Accumulation of $O^6$-Methylguanine in Human Blood Leukocyte DNA during Exposure to Procarbazine and Its Relationships with Dose and Repair

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ABSTRACT

$O^6$-Methylguanine was measured by a competitive repair assay in blood leukocyte DNA of seven patients with Hodgkin's or non-Hodgkin's lymphoma during therapeutic exposure to procarbazine involving three daily p.o. doses (50 mg each) for 10 days (corresponding to 2.1 mg/kg/day for a 70-kg human). Adduct accumulation was observed in all seven cases, reaching levels up to 0.28 fmol/μg of DNA (0.45 μmol/mol of guanine). In one individual, maximal levels of adduct were reached after 7 days of exposure, followed by a steady decline, whereas in all other individuals continuous accumulation was observed throughout the exposure period. In four individuals for which data were available for Day 11 (12 to 16 h after the final intake of procarbazine), decreased amounts of $O^6$-methylguanine were observed relative to the last previous measurements. The accumulation of $O^6$-methylguanine was linearly correlated ($P < 0.01$) with the cumulative dose of procarbazine, with a slope of 0.011 fmol of $O^6$-methylguanine/μg of DNA per mg/kg of body weight or 2.68 × 10⁻⁴ fmol of $O^6$ methylguanine DNA per mg/m². Two h after the administration of single p.o. doses of 1 to 10 mg/kg of procarbazine to rats, $O^6$-methylguanine formation in leukocyte DNA was just under half that in liver DNA and showed a linear relationship with dose with a slope of 0.017 fmol/μg of DNA per mg/kg of body weight or 5.67 × 10⁻⁴ fmol of $O^6$-methylguanine/μg of DNA per mg/m². A negative correlation ($P < 0.05$) between the rate of accumulation of $O^6$-methylguanine in different individuals and lymphocyte $O^6$-alkylguanine-DNA alkyltransferase (AGT) was observed, demonstrating a probable protective effect of AGT against the accumulation of $O^6$-methylguanine during exposure to methylating agents. This observation supports the suggestion of a possible role of procarbazine-induced $O^6$-methylguanine in the pathogenesis of acute nonlymphocytic leukemia appearing after treatment with chemotherapeutic protocols which include procarbazine, based on the finding of low lymphocyte AGT levels in patients with such therapy-related neoplastic disease (Sagher et al., Cancer Res., 48: 3084-3089, 1988). Lymphocyte AGT levels were mainly in the range of 5 to 10 fmol/μg of DNA and showed no consistent variation during procarbazine exposure.

INTRODUCTION

PCZ (n-isopropyl-a-(2-methylhydrazino)-p-toluamide hydrochloride) is a cytotoxic drug used with considerable success in the chemotherapy of a number of human neoplasias, particularly Hodgkin’s and non-Hodgkin’s lymphomas (1, 2). The use, however, of combination chemotherapy protocols which include PCZ is also associated with an increased frequency of secondary acute nonlymphocytic leukemia (3–5). Although this carcinogenic effect cannot be attributed specifically to PCZ, it is possible that this drug, which is a known animal carcinogen (6–8), may contribute to it.

The bioactivation of PCZ involves its initial oxidation into azoprocabazine (9). This is further metabolized into a mixture of methylazoxypocarbazine derivatives which can ultimately lead to the generation of methylating or arylating diazonium ions. A separate metabolic pathway probably involving free radicals and resulting in the formation of methane also exists. Following the administration of radiolabeled PCZ to experimental animals, the formation of methylated adducts in DNA has been demonstrated (10, 11). After p.o. exposure of rats, high levels of methylated adducts have been found in the liver and mammary tissue, while lower levels have been observed in the lung and gastrointestinal tissues. Among the products of the methylation of DNA by the methylidiazonium ion or related intermediates, $O^6$-mGua has been implicated in the mechanism of mutagenesis and carcinogenesis. It is a directly miscoding lesion whose presence in DNA during cell replication can result in the generation of G:A transitions which can, in turn, lead to the activation of oncogenes (12). Furthermore, the accumulation of $O^6$-mGua in particular types of tissues or cells during experimental carcinogenesis has been shown to correlate with the appearance of cancer (13, 14). It is possible, therefore, that $O^6$-mGua may play a causative role in any carcinogenic activity of PCZ in humans. Evidence supporting the possibility of $O^6$-mGua involvement in the pathogenesis of acute nonlymphocytic leukemia following therapeutic exposure to PCZ was recently presented by Sagher et al. (15) who have reported that patients with therapy-related neoplasia have lower lymphocyte levels of AGT, the enzyme responsible for repair of $O^6$-mGua. This implies that $O^6$-mGua may accumulate to an increased extent during PCZ exposure in individuals with low AGT and thus result in increased risk of subsequent carcinogenesis.

There is evidence that the mechanism of the cytotoxic action of PCZ may also be mediated by the methylating metabolic pathway. A significant recent finding supporting this view is that methylazoxypocarbazine, an intermediate on this pathway, is more cytotoxic than PCZ itself, while benzylazoxypocarbazine (the corresponding arylating metabolite) lacks cytotoxic activity (16, 17). Although the cytotoxic activity of simple methylating agents has for a long time been attributed to adducts other than $O^6$-mGua, there has been mounting evidence during recent years that the latter may also contribute to cytotoxicity (18–20). Evidence supporting the involvement of $O^6$-mGua in the cytotoxic (and antineoplastic) activity of PCZ was recently produced by Schold et al. (21) who showed that administration of PCZ to mice bearing xenografts of human brain tumors results in greater growth delay of those tumors which are of the mer⁻ phenotype, i.e., deficient in AGT.

In view of the evidence that $O^6$-mGua may contribute to the carcinogenic as well as the cytotoxic activity of PCZ, it seems possible that the measurement of the accumulation of this adduct in humans during therapeutic exposure to PCZ may serve as a useful index of individual biologically significant exposure (22), with possible implications in the development of improved therapeutic protocols. We wish to report here the results of a pilot study in which $O^6$-mGua and AGT were...
measured in leukocyte DNA of individuals through a 10-day course of treatment with PCZ. For purposes of comparison, O6-mGua formation was also measured in rat liver and leukocyte DNA following p.o. administration of PCZ at doses comparable to those to which humans are exposed. Measurement of O6-mGua was achieved using a recently developed sensitive CRA based on the use of Escherichia coli AGT for the suicide repair of O6-mGua in DNA in competition with a radiolabeled O6-mGua-containing oligonucleotide (23).

MATERIALS AND METHODS

Oligonucleotides and Antibodies. The oligonucleotide d(CGC(O6-mGua)AGCTCGCG), synthesized by the phosphotriester method and purified by HPLC, was generously provided by Dr. B. F. Li and Dr. P. F. Swann, Department of Biochemistry, The University College and Middlesex Hospital Medical School, London, United Kingdom (24). 5'-Labeling with [3H]adenosine 5'-[7-thio]triphosphate (800 to 1400 Ci/mmol) (New England Nuclear, Dreieich, Federal Republic of Germany) was carried out using T4-polynucleotide kinase under the conditions of the exchange reaction (25), except that two additions of enzyme (10 units each) were used, one at the beginning of the reaction and one 30 min later. Typical specific activities achieved were 100 to 250 cpm/nmol. The labeled oligonucleotide was purified by gel filtration through Sephadex G15 using for elution 50 mM ammonium bicarbonate/10 mM DTT. Its specific activity was determined by titration with AGT preparations of known activity (measured using as substrate [3H]-adenosine 5'-[7-thio]triphosphate (New England Nuclear, Dreieich, Federal Republic of Germany)).

Calf thymus DNA, containing 0.38 μmol of O6-mGua/mol of guanine, was prepared by reaction in vitro with N[3H]methyl-N-nitrosourea (5 Ci/mmol; Amersham) and analyzed for methylated adducts by acrylamide and HPLC. The preparation and properties of rabbit anti-serum against O6-methyldeoxyguanosine have been previously described (26). Purified AGT from E. coli (ada protein) (specific activity, 3000 pmol/mg of protein) was purchased from Applied Genetics, F. Swann, Department of Biochemistry, The University College and Middlesex Hospital Medical School, London, United Kingdom. All other reagents were research grade materials obtained from Sigma, Poole, United Kingdom.

PCZ Treatment and Collection of Blood. Blood samples were collected after informed consent from 7 patients with Hodgkin's or non-Hodgkin's lymphoma on treatment with PCZ. Details of the individuals studied are shown in Table 1. PCZ treatment involved intake of a p.o. dose of 50 mg of PCZ, 3 times per day, during a 10-day cycle. Patients on PCZ were also treated with vincristine, prednisone, and either cyclophosphamide or mechlorethamine hydrochloride. Vincristine and on PCZ were also treated with vincristine, prednisone, and either prednisone; HD. Hodgkin's lymphoma; M. mechlorethamine hydrochloride.

Animal Experiments. PCZ, freshly dissolved in water and kept away from light, was administered by stomach tube at doses 1, 2.5, 5, and 10 mg/kg to pairs of female Sprague-Dawley rats, weighing 150 g each. Controls received 0.2 ml of water. Two h later the animals were anesthetized with ether, and blood was collected through the hepatic artery. Approximately 4 to 6 ml of blood were collected from each animal and immediately frozen in liquid N2. Subsequently, the livers were also excised and frozen in the same way. Tissues from similarly treated animals were pooled and used for the extraction of DNA. DNA extraction from rat whole blood was carried out as described in the next paragraph. DNA was extracted from rat liver by multiple extractions with phenol and chloroform and treatments with proteinase K and RNase A as previously described (23).

Extraction of DNA from Whole Blood. Each blood sample was rapidly thawed under running cold water and immediately mixed with an equal volume of PBS [1 mM Na2HPO4 (pH 7.2):0.15 M NaCl]. It was then centrifuged for 15 min (6000 × g), the supernatant was decanted, and the precipitate was suspended in 6 ml of TE buffer. SDS (0.5%) and proteinase K (100 μg/ml) were added, and the mixture was incubated at 37°C for 2 h. It was subsequently extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform, and the DNA was precipitated with ethanol. After brief drying, the DNA was resuspended in 400 μl of TE buffer and sequentially treated with RNase A (50 μg/ml) and proteinase K (100 μg/ml)/SDS (0.5%) for 1 h each at 37°C. Finally, the mixture was again extracted twice with the above phenol:chloroform mixture and once with chloroform, precipitated with ethanol, washed with 70% ethanol, lyophilized, and redissolved in 100 μl of TE buffer for analysis of O6-mGua content. The ratio A260/A280 was >1.8. For purposes of quantitation of DNA, 1 A × ml was taken as 50 μg.

Isolation of Lymphocytes and Extraction of AGT. Lymphocytes (mononuclear cell fraction) were isolated from human blood by centrifugation on Ficoll (Pharmacia, Uppsala, Sweden) or Lymphoprep (Nycomed, Oslo, Norway) gradients and washed once with PBS. They were subsequently used for the extraction of AGT by sonication (2 times for 5 s each) in 100 μl of cold TE buffer containing 2 mM DTT, followed by centrifugation (15,000 rpm for 5 min). The supernatant was used for determination of AGT. The precipitate was subsequently treated with 200 μl of 0.4 M perchloric acid (70°C, 30 min), and the pooled extracts were used for the determination of DNA by the Burton diphenylamine method (27).

Determination of O6-mGua in DNA using the CRA. The CRA for O6-mGua contained in DNA was used as described in Table 1. To a series of microcentrifuge tubes containing standard, methylated DNA (0 to 10 fmol of O6-mGua) or unknown DNA (10 μg of total DNA) in total volume (50 μl of (2-hydroxyethyl)-1-piperazinethanesulfonic acid (pH 7.8):1 mM EDTA:10 mM DTT:50 μM spermidine:5% glycero:50 μg/ml of bovine serum albumin), 7 fmol of E. coli AGT were added, and the mixture (total volume, 36 μl) was incubated for 2 h at 37°C. Ten fmol of labeled, O6-mGua-containing oligonucleotide in 4 μl of transferase buffer were subsequently added, and the incubation was continued overnight. Any unrepaired, methylated oligonucleotide was finally immunoprecipitated with 25 μl of rabbit anti-O6-methyldeoxyguanosine antisera (at a dilution found to result in practically complete and selective precipitation of methylated oligonucleotide) and washed with 50% saturated ammonium sulfate solution. The precipitate was dissolved in 0.1 N sodium hydroxide and mixed with scintillation cocktail, and its radio-

Table 1 Individuals examined

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</table>

* NHL, non-Hodgkin's lymphoma; C, cyclophosphamide; V, vincristine; P, prednisone; HD, Hodgkin's lymphoma; M, mechlorethamine hydrochloride.
activity was measured in a scintillation counter. Each assay was carried out in triplicate. A typical standard curve is shown in Fig. 1. The limit of detection of O'-mGua by this assay was 0.5 fmol which, for 10 µg of DNA per assay, corresponds to a sensitivity of 50 fmol of O'-mGua/mg of DNA (8 x 10^-8 mol of O'-mGua/mol of guanine). As a control against false-positive results, samples of DNA found positive by the CRA were occasionally subjected to overnight pretreatment with excess E. coli AGT, followed by heat inactivation of the latter, prior to reanalysis by the CRA (23). In all cases negative results were found after such pretreatment.

Assay of AGT. AGT in extracts of lymphocytes was determined by a recently described microassay utilizing the same oligonucleotide as described above as a substrate for the enzyme and immunoprecipitation for the separation of repaired from unrepaired substrate (28). Preliminary experiments indicated that incubation of lymphocyte extracts with the labeled oligonucleotide did not result in any nonspecific degradation of the latter, thus permitting the use of the assay for the reliable determination of lymphocyte AGT (29). For the assay of AGT, to 4 µl of 10 x transferase buffer in a 1.5-ml conical microcentrifuge tube were added 4 µl of TE buffer containing labeled oligonucleotide (usually 4 fmol, 300 to 1000 cpm), followed by TE buffer and AGT extract to give a total volume of 40 µl. Following incubation at 37°C for 2 h, immunoprecipitation and radioactivity counting of the remaining methylated oligonucleotide were carried out as described in the previous paragraph. At least three different amounts (containing less than 30 µg of protein) from each extract were analyzed in duplicate. The use of 3H-labeled substrate precluded the routine conversion of cpm data to dpm for the determination of the stoichiometric concentrations of AGT. Instead, a standard curve was constructed using an AGT preparation of known activity in parallel with the analysis of unknown samples and used for the calculation of AGT activities.

RESULTS

O'-mGua in Lymphocytes of Individuals Exposed to PCZ. The formation of O'-mGua in leukocyte DNA of PCZ-treated individuals is shown in Fig. 2. All 7 individuals examined showed detectable amounts of O'-mGua at some time during the treatment period, reaching levels as high as 0.28 fmol/µg of DNA (0.45 µmol of O'-mGua/mol of guanine). Individual 1 (a smoker) had detectable amounts of adduct even prior to exposure to PCZ, probably due to exposure to other, unknown methylating agents. This individual began to accumulate O'-mGua from the beginning of exposure up to Day 7, thereafter showing a steady decline. The remaining individuals did not show detectable levels of O'-mGua until later during exposure. However, once O'-mGua became detectable, it continued to accumulate throughout the course of observation. In 5 cases, data for Day 11 (i.e., approximately 12 to 16 h after the final intake of PCZ) were also available, and in all of them detectable amounts of adduct were still present. Of these, Individuals 2 and 7 showed a decline in O'-mGua levels on Day 11 as compared with those of Day 10. (No such comparison was possible for Individuals 1, 4, and 5, owing to lack of data for Day 10).

Fig. 3 shows that the accumulation of O'-mGua correlates linearly with the cumulative dose of PCZ received, with a slope of 0.011 fmol of O'-mGua/µg of DNA per mg/kg of body weight (0.018 µmol of O'-mGua/mol of guanine per mg/kg of body weight). If the cumulative dose of PCZ based on individual surface area, rather than body weight, is used, the corresponding
O'-METHYLGUANINE IN LEUKOCYTE DNA FOLLOWING EXPOSURE TO PROCARBAZINE

slope is calculated as $2.68 \times 10^{-4}$ fmol of O'-mGua/µg of DNA per mg/m².

Clinical Observations. Increased numbers of blood leukocytes were observed on Day 8 in 5 of the 7 patients and decreased numbers in 2 (Nos. 2 and 4) (Table 2). The leukocyte count was reduced at the time of the start of the second cycle of treatment for all patients except for No. 1 who showed a small increase. At neither time did the leukocyte count show any correlation with the measured extent of O'-mGua accumulation. At the end of the first cycle of treatment, complete response was noted in all patients except Patient 2 (a non-Hodgkin’s lymphoma) in whom only partial response was observed. Furthermore, in all complete responders the results were sustained after further treatment, while in Patient 2, disease progression was evident.

AGT in Lymphocytes of Individuals Exposed to PCZ. The levels of lymphocyte AGT during PCZ treatment are shown in Fig. 4, where it can be seen that no consistent changes occurred. While AGT remained relatively stable in Individuals 1, 3, 5, 6, and 7, Individuals 2 and 4 showed significant fluctuations. Individual 2 showed a steady decline in AGT up to Day 7, when AGT reached very low levels. In contrast, AGT in Individual 4 more than doubled (relative to its preexposure value) on Day 5, reaching the highest levels observed in the present study, and remained high even up to Day 11.

Relationship between AGT and O'-mGua Accumulation. Inspection of Figs. 2 and 4 shows a striking qualitative correlation between the accumulation of O'-mGua in different individuals and the corresponding AGT levels. Thus, Individual 4, who was the most refractive toward O'-mGua accumulation, had the lowest levels of AGT. Conversely, Individual 2, who accumulated the highest levels of O'-mGua, had low AGT. The effect of AGT on the accumulation of O'-mGua is examined systematically in Fig. 5, where the individual rates of accumulation of O'-mGua are plotted against the corresponding values of AGT prior to PCZ exposure. A significant negative correlation is indicated ($r = -0.76$, $P = 0.049$).

O'-mGua in Liver and Leukocyte DNA of Rats Treated p.o. with PCZ. Fig. 6 shows the levels of O'-mGua in liver and leukocyte DNA of rats, 2 h after p.o. dosage with 1, 2.5, 5, and 10 mg/kg. The levels found in liver DNA were 0.063 to 0.33 fmol/µg of DNA (0.10 to 0.53 µmol of O'-mGua/mol of guanine) [which compare well with the values reported, for significantly higher doses, by Wiestler et al. (10) and, more recently, Fong et al. (11)] and were linearly related with the dose used. O'-mGua in leukocyte DNA was detectable at the two highest doses administered and was just under one half of that formed in liver DNA. It too was linearly related to the PCZ dose used.
**DISCUSSION**

O₆-mGua is an important precarcinogenic lesion induced in DNA by methylating carcinogens, including some nitrosamines to which there is widespread human exposure and which may therefore be of relevance to cancer in humans. While the presence of O₆-mGua in human DNA (as a result of exposure to unknown methylating agents) has been reported in the past (30, 31), the present study constitutes the first report of the dosimetry of its formation following controlled human exposure to a specific methylating agent and known animal carcinogen. A steady accumulation of O₆-mGua in leukocyte DNA was observed throughout a 10-day course of repeated exposure to PCZ in 6 individuals. In a seventh individual examined, maximum accumulation was observed on Day 7, followed by a steady decline. It is notable that this decline occurred in that individual in which the highest levels of O₆-mGua were observed, implying the possibility of selective loss of cells containing high levels of adduct. While (consistent with this suggestion) a significant decrease in leukocyte count was observed in this individual (Table 2), no overall correlation of leukocyte counts or response to therapy with O₆-mGua accumulation was noted in the small number of patients examined.

Comparison of the levels of O₆-mGua on Days 10 and 11 for 2 individuals (Fig. 2, Nos. 2 and 7) indicates that significant loss of O₆-mGua (at least part of which is likely to be due to DNA repair) takes place in 12 to 16 h (the interval between the final intake of PCZ and the collection of a blood sample). This indication is also supported by observations of leukocyte O₆-mGua following single dose exposure to the methylating drug dacarbazine.⁴

The daily intake (150 mg) received by a 70-kg human on treatment with PCZ corresponds to a dose of 2.1 mg/kg of body weight. Repeated, daily exposure to this dose results in the accumulation of O₆-mGua in human leukocyte DNA at a rate of 0.011 fmol/μg of DNA per mg/kg of body weight or 2.68 × 10⁻⁴ fmol/μg of DNA per mg/m² (Fig. 3). The slope of the linear regression describing the formation of O₆-mGua in leukocyte DNA of rats 2 h after single doses of PCZ (1 to 10 mg/kg) is calculated from Fig. 6 as 0.017 fmol of O₆-mGua/μg of DNA per mg/kg of body weight, corresponding to 5.67 × 10⁻⁴ fmol/μg of DNA per mg/m²; i.e., it is slightly larger than that observed in humans. However, taking into account the fact that the latter was based on the total exposure accumulated over a period of 10 days (during which significant amounts of O₆-mGua were lost by repair), it seems likely that human leukocyte DNA may be at least as susceptible to PCZ-induced formation of O₆-mGua as rat leukocyte DNA.

The O₆-mGua levels induced in rat leukocyte DNA by procarbazine were only 2-fold lower than those found in liver DNA. This finding is similar to the corresponding observation with dimethylnitrosamine (23, 32) and supports the suggestion that adduct formation in circulating leukocytes may be a sensitive means of monitoring human exposure to alkylating agents.

The measurement of AGT in lymphocytes and of O₆-mGua in total blood leukocytes (of which approximately 70% are neutrophils and only approximately 30% lymphocytes) constitutes a complication in the present study imposed by practical considerations. However, in view of the relatively short time which neutrophils spend in blood (8 to 12 h), it is likely that the relationships observed between AGT and the accumulation of O₆-mGua apply primarily to lymphocytes (whose presence in blood is much longer-lived). The negative correlation observed between AGT prior to the start of PCZ exposure and the rate of O₆-mGua accumulation (Fig. 5) indicates a probable protective effect of AGT against the accumulation of O₆-mGua in human DNA during exposure to a methylating agent. It also suggests that interindividual variations of AGT within the range normally observed in human populations (33, 34) may result in significant differences in the accumulation of O₆-mGua and underlines the importance of studies of the epidemiology of AGT and its relationship to cancer risk. Following their observation of reduced lymphocyte AGT in patients with therapy-induced disease, Sagher et al. (15) have suggested that PCZ may contribute significantly to the pathogenesis of acute non-lymphocytic leukemia following PCZ-based therapy for Hodgkin's disease. This suggestion was based on the hypothesis that (a) low AGT results in increased accumulation of O₆-mGua during PCZ exposure and (b) such increased accumulation of O₆-mGua leads to increased risk of neoplasia. Our observations directly confirm the first part of this hypothesis.

The suicide mechanism of repair of O₆-mGua by AGT would be expected to result in depletion of the enzyme in cells undergoing DNA methylation and subsequent repair (33). Such depletion has been reported by, among others, Meer et al. (35) following administration of large doses of PCZ to rats and by Gerson (36) in the lymphocytes of humans exposed to the methylating drug streptozotocin. While the amounts of O₆-mGua observed in the present study (under 0.3 fmol/μg of DNA) are much lower than the corresponding AGT levels (usually 5 to 10 fmol/μg of DNA), it should be borne in mind that they represent only the residual amounts of adduct present in DNA at the time of sampling and which were not repaired by AGT within the limited time period since PCZ exposure. As already noted, significant loss of O₆-mGua from lymphocyte DNA occurs within 12 to 16 h after PCZ intake. Furthermore, it is established that extensive repair of O₆-mGua can take place within as little as 1 h of low-level exposure of experimental animals to methylating agents, resulting in removal of as much as 50 to 80% of the adduct initially formed (32, 37). It seems likely, therefore, that the overall amount of O₆-mGua formed in human DNA during repeated exposure to PCZ was greater than that actually measured, the rest having been removed by repair with a concomitant stoichiometric loss of AGT. The measurements of lymphocyte AGT during the 10-day course of PCZ exposure did not reveal a systematic decrease of this enzyme (Fig. 3). This indicates that either the extent of repair of O₆-mGua which occurred was too small to significantly affect the cellular AGT pool or that de novo synthesis of AGT was sufficient to largely make up for such loss. While it has been noted that no significant recovery of AGT occurs in resting human lymphocytes in vitro for at least 72 h following AGT depletion (36), no information currently exists regarding the rate at which such recovery may take place in vivo.

The results reported here show that it is possible to measure O₆-mGua in a readily accessible tissue of humans exposed to PCZ and indicate a possibility of examining the quantitative aspects governing its accumulation and its relationships with biological end-points, including therapeutic efficacy and chronic toxicity.

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⁴ Unpublished results.
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