

RESULTS

Time-Course of Hepatocarcinogenesis in DMN-Ac-Treated Rats

In DMN-Ac-treated rats fed a diet containing 0.05% phenobarbital, yields of hepatocytic neoplasms increased continuously after a latency of 20 weeks (Fig. 1). By 60 wk after treatment, livers contained an average of 5.5 neoplasms, both nodules and carcinomas. In these animals, the numbers of glycogen-storage islands also appeared to increase continuously between 20 and 60 wk after treatment (Fig. 2). By 60 wk, livers were estimated to contain about 10,000 of these islands. For this 60-wk group, the diameters of liver neoplasms identified in histologic sections were measured (n = 33). The volumes of these neoplasms were estimated assuming that the neoplasms were spheroids. Within individual livers neoplasms were estimated to occupy from 1–20% of the liver volume with an average of 5% of the liver occupied by neoplasm. Consequently, for the estimation of island numbers in nonneoplastic tissue, the use of the total liver volume [12] did not significantly alter the results. In the rats fed the diet containing phenobarbital as promoter, neoplasms and islands appeared to maintain a constant numerical relationship over time, with one neoplasm emerging for every 1,450 islands (Fig. 1, insert). In treated rats not fed the promoter the islands emerged later, but by 45 and 60 wk their numbers approximated those seen in promoted rats (Fig. 2). However, the yields of hepatocytic neoplasms were significantly less in rats fed control diet than in rats fed phenobarbital-containing diet (Fig. 1). At 45 and 60 wk after treatment with DMN-Ac, the ratios of yields of neoplasms for promoted versus nonpromoted rats were 6.1 and 8.3, respectively. The ratio of islands to neoplasms in nonpromoted livers (7,750:1, Table I) was somewhat

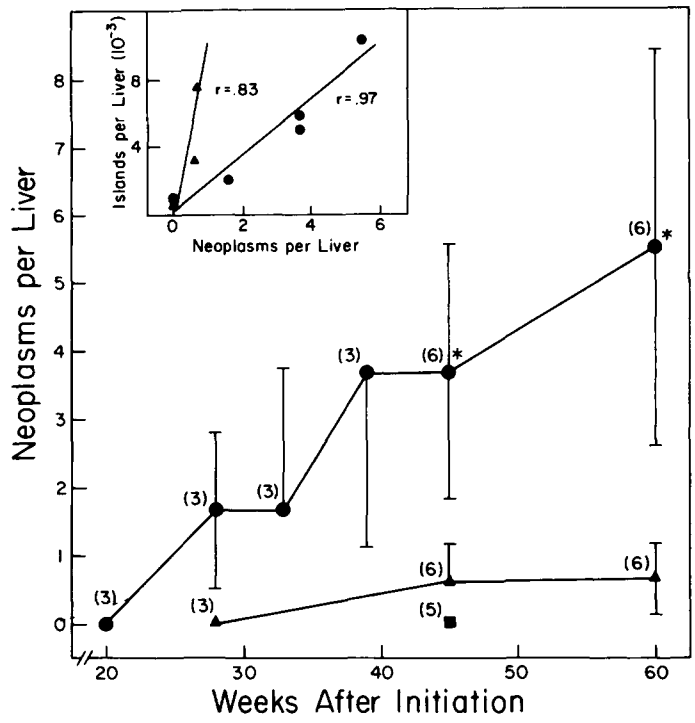

Fig. 1. Kinetics of emergence of hepatocytic neoplasms following initiation with DMN-Ac. The mean yields of hepatocytic neoplasms (±SD) were enumerated in groups of rats (numbers in parentheses) at various times after treatment with 0.1 mmole/kg DMN-Ac. (●), DMN-Ac-treated rats fed diet containing 0.05% phenobarbital; (▲), DMN-Ac-treated rats fed control diet; (■), hepatectomized rats fed diet containing 0.05% phenobarbital.(*), $P < .025$ for carcinogen-treated rats fed phenobarbital diet vs carcinogen-treated rats fed control diet (Student's t test). Insert: For each experimental time-point, the average yields of neoplasms were plotted against the estimated yields of glycogen-storage islands as illustrated in Figure 2. (Reprinted from [12] with permission.)

less than the 11,000:1 value that was calculated previously by linear regression [12]. No hepatocytic neoplasms and few glycogen-storage islands were seen in solvent-treated control rats fed the phenobarbital-containing diet for 42 wk.

Dose-Responses for Initiation of Hepatocarcinogenesis by DMN-Ac, BPDE, and Gamma Radiation

Rats were treated once with DMN-Ac, BPDE, or gamma radiation at the peak of DNA synthesis after partial hepatectomy, then 3 wk after treatment, phenobarbital was added to their diet at 0.05%. Based on the results of the time-course study described above, animals were held for 39–45 wk after treatment with carcinogen to allow sufficient time for expression and growth of islands and neoplasms. Dose-response curves for induction of glycogen-storage islands and for initiation of neoplasms are shown in Figure 3. Whereas a single dose of all three carcinogens appeared to be able to induce islands of cellular alteration, the carcinogens varied in their abilities to induce neoplasms. At doses of carcinogen that induced approximately

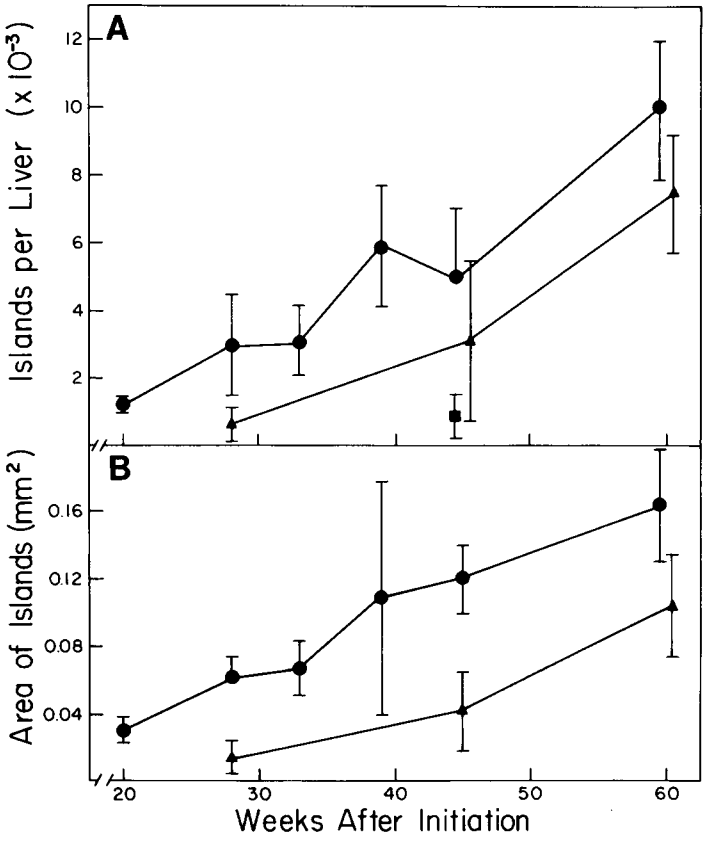

Fig. 2. Kinetics of emergence of glycogen-storage islands following initiation with DMN-Ac. A) The mean yields of islands displaying abnormal retention of glycogen upon fasting (±SD) were estimated from transections by quantitative stereology. Symbols are as in Figure 1. B) The mean areas of islands (±SD) at various times after initiation. Symbols are as in Figure 1. (Reprinted from [12] with permission.)

TABLE I. Island to Neoplasm Ratio

Carcinogen	Protocol	Dietary phenobarbital	Rats	No. per liver[a]		Ratio[b]
				Islands	Neoplasms	
DMN-Ac	Time-course	+	37	2,986	2.05	1,456
		–	15	3,430	0.47	7,751
DMN-Ac	Dose-response	+	15	3,827	2.67	1,433
BPDE	Dose-response	+	9	4,828	0.67	7,206
Gamma rays	Dose-response	+	6	2,650	0	(>15,900)
Gamma rays	6 Gy at 4 hr after partial hepatectomy	+	9[c]	2,694	0.22	12,245

[a]The average Nos. of induced islands and neoplasms were determined for the data presented in Figures 1–3.

[b]Ratio = the average No. of islands per liver divided by the average No. of neoplasms.

[c]Six livers in this group were evaluated to determine the average Nos. of glycogen-storage islands.

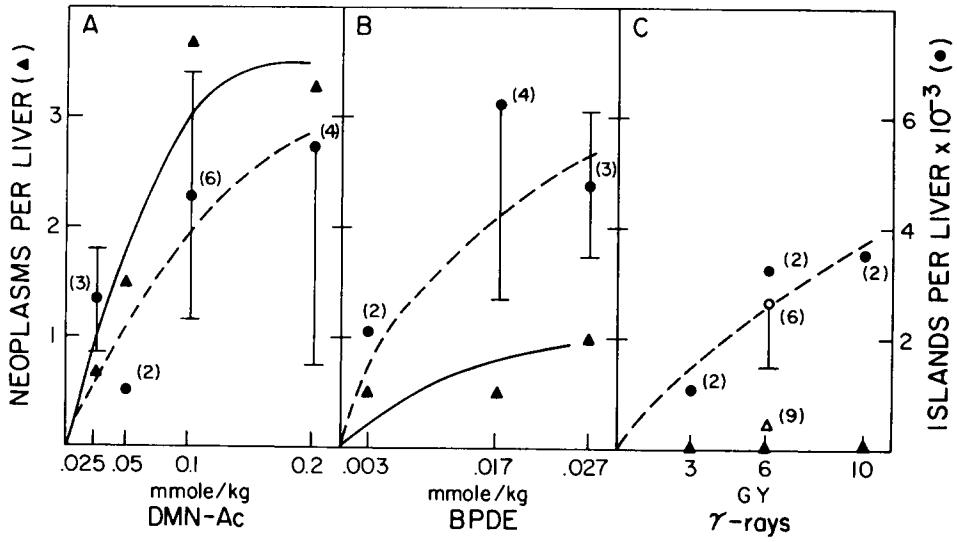

Fig. 3. Dose-responses for initiation of hepatocytic neoplasms and for induction of glycogen-storage islands following a single treatment with carcinogen. The yields of neoplasms (triangles) and islands (circles) were enumerated in livers of rats fed 0.05% phenobarbital for 36–42 wk after a single initiating treatment with various doses of (A) DMN-Ac, (B) BPDE, or (C) gamma radiation. In parentheses are the numbers of livers analyzed at each experimental point. In C, the open symbols depict yields of islands and neoplasms in a group of rats irradiated at 4 hr after partial hepatectomy. Error bars enclose one standard deviation about the mean.

equal numbers of islands, DMN-Ac produced several neoplasms per liver, BPDE produced 0.5–1 neoplasm per liver, and for the sample of livers treated at the peak of DNA synthesis in regenerating livers, gamma rays failed to induce any neoplasms. This result is summarized in Table I in which the total number of induced islands in our sample was compared with the total number of induced neoplasms. As noted above, the ratio of islands to neoplasms was about 1,450:1 for DMN-Ac-treated livers. For BPDE-treated livers, this ratio was about 7,200:1. The ratio could not be computed for the gamma-irradiated livers owing to the lack of induced neoplasms. These results suggested a lower limit of about 16,000:1 for this ratio in livers irradiated at the peak of DNA synthesis after partial hepatectomy. In another experiment, the timing of irradiation after partial hepatectomy was varied. In a group of nine rats irradiated with 6 Gy at 4 hr after partial hepatectomy and then promoted with phenobarbital, two hepatocytic neoplasms were observed 45 wk after irradiation. The average number of glycogen-storage islands estimated for livers in this group was 2,694 (n = 6, SD 1,186). Consequently, the computed ratio of islands to neoplasms was 2694/0.22 or 12,245 in this group of irradiated livers (Table I).

DISCUSSION

Islands of histochemically altered hepatocytes are generally seen in livers soon after an exposure to a carcinogen that produces hepatocytic neoplasms after a longer observation period [12,13]. Consequently, it appears that the observation of such

islands long after a test compound had decayed gives at least qualitative evidence of initiating activity [7,8]. Our current results confirm observations that islands appear in far greater numbers that neoplasms [12–15] and demonstrate that for livers treated with DMN-Ac, islands are produced in a constant ratio to initiated hepatocytes of about 1,450:1. However, in livers treated with BPDE or gamma rays and then promoted with phenobarbital, the observed ratios of the yields of induced islands of glycogen retention to hepatocytic neoplasms were 7,200:1 and 12,200:1, respectively. These results suggest that the population of glycogen storage islands may not be the progeny of initiated hepatocytes.

Previous studies have shown that phenobarbital may increase the frequency of observation of islands of cellular alteration following a single initiating treatment with chemical carcinogens [14]. Our results discussed in more detail in [12] suggested that at 28 wk after treatment with DMN-Ac, promoted livers contained about five times as many islands as nonpromoted livers. However, at 45 and 60 wk after treatment, the numbers of islands in promoted and nonpromoted livers were nearly equivalent. Others have reported results similar to these [15,16] or results indicating that island frequencies were not appreciably affected by the promoter [17]. It has recently been reported that in some islands of cellular alteration, expression of gamma glutamyl-transpeptidase may be reversibly induced by phenobarbital [18]. In the absence of the promoter, these islands, although present, would not be scored owing to the lack of marker enzyme activity. Altered cell islands that display abnormal retention of glycogen on fasting presumably have a defect in the complex pathways of carbohydrate metabolism, such as a deficiency in glucose-6-phosphatase. Expression of this deficiency may not be affected by the inductive effects of phenobarbital. Our studies suggest that phenobarbital may speed the initial emergence and growth of altered cell islands expressing the glycogen-storage phenotype, but it does not affect the numbers of these islands that ultimately appear in damaged livers. In contrast, the promoter does not appear to speed the emergence or growth of neoplasms [19], but rather it enhances the expression of the neoplastic phenotype by initiated cells that would otherwise remain latent.

Ionizing radiation has been reported to increase the incidence of hepatic neoplasia when administered to mice with regenerating livers [20,21]. We also are aware of a preliminary report of the induction of altered cell islands in regenerating livers of rats exposed to ionizing radiation and 3-aminobenzamide [22]. To our knowledge, there have been no previous reports of radiation-induced hepatic neoplasia in the rat. The results of an extensive analysis of hepatocarcinogenesis in gamma-irradiated livers are currently being quantified and will be presented elsewhere. Our preliminary results suggest that gamma radiation is indeed capable of inducing altered cell islands and initiating hepatocytic neoplasia. However, in comparison to BPDE and DMN-Ac, this radiation appears to be less efficient in producing initiated hepatocytes that can be promoted to form neoplasms. Neoplasms initiated by gamma rays may have an unusually long latency so that they might not be readily detected as a result of our study design. This possibility would require that in the initiated cell populations that are produced by different carcinogens there are differences that affect the speed with which initiated cells can be promoted to form neoplasms. Alternatively, gamma rays may be able to induce efficiently only a subset of the genetic alterations that initiate hepatocarcinogenesis in the rat. Support for this concept may be found in the recent observation that, in combination with the treatment of neonatal rats with diethylnitro-

samine, gamma radiation produced a synergistic increase in the frequency of altered cell islands [23].

DMN-Ac and BPDE both appeared to be effective initiators of hepatocarcinogenesis when given as a single dose after partial hepatectomy. When viewed on a molar basis, BPDE appeared to be a more efficient inducer of islands of cellular alteration than DMN-Ac. However, in the absence of information on the levels of specific DNA adducts produced in damaged livers and of the effects of these adducts on oncogenetic alterations, comparisons of relative efficiencies of induction of islands or initiation of neoplasms must be viewed with caution. A more thorough analysis of the effects of dose of carcinogen and of the time after treatment on the yields of induced islands and neoplasms also is required before comparisons of carcinogenic effectiveness can be attempted.

In summary, we found that in livers given a single treatment with one of three known carcinogens followed by promotion with phenobarbital there were carcinogen-dependent differences in the ratios of yields of islands of cellular alteration to yields of neoplasms. Comparison of the hepatocellular alterations induced by the three carcinogens evaluated here may reveal a specific subset of alterations associated with elevated risk of developing cancer in the liver. The present results suggest that the cellular alteration(s) associated with retention of glycogen on fasting may not be directly associated with the formation of initiated cells that can be promoted to form neoplasms.

ACKNOWLEDGMENTS

We are grateful to Michael Judge and Scott Stroup for their excellent technical assistance in these studies. This work was supported by PHS grant △CA32238 awarded by the National Cancer Institute, DHHS. D.G.K. was the recipient of a Research Career Development Award (△CA00431) from the NCI. R.J.R. was supported by an Environmental Pathology training grant (△ES07017) from the NIEHS.

REFERENCES

1. Berenblum I, Shubik PA: Br J Cancer 3:384, 1949.
2. Boutwell RK: Prog Exp Tumor Res 4:207, 1964.
3. Kaufmann WK, Kaufman DG, Rice JM, Wenk ML: J Cell Biochem 8A:137, 1983.
4. Kaufmann WK, Kaufman DG, Rice JM, Wenk ML: Cancer Res 41:4653, 1981.
5. Craddock VM, Frei JV: Br J Cancer 30:503, 1974.
6. Grisham JW, Kaufman DG, Kaufmann WK: Syn Survey Path Res 1:49, 1983.
7. Tatematsu M, Hasegawa R, Iwaida K, Tsuda H, Ito N: Carcinogenesis 4:381, 1983.
8. Pitot HC, Sirica AE: Biochim Biophys Acta 605:191, 1980.
9. Bannasch P, Mayer D, Hacker HJ: Biochim Biophys Acta 605:217, 1980.
10. Roller PP, Shimp DR, Keefer LK: Tetrahedron Let 25:2065, 1975.
11. Pugh TD, King JH, Koen H, Nychka D, Chover J, Wahba G, He Y, Goldfarb S: Cancer Res 43:1261, 1983.
12. Kaufmann WK, MacKenzie SA, Kaufman DG: Am J Pathol 119:171, 1985.
13. Peraino C, Staffeldt EF, Carnes BA, Ludeman VA, Blomquist JA, Vesselinovitch SD: Cancer Res 44:3340, 1984.
14. Pitot HC, Barsness L, Goldsworthy T, Kitagawa T: Nature 271:456, 1978.
15. Kitagawa T, Sugano H: Gann 69:679, 1978.
16. Herren SL, Pereira MA: Envir Health Perspect 50:123, 1983.

17. Moore MA, Hacker HJ, Kunz HW, Bannasch P: Carcinogenesis 4:473, 1983.
18. Sirica AE, Jicinsky JK, Heyer EK: Carcinogenesis 5:1737, 1984.
19. Peraino C. Staffeldt EF, Haugen DA, Lombard LS, Stevens FJ, Fry RJM: Cancer Res 40:3268, 1980.
20. Cole LJ, Nowell PC: Science 150:1782, 1965.
21. Wiley AL, Vogel HH, Clifton KH: Radiat Res 54:284, 1973.
22. Enomoto K, Dempo K, Oyamada M, Suzuki J, Sakurai T, Mori M: Proc Am Assoc Cancer Res 25:125, 1984.
23. Peraino C, Grdina D, Carnes B: Proc AACR 26:74, 1985.