Purine N-Oxides
XVI. Oncogenic Derivatives of Xanthine and Guanine

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SUMMARY

The oxidation of guanine to an N-oxide and its hydrolysis to a xanthine N-oxide are reported. The s.c. administration of the xanthine N-oxide to rats for 6 months resulted in development of a variety of tumors. There were 1 liposarcoma, 3 fibrosarcomas, 1 mammary adenocarcinoma, 3 fibroblastic tumors, 2 rhabdomyosarcomas, and 1 epidermoid carcinoma at the site of injection and elsewhere in 11 out of 13 rats, or an incidence of 85%. The 1st measurable tumor appeared at 230 days and the last at 442 days after the 1st injection of the compound.

The guanine N-oxide, injected s.c. for 6 months, but at a lower concentration, induced 2 fibrosarcomas and 2 fibroblastic tumors in 15 rats; the 1st tumor appeared at 334 days and the last at 445 days after the 1st injection of the compound.

Administration of adenine 1-N-oxide for 6 months failed to produce tumors in any tissues of 13 rats during the experimental period of 15 months.

Of 8 tumors induced by the xanthine N-oxide, 7 were transplantable, and serial transplantation has been successful for 15 generations with one fibrosarcoma and for 5 generations with another.

Thousands of derivatives of purines have been tested as cancer chemotherapeutic agents, whereas but few have been tested for oncogenicity. We have now found that certain N-oxide derivatives of xanthine and guanine, but not adenine 1-N-oxide, can induce a variety of tumors in rats. These assays were prompted by the fact that N-hydroxy derivatives have been associated with the oncogenicity of several aromatic amines (13, 16, 17, 19) and that it is possible for certain heterocyclic N-oxides to exist in tautomeric forms which are heterocyclic N-hydroxy derivatives.

MATERIALS AND METHODS

Biological Methods

This study included groups of 15 female rats, Wistar strain (Carworth Farms), for controls and for each compound. The animals, initially about 80-100 gm and 6 weeks of age, were maintained on a standard pellet diet (Purina Laboratory Chow) and water ad libitum. They were weighed and examined for subcutaneous tumors weekly.

Injections (s.c.) of the compounds at less than maximum tolerated doses (10 mg/rat of adenine 1-N-oxide, 1.0 mg/rat of guanine x-N-oxide, and 7.0 mg/rat of xanthine x-N-oxide·H$_2$O) were made within a cm of the same site in the midline subscapular region once weekly for 6 months. Preliminary tolerance tests were run on mice to determine the acute toxicity, and an arbitrary dosage was selected.

All compounds were homogenized with carboxymethylcellulose (CMC) solution (0.5% CMC in 0.85% NaCl); 0.5 ml contained the amount for each weekly injection. All tumors were examined histologically and diagnosed. Tissues and visceral organs of all rats were carefully examined grossly at autopsy for tumors, and most were examined histologically.

The transplantability into young adult Wistar rats of the chemically induced tumors was also determined. Small pieces of tumor, approximately 2.5 cu mm, were dissected from non-necrotic portions of solid tumors and implanted s.c. by trocar into the region of the right axilla of 10 healthy young Wistar rats. Evaluation of tumor growth was made 3 weeks after tumor inoculation. Transplants which proliferated for several days but completely regressed within 21 days were considered to be negative; those still growing after 3 weeks were considered to be positive.

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The age of the rats had a definite influence upon the continued growth of transplanted rat tumors (15). In the suckling rats, of ages 1 to 20 days, the implants were successful and grew progressively until subsequent tumor ulceration. Spontaneous regression of the growths in suckling rats was rare. On the other hand, the percentage of tumor "takes" in rats approximately 1 year old and 2 months old was the same as that of suckling rats, but regression of tumors was more frequent in older animals.

SYNTHETIC METHODS

Adenine 1-N-oxide.—This was prepared as described (14).

Guanine x-N-oxide.—A mixture of 15 ml of trifluoro-acetic acid and 10 ml of 30% H2O2 was stirred magnetically, and 5 gm of powdered guanine were added in portions. The mixture dissolved slowly, and stirring was continued overnight. About 10 mg of 10% Pd-C was added to decompose the excess H2O2. The stirring was continued the following day, and solids were separated. The mixture was cooled overnight, and the solids were collected and dissolved in 50 ml of hot 2 N HCl. The Pd-C was removed by filtration of the hot solution, which was then cooled. The 3.5 gm of needles were recrystallized from 50 ml of hot 2 N HCl.

Analysis.—Calculated for C12H12N2O4.HCl: C, 32.39; H, 2.99; N, 37.73; Cl, 9.52. Found: C, 32.41; H, 3.20; N, 37.48; Cl, 9.56.

A 3-gm sample was dissolved in 135 ml of water and 50 ml of 1 N sodium hydroxide and was precipitated by slow addition of dilute acetic acid. At the centrifuge it was washed 3 times by suspension in 200 ml of water, and the product was dried in a vacuum to yield 2.5 gm of analytically pure anhydrous material.

Analysis.—Calculated for C12H12N2O4: C, 35.93; H, 3.01; N, 41.90. Found: C, 35.71; H, 3.01; N, 41.75.

Reduction of guanine x-N-oxide.—The reduction of 184 mg in 20 ml of 0.1 N NaOH with Raney nickel and hydrogen resulted in the uptake of slightly more than 1 mole of hydrogen. The solution was filtered and made acid with H2SO4. The material that precipitated was identical in spectra (2) with guanine and showed the same Rf's in isoamyl alcohol-sodium phosphate (5) and in isopropanol-hydrochloric acid (20).

Analysis.—Calculated for (C6H5N5O)2•H2SO4-2H2O: N, 32.2. Found: N, 32.3.

Xanthine x-N-oxide.—A solution of 5 gm of guanine x-N-oxide hemihydrochloride in 75 ml of 18% HCl was refluxed for 17 hr. It was then cooled, and 4 gm of yellow prisms were collected. These were dissolved in 100 ml of 1 N NaOH, charcoal was added, and the solution was filtered. About 5 ml of acetic acid were added slowly with stirring; the mass of fine white needles was collected, washed by suspension in water, and dried in a vacuum at room temperature to yield 3 gm.

Analysis.—Calculated for C12H12N2O4.1H2O: C, 32.79; H, 3.11; N, 30.11. Found: C, 32.3; H, 3.25; N, 30.10.

This hydrate was used in the biologic testing.

A sample dried at 118°C lost 9.7% weight, corresponding to the loss of 1 mole of water. It was again analyzed.

Analysis.—Calculated for C12H12N2O4: C, 35.72; H, 2.40; N, 33.33. Found: C, 35.78; H, 2.46; N, 33.44.

The reduction of xanthine x-N-oxide.—A solution of 165 mg in 20 ml of 0.1 N NaOH was reduced with Raney nickel and hydrogen. The hydrogen consumption was slow and the reduction was continued for 18 hr, when the uptake was slightly more than 1 mole. The solution was filtered and adjusted to pH 3 with HCl. The precipitate collected had Rf's (5, 20) and spectra (6) identical with those of xanthine. A trace of unreduced xanthine x-N-ox ide was observed on paper chromatograms.

RESULTS

Controls.—All 15 of the rats treated with CMC survived 15 months. Nononcogenicity of carboxymethylcellulose under the conditions used was indicated by the lack of tumors at the site of injection and the normal appearance of all visceral organs, both grossly and histologically.

Various investigators have reported that the incidence of spontaneous tumors in Wistar strain rats was low, less than 5% (12). One of us (Sugiura) has observed 100 Wistar rats during periods up to 15 months and noted a 2% incidence of tumors—1 mammary carcinoma and 1 sarcoma.

Adenine 1-N-oxide.—Of 15 rats treated with the adenine N-oxide, 2 died on the 21st and 35th days with congestion of the lung. Two died on the 140th day and 1 on the 175th day with tracheobronchitis with associated abscesses of the lung. The last 3 rats which died and 10 remaining rats sacrificed at 470 days had no subcutaneous tumor at the site of injection or elsewhere. This indicates that under the experimental conditions the adenine N-oxide is not oncogenic, although the number of animals treated was small.

Guanine x-N-oxide.—Of 15 rats treated with guanine N-oxide, 12 survived the experimental period of 15 months (Table 1). Four tumors developed subcutaneously at the site of injection on the 334th, 401st, 439th, and 446th day after the 1st injection. One tumor grew rapidly and the animal was sacrificed for tumor transplantation. The tumor was solid and medium firm, with small hemorrhagic and necrotic areas, and microscopic examination showed a fibrosarcoma. A careful examination showed that the internal organs and tissues were free from any metastatic growth or other primary tumors. The tumor was transplantable for 1 generation only, but with 70% regressions. Three other tumors grew slowly. These were in the subcutaneous fat tissue, were firm, white, homogeneous, and were a fibrosarcoma and 2 fibroblastic tumors. One was successfully transplanted without regression and has been transplanted again.

Xanthine x-N-oxide.—Xanthine N-oxide induced tumors in rats, usually at the site of injection, in 11 of 13 animals. Two of the initial 15 rats died with congestion of the lung 2 days after the first injection of the compound and therefore were not included in the evaluation of results (Table 2).

The 1st tumor, a liposarcoma, developed on the 230th day, grew rapidly, and killed in 48 days. The lung was inflamed, but other organs were normal.

The 2nd tumor (Fig. 1) was a homogeneous and very anaplastic fibrosarcoma (Fig. 2). The animal was sac-
TABLE 1
TUMORS INDUCED BY GUANINE N-OXIDE
Dose: 1.0 mg/rat/wk

<table>
<thead>
<tr>
<th>Appearance of tumor (days)</th>
<th>Animal sacrificed (days)</th>
<th>Body wt final, with tumor (gm)</th>
<th>Tumor size (cm)</th>
<th>Tumor wt (gm)</th>
<th>Histologic diagnosis</th>
<th>Transplantability</th>
</tr>
</thead>
<tbody>
<tr>
<td>334</td>
<td>367</td>
<td>265</td>
<td>3.0 x 2.8 x 1.5</td>
<td>10.0</td>
<td>Fibrosarcoma</td>
<td>Positive</td>
</tr>
<tr>
<td>401</td>
<td>447</td>
<td>331</td>
<td>0.8 x 0.6 x 0.4</td>
<td>0.11</td>
<td>Fibroblastic tumor</td>
<td>Positive</td>
</tr>
<tr>
<td>439</td>
<td>490</td>
<td>350</td>
<td>2.0 x 1.8 x 1.1</td>
<td>3.9</td>
<td>Fibrosarcoma</td>
<td>Positive</td>
</tr>
<tr>
<td>446</td>
<td>479</td>
<td>294</td>
<td>0.5 x 0.4 x 0.4</td>
<td>0.08</td>
<td>Fibroblastic tumor</td>
<td></td>
</tr>
</tbody>
</table>

* Found dead.

rificed 94 days later, and all organs were normal. This tumor was successfully transplanted into Wistar rats for 15 generations (to January 19, 1965). At first all transplants grew, but subsequently 40% regressed. Malignancy increased; 80% of the implants grew in the 2nd transplant generation and 90–100% thereafter. Transplants grew rapidly and killed animals in 4–6 weeks. The 5th and 10th tumors were also fibrosarcomas in the subcutaneous fat tissue at the site of injection. The organ were normal, except for blood extravasations in the livers. One of these fibrosarcomas was successfully transplanted for 5 generations (to January 7, 1965).

The 3rd, a mammary adenocarcinoma, was a slow growing one at the approximate site of injection. It had infiltrated fat and muscle tissue and was not encapsulated, and there was extensive vascularization and partial necrosis. Transplants were positive, but 2nd transplantations failed to grow. The inner inferior margin of the middle lobe of the liver had a 2nd loosely implanted tumor measuring 1.1 x 1.0 x 0.6 cm in size. Histologic examination revealed extensive blood extravasations and well-differentiated hyperplastic nodules.

A slow-growing fibroblastic tumor, which was histologically potentially malignant, next developed at the site of injection. It was in the subcutaneous fat tissue, encapsulated, and homogeneous. The liver showed degeneration and blood extravasations; other organs were normal. Four of 10 transplants grew slowly, and 2nd transplantations failed. The 5th and 7th were also fibroblastic tumors at the site of injection.

TABLE 2
TUMORS INDUCED BY XANTHINE N-OXIDE
Dose: 7.0 mg/rat/wk

<table>
<thead>
<tr>
<th>Appearance of tumor (days)</th>
<th>Animal sacrificed (days)</th>
<th>Body wt final, with tumor (gm)</th>
<th>Tumor size (cm)</th>
<th>Tumor wt (gm)</th>
<th>Histologic diagnosis</th>
<th>Transplantability</th>
</tr>
</thead>
<tbody>
<tr>
<td>230</td>
<td>278*</td>
<td>330</td>
<td>6.4 x 4.6 x 3.0</td>
<td>48.2</td>
<td>Liposarcoma</td>
<td>Positive</td>
</tr>
<tr>
<td>235</td>
<td>329</td>
<td>335</td>
<td>6.1 x 5.3 x 4.1</td>
<td>72.5</td>
<td>Fibrosarcoma</td>
<td>Positive</td>
</tr>
<tr>
<td>281</td>
<td>415</td>
<td>280</td>
<td>2.4 x 2.5 x 1.6</td>
<td>7.0</td>
<td>Mammary adenocarcinoma</td>
<td>Positive</td>
</tr>
<tr>
<td>291</td>
<td>404</td>
<td>520</td>
<td>2.0 x 1.8 x 1.0</td>
<td>8.8</td>
<td>Fibroblastic tumor</td>
<td>Positive</td>
</tr>
<tr>
<td>297</td>
<td>413</td>
<td>464</td>
<td>3.7 x 3.2 x 1.5</td>
<td>12.8</td>
<td>Fibroblastic tumor</td>
<td>Positive</td>
</tr>
<tr>
<td>306</td>
<td>364*</td>
<td>380</td>
<td>4.8 x 5.4 x 3.0</td>
<td>68.0</td>
<td>Rhabdomyosarcoma</td>
<td>Positive</td>
</tr>
<tr>
<td>306</td>
<td>373</td>
<td>445</td>
<td>2.5 x 2.3 x 1.3</td>
<td>7.5</td>
<td>Fibroblastic tumor</td>
<td>Negative</td>
</tr>
<tr>
<td>306</td>
<td>406</td>
<td>385</td>
<td>5.1 x 7.2 x 3.0</td>
<td>82.8</td>
<td>Fibrosarcoma</td>
<td>Positive</td>
</tr>
<tr>
<td>341</td>
<td>360</td>
<td>326</td>
<td>5.4 x 4.9 x 3.1</td>
<td>51.0</td>
<td>Rhabdomyosarcoma</td>
<td>Positive</td>
</tr>
<tr>
<td>418</td>
<td>461</td>
<td>276</td>
<td>2.1 x 2.3 x 2.0</td>
<td>8.0</td>
<td>Fibrosarcoma</td>
<td>Positive</td>
</tr>
<tr>
<td>442</td>
<td>463</td>
<td>279</td>
<td>3.4 x 3.3 x 1.2</td>
<td>9.5</td>
<td>Epidermoid carcinoma</td>
<td></td>
</tr>
</tbody>
</table>

* Found dead.

When the 7th tumor was sacrificed 94 days later, and all organs were normal. This tumor was successfully transplanted into Wistar rats for 15 generations (to January 19, 1965). At first all transplants grew, but subsequently 40% regressed. Malignancy increased; 80% of the implants grew in the 2nd transplant generation and 90–100% thereafter. Transplants grew rapidly and killed animals in 4–6 weeks. The 8th and 10th tumors were also fibrosarcomas in the subcutaneous fat tissue at the site of injection. The organ were normal, except for blood extravasations in the livers. One of these fibrosarcomas was successfully transplanted for 5 generations (to January 7, 1965).

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The 6th and 9th were rapidly growing rhabdomyosarcomas at the sites of injection. Internal organs were normal except that 1 liver was hemorrhagic and 1 lung showed bronchial pneumonia. Transplantation of 1 of these was successful to the 3rd generation.

The 11th tumor developed between the thighs mostly to the left of the abdominal midline on the 442nd day after the 1st injection. It grew rapidly, and 21 days later it (Fig. 3) was rounded, moderately firm, and pliable. The largely subcutaneous mass had a rounded area of ulceration measuring 1.4 x 1.4 cm in diameter. Transsections showed a grayish necrotic mass with very little firm tissue, and histologic examination showed epidermoid carcinoma (Fig. 4). The parenchymatous organs were normal, with the exception of the lower part of the liver lobes, which had several large blood extravasations.

Growth of the hosts.—The control animals receiving CMC reached a maximum weight of 340 gm within 6 months, and none weighed less than 220 gm, a usual range for these rats. During the same period, animals treated with adenine 1-N-oxide weighed from 250 to 330 gm, with guanine N-oxide from 230 to 320 gm, and with xanthine N-oxide from 240 to 390 gm. At 15 months the control animals weighed from 272 to 480 gm, adenine 1-N-oxide animals weighed from 239 to 500 gm, guanine N-oxide animals from 265 to 470 gm, and xanthine N-oxide animals from 276 to 520 gm. All appeared in good health except the approximately 70% of the xanthine N-oxide-treated rats with liver damage.

DISCUSSION

The 85% incidence of tumors and the variety of cell types from which they arose place the xanthine N-oxide among the potent oncogenic chemical agents. The guanine N-oxide induced fewer tumors—27% incidence—but because the dosage level administered was only 4 as much on a molar basis, no direct comparison of the oncogenities of the N-oxides should yet be made. There was no indication of oncogenicity with the adenine 1-N-oxide, which, on a molar basis, was administered at nearly 2-fold the level of the xanthine N-oxide.

In the only previous experimental reference to oncogenicity of a simple purine derivative, Allen et al. (1) reported, in 1957, that xanthine, when implanted in cholesterol pellets in mouse bladder epithelium, induced 2 papillomas and 2 carcinomas in 15 mice. They (1), and also Boyland and Watson (3) in 1956, mention a personal communication from A. Haddow that xanthine induced tumors. Nothing further has been published, but Haddow states (recent personal communication): "Xanthine given in large doses subcutaneously [in arachis oil] at weekly intervals for a few months produced frequent granuloma, often with a significant frequency of mitotic figures. A small portion eventually underwent malignant change and became sarcoma." Walpole et al. (18) have shown that arachis oil may induce sarcomas.

Complete controls with xanthine under our conditions will not be available for some months.

The structure of the adenine 1-N-oxide tested is firmly established (14), but the position of the N-oxide function in the guanine and xanthine N-oxides is uncertain. Guanine was oxidized with trifluoroperoxyacetic acid, and since the nitrogen which protonates and alkylates is the one which may be expected to be oxidized, it may well be the N-7 (or N-9) which was oxidized. The structurally analogous xanthine N-oxide was obtained by hydrolysis of the guanine derivative. We (9) have now prepared xanthine 3'-N-oxide by total synthesis, and it is being tested for oncogenicity.

The creation of oncogenic derivatives of ubiquitous purines by the introduction of an N-oxide grouping, a functional group of a type which can arise (7, 10) and can be reduced (7, 11) metabolically, and which can possibly be produced by ionizing radiation (4, 8), raises the possibility that such derivatives could be natural oncogenic agents. Extensive explorations of the oncogenicity and metabolism of these and related purine derivatives, and of the tautomeric structures of the N-oxides are needed before an attempt can be made to assign the structural features associated with their oncogenicity.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. Greta Stohr for the gross and histologic examination of the chemically induced tumors.

REFERENCES


Fig. 1.—A large tumor arising at the site of administration of xanthine N-oxide, photographed on the 235th day after beginning of treatment, the 68th day after detection of the tumor.

Fig. 2.—Histologic section of the above tumor showing a fibrosarcoma. × 250.

Fig. 3.—A large ulcerated tumor arising at the abdominal midline between the thighs of a rat receiving xanthine N-oxide, photographed on the 463rd day after beginning treatment, the 21st day after detection of the tumor.

Fig. 4.—Histologic section of the above tumor showing the change from regular epidermal epithelium into malignant form. × 250.
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