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Effects of bacterial and presystemic nitroreductase metabolism of 2-chloro-5-nitro-N-phenylbenzamide on its mutagenicity and bioavailability

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Abstract

2-Chloro-5-nitro-*N*-phenyl-benzamide (GW9662), a potent irreversible PPAR- γ antagonist, has shown promise as a cancer chemopreventive agent and is undergoing preclinical evaluations. Studies were initiated to assess its bacterial mutagenicity and pharmacokinetic profile in two animal species prior to subchronic oral toxicity evaluations and the results are reported here. GW9662 was mutagenic in both TA98 and TA100 bacterial strains with and without metabolic activation but was negative in the nitroreductase-deficient strains (TA98NR and TA100NR) also with and without metabolic activation, indicating that GW9662 mutagenicity is dependent on nitroreduction. The mutagenic activity was predominantly via a base-substitution mechanism. Following oral dosing in rats and dogs, the parent compound, GW9662, was virtually absent from plasma samples, but there was chromatographic evidence for the presence of metabolites in the plasma as a result of oral dosing. Metabolite identification studies showed that an amine metabolite ACPB (5-amino-2-chloro-*N*-phenyl benzamide), a product of nitro reduction, was the predominant species exhibiting large and persistent plasma levels. Thus systemic circulation of GW9662 has been attained largely in the form of its reduced metabolite, probably a product of gut bacterial metabolism. GW9662 was detectable in plasma of rats and dogs after intravenous dose albeit at low concentrations. Pharmacokinetic analysis following intravenous dosing in rats showed a rapid clearance and an extensive tissue distribution which could have accounted for the very low plasma levels. Of note, the amine metabolite was absent following intravenous dosing in both rats and dogs, confirming it being a product of presystemic metabolism. The potential utility of GW9662 as a chemopreventive agent, especially as an Estrogen Receptor- α (ER α) inducer in an otherwise ER α negative breast tissue, is of great interest. However, the results shown here suggest that additional animal toxicological and bioavailability studies are required to establish a role of GW9662 as a chemopreventive agent.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Keywords

Nitroreductases; Ames assay; nitro compounds; rat; dog; chemoprevention

1. INTRODUCTION

Peroxisome-proliferator-activated receptors (PPARs) are involved in the regulation of lipid and glucose metabolism and cell differentiation and proliferation [1, 2]. PPAR- γ (NR1C3), which is a member of the nuclear hormone receptor superfamily, is considered a potential target for the treatment of obesity, diabetes, and cancer. Several natural ligands that activate PPAR have been identified, including 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), linoleic acid, and lysophosphatidic acid [3]. Synthetic ligands shown to activate PPAR include members of the thiazolidinedione (TZD) family such as troglitazone, pioglitazone, rosiglitazone and ciglitazone. Significantly, both members of the TZD family as well as the non-thiazolidinedione tyrosine based PPAR γ agonist GW7845 have been shown to inhibit breast cancer both *in vitro* and *in vivo* [4]. However, these effects by PPAR agonists are apparently, largely, context – dependent, as for example cancer cell type [5, 6]. Of note, PPAR- γ receptors are differentially expressed in tumor relative to normal tissues [7]

Initially, most of the anti-cancer effects have been ascribed to PPAR- γ agonists. Subsequently, it has been recognized that the PPAR- γ antagonists also exhibit anti-proliferative and pro-apoptotic effects [8]. GW9662 is a potent irreversible PPAR- γ antagonist [9] and has shown anticancer potential *in vitro* against human mammary cancer cell lines [10]. However, the mechanisms through which GW9662 effects its anti-cancer activity are not entirely clear [10]. More recently, the anticancer activity of GW9662, including potential mechanisms, has been demonstrated in DMBA-treated mouse mammary organ cultures [11] and in the DMBA/progestin mouse mammary cancer model *in vivo* (manuscript in preparation). To better evaluate the utility of GW9662 as a potential chemopreventive agent, here we focused on preclinical testing including bacterial mutagenesis and pharmacokinetic evaluation.

2. MATERIALS AND METHODS

2.1 Chemicals and animals

GW9662 (2-Chloro-5-nitro-*N*-phenyl-benzamide) and its putative amine metabolite, 5-amino-2-chloro-*N*-phenyl benzamide (ACPB), were purchased from Alchem Laboratories Corporation (Alachua, FL) and Molport Laboratories (Riga, Latvia), respectively. The analytical internal standard benzyl nicotinate was purchased from Fluka, (Buchs, Switzerland). Labrasol was from Linden Warehouse and Distribution (Linden, NJ); polyethylene glycol (NF, PEG400), methyl cellulose (MC), DMSO, sterile water for injection from VWR International (Brisbane, CA); 2-aminoanthracene and sodium azide from Sigma-Aldrich (Milwaukee, WI); 2-nitrofluorene from Acros Organics (Fair Lawn, NJ); 2-aminofluorene and 2,4,6-trinitrotoluene (TNT) from Chem Service, Inc. (West Chester, PA).

2.2 Bacterial mutagenesis

The *S. typhimurium* tester strains TA98, TA100, TA98NR and TA100NR were received from BioReliance (Maryland, USA). Aroclor 1254-induced rat liver post mitochondrial supernatant (S9) were obtained from Moltox (North Carolina, USA).

The tester strains included the *S. typhimurium* histidine auxotrophs TA98 and TA100 as described by Ames *et al.* [12], and the *S. typhimurium* nitroreductase-deficient TA98NR and TA100NR as described by Rosenkranz *et al.* [13]. Based on preliminary experiments, dimethylsulfoxide (DMSO) was selected as the solvent of choice and all dosing formulations appeared as clear solutions. Selection of dose levels for the confirmatory mutagenic assay was based upon the toxicity and precipitation profile of the test article assessed in an initial mutation assay. Test article dosage formulations were prepared once prior to dosing based upon the results of the stability determination of dosing formulations. Formulations were prepared by serial dilutions of the stock. The concentration of the test article in the stock solution was confirmed analytically by HPLC. All dose levels of the test article, negative control, positive controls and reference control were plated in triplicate. The test system was exposed to the test article via the plate incorporation methodology originally described by Ames *et al.* [12] and updated by Maron and Ames [14]. This test system has been shown to detect a wide range of classes of chemical mutagens [15, 16]. On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assay, all tester strain cultures were checked for the appropriate genetic markers. Histidine dependence for all *S. typhimurium* strains was demonstrated by growth on selective agar plates (biotin, biotin/histidine and biotin/histidine/tryptophan control plates). Test for crystal violet sensitivity (*rfa* mutation) was performed using nutrient agar plates supplemented with biotin/histidine. Test for ultraviolet light sensitivity was conducted to check *uvrB* mutation in *S. typhimurium* tester strains. Test for ampicillin resistance (R-factor, presence of plasmid pKM101) was performed by using sterile filter paper discs with ampicillin that were placed to bacterial streak. Nitroreductase deficiency for TA98NR and TA100NR tester strains was confirmed by responses to reference control (2,4,6-trinitrotoluene) at different concentrations. The condition of the bacterial background lawn was evaluated for evidence of test article toxicity by using an invertoscope. Evidence of toxicity and degree of precipitation were scored relative to the negative control plate and recorded along with the revertant count for that plate. Toxicity was evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation was evaluated after the incubation period by visual examination without magnification. Revertant colonies for a given tester strain and activation condition were counted by hand using a counter pen. GW9662 was considered to be positive if it caused a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations, if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value and if the mean, positive control value was exhibited at least 3.0-fold increase over the respective mean, negative control value (vehicle) for the respective tester strain [17]. As a measure of GW9662 mutagenic potency, the initial slope of the dose-response curve was used [18].

2.3 In vivo rat and dog pharmacokinetics

Male 9–11 week old Sprague-Dawley rats were from Charles River (Hollister, CA). For intravenous study, the rats with jugular vein catheterized by the vendor were used. Male and female 9–10 months old Beagle dogs were from Marshall BioResources (North Rose, NY). GW9662 was administered to Sprague Dawley rats and Beagle dogs to investigate pharmacokinetics and metabolite formation. In the rat pharmacokinetic study, the effect of vehicle on absorption was studied by comparing GW9662 formulated as a suspension in 1% methylcellulose or in PEG400:Labrosol, 1:1. GW9662 was administered to three male rats per treatment group by either oral gavage of 500 mg/kg in 1% methyl cellulose, 500 mg/kg in PEG400:Labrosol, 1:1, or 2000 mg/kg in 1% methyl cellulose or i.v. of 2.5 mg/kg in 5% DMSO:95% PEG400. In the dog pharmacokinetic study, GW9662 was administered to 2 male and 2 female dogs per treatment group by either oral gavage of 300 or 700 mg/kg in 1% methyl cellulose or i.v. of 1, 2 or 7.5 mg/kg. An i.v. dose of 20 mg/kg was targeted for

the dog study, but adverse effects were observed during dose administration to the first dog, which resulted in administration of only 7.5 mg/kg to this dog. The remaining dogs in this treatment group were administered either 1 or 2 mg/kg.

The bioanalytical method for analysis and quantification of GW9662 in plasma (sample volume 50 µl) entailed the addition of 200 µl of acetonitrile to precipitate plasma proteins. The samples were vortex-mixed for 10 sec, and then the suspensions were clarified by centrifugation (18000 g, 10 min). The resulting supernatants (100 µl) were transferred to another set of microfuge tubes containing 200 µl of a solution of 75 ng/ml benzyl nicotinate (internal standard) in 35% acetonitrile: 65% water (v:v). These mixtures were briefly vortexed, and then transferred to HPLC vials fitted with glass inserts for LC-MS/MS analysis. Study samples were quantitated using a set of calibration standards containing both GW9662 and ACPB that were prepared in blank matrix, and were processed in parallel. The LC-MS/MS system consisted of autosampler (CTC-PAL, Leap Technologies, Carrboro, NC), pumps (LC-20AD, Shimadzu, Columbia, MD), Phenomenex Luna C18(2) column (100A, 50 × 2 mm; 3 µm) (Torrance, CA), and triple quadrupole mass spectrometer (API Sciex 4000™, AB Sciex, Foster City, CA). Samples (10 µl) were injected onto the LC-MS/MS system and eluted from the column at a flow rate of 0.5 ml/min with a gradient of mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The gradient conditions were as follows: 0 to 1 min 75% A and 25% B; 1 to 2.5 min 0% A and 100% B; 2.5 to 4.5 min 0% A and 100% B; 4.5 to 4.6 min 75% A and 25% B, and 4.6 min to 6 min 75% A and 25% B. The mass spectrometer was operated in the electrospray, positive ion mode with unit mass resolution in both mass analyzer quadrupoles. The desolvation temperature was 550° C, the ion spray voltage was 5.5 kV, the declustering potential was 56 V, the entrance potential was 10 V, the exit potential was 28 V, and the collision energy was 31 eV for all three analytes. The following MRM transitions were used in the analyses: GW9662 277.0→184.0 (retention time ~2.18 min), ACPB 246.8→153.5 (retention time ~1.84 min), and internal standard benzyl nicotinate 214.0→91.0 (retention time ~2.02 min). Integration and quantitation was done by Analyst Software ver 1.5.1. The lower limit of quantitation (LLOQ) was 3 ng/ml for GW9662 and ACPB. Determination of the LLOQ was based on the FDA Guidance, Guidance for Industry, Bioanalytical Method Validation [19].

The plasma drug level data were analyzed using WinNonlin® version 5.2 Professional (Cary, NC) by noncompartmental modeling using the sparse sampling feature. The following parameters and constants were determined: maximal plasma concentration (C_{max}), area under the plasma concentration-time curve to the last time point (AUC_{last}) and to infinity (AUC_{inf}), apparent volume of distribution (V), apparent clearance (Cl) and terminal elimination half-life ($t_{1/2}$).

2.4 Metabolite Identification Studies

Residual plasma samples from 2 rats administered 2000 mg/kg GW9662 by oral gavage were processed identically as for the quantitative analysis (above), and then they were examined by LC-MS/MS in 4 separate detection modes. Each of these modes used the same HPLC conditions as described above for the quantitative analysis, but the triple quadrupole mass spectrometer was operated in 4 different modes. In each of these modes, an Information Dependent Acquisition (IDA) was established to collect enhanced product ion spectra (using the linear ion trap feature of the AB Sciex the line 4000 Q trap instrument) of potential metabolites that were found during the run that exceeded a particular threshold above background. In this way it is possible to simultaneously obtain both LC-MS and LC-MS/MS data from the same analytical run.

The first mode used was an enhanced MS positive ion full scan ranging from m/z 90 to 750. This is the broadest scanning method, designed to evaluate all masses over this range, but it also will have the poorest signal-to-noise ratio of the 4 methods due to the high background chromatograms for the endogenous component of the plasma samples. The other 3 modes used took advantage of the major collision-induced fragmentation observed for GW9662, which can be depicted as follows:

Thus 2 of the modes were designed to detect precursor molecules (i.e., potential metabolites) that resulted that were evident either (1) as having a neutral loss of 93 or (2) was a precursor of the formation of an ion at m/z 184. The neutral loss method will monitor changes occurring on the 5-nitro-2-chloro benzoyl portion of the molecule, while the precursor ion scans will monitor changes occurring on the aniline portion of the molecule. Finally, a series of predictive MRMs based on the above fragmentation process were established on the basis of biotransformations occurring to either the 5 nitro-2-chloro benzoyl portion or the aniline portion of the molecule. These MRM scans will not be able to detect any unexpected biotransformations, but they do have the advantage of having potentially the best signal-to-noise for any of the scans. The predictive MRMs (only shows precursor ion mass sought) were as follows:

Mass Sought	Mass Shift from Parent	Biotransformation
247	-30	NO ₂ reduction to NH ₂
293	16	oxidation
309	32	di-oxidation
325	48	tri-oxidation
242	-35	dechlorination
291	14	methylation dimethylation or acetylation of reduction product plus oxygen
305	28	
469	192	oxidation + glucuronidation
582	305	glutathione addition
94	-183	hydrolysis to aniline

The data obtained in all of these analytical runs were then evaluated using the LightSight software program that allows pairwise comparison between runs, specifically between a control run (predose samples) and one of the experimental samples (obtained after dose administration). This enables us to distinguish those LCMS peaks that are a result of administration of the drug as opposed to those which may be endogenous to the rat plasma, but which happens to have sufficiently similar molecular weights to the putative metabolites.

3. RESULTS

3.1 Bacterial Mutagenesis

Here we used standard bacterial TA98 and TA100 strains of *Salmonella typhimurium* and their corresponding nitro- reductase deficient counterparts, TA98NR and TA100NR. Standard bacterial strains, TA98 and TA100, showed dose dependent increase in revertants by GW9662, in the absence or presence of rat liver post mitochondrial supernatant (S9 fraction) oxidative metabolic activation system (Tables 1 and 2, respectively). Conversely, no increase in revertants was seen upon exposure of TA98NR and TA100NR bacterial strains to GW9662 in the absence or presence of rat liver post mitochondrial supernatant metabolic activation system (Tables 1 and 2, respectively). Concurrent vehicle, negative and positive controls yielded the expected data confirming the validity of these assays.

GW9662 was clearly mutagenic in both TA98 and TA100 tester strains with and without metabolic activation but was negative in the nitroreductase-deficient strains (TA98NR and TA100NR) also with and without metabolic activation, indicating that GW9662 mutagenicity is dependent on nitroreduction. In the absence of metabolic activation, GW9662 had 34 times more base-substitution potency (TA100) than frameshift potency (TA98) (17.0 rev/ μ g vs. 0.5 rev/ μ g, respectively). In the presence of metabolic activation, GW9662 showed 12 times more base-substitution potency than frameshift potency (7.3 rev/ μ g vs. 0.6 rev/ μ g, respectively). GW9662 has the same frameshift potency in the presence or absence of metabolic activation (0.6 rev/ μ g vs. 0.5 rev/ μ g, respectively); however, the drug had twice the base-substitution potency in the absence of metabolic activation compared with the presence of metabolic activation (17.0 rev/ μ g vs. 7.3 rev/ μ g, respectively).

3.2 Pharmacokinetic in rats in vivo

Initial pilot toxicology studies in rats and dogs showed very low detectable plasma levels of GW9662 at different time points following oral gavage dosing (data not shown). Therefore studies were undertaken in rats and dogs to measure plasma levels following oral and i.v. dosing with GW9662. Oral gavage dosing of rats with GW9662 (500 or 2000 mg/kg in 1% methyl cellulose or 500 mg/kg in PEG400:Labrasol, 1:1, v/v) did not produce detectable levels of the parent GW9662 using a sensitive and specific LC-MS/MS assay that has a lower limit of quantitation of 3 ng/ml. However, LC-MS/MS chromatograms of samples from dosed animals did indicate the presence of metabolites in the plasma from animals that had received a dose of GW9662.

Metabolite identification studies were thus undertaken, and the results were as follows. The predominant molecule found by these approaches was a positive ion at m/z 247 that produced a fragment ion at m/z 154. This is consistent with a metabolite that is the result of nitrate reductase activity, the structure of which is given in Figure 1. The retention time for this was ~1.89 min. This peak is seen at all time points from 1 hr on, and the peak area increases with time after dose. By 4 hr after dosing there is another strong peak seen in both the full scan and neutral loss modes at m/z 289 (retention time ~1.93 min), which would be consistent with the N-acetylation product of the above compound, and then in the 8 hr and 24 hr samples there appears another strong peak at m/z 305 (retention time ~1.2 min), which would be consistent with the N-acetylated product receiving the addition of oxygen. Since this peak appeared in the neutral loss 93 chromatograms, which would indicate that oxygen was probably added to the aromatic ring containing the Cl and NH₂ groups.

Subsequently a reference standard for this metabolite was synthesized, and the mass chromatographic and mass spectral properties matched those of the metabolite in plasma extracts, thus confirming the structure of the metabolite to be 5-amino-2-chloro-N-phenyl benzamide (ACPB), an amine due to a nitro-reduction of the parent compound. This

reference standard was then used to quantify ACPB plasma levels in subsequent pharmacokinetic studies.

The GW9662 plasma concentration-time profile after i.v. dosing (2.5 mg/kg of GW9662) in rats is shown in Figure 2 and the resultant pharmacokinetic parameters are summarized in Table 3. High values for the apparent clearance and the apparent volume of distribution (51 L/hr/kg and 83 L/kg, respectively) were noted. The putative major metabolite, ACPB, shown following oral dosing was not detectable by i.v. dosing.

3.3 Pharmacokinetic in dogs in vivo

After both oral and i.v. administration of GW9662 to dogs, the parent drug was detected only sporadically in plasma samples and at low concentrations, 39.6 ng/ml or less. Thus there was insufficient data for calculation of pharmacokinetic parameters of GW9662. None of the samples that were obtained by i.v. dosing contained the putative metabolite ACPB. On the other hand, exposure to ACPB, based on AUC_{last} was as high as 130481 ng $\times$ hr/ml (male dog, 700 mg/kg) following oral dosing with GW9662. ACPB remained high throughout the 24-hour sample collection (Fig. 3).

DISCUSSION

GW9662, a PPAR- γ antagonist, has shown anti-cancer activity and is being evaluated preclinically as a potential cancer chemopreventive agent. For example, it has been previously demonstrated that inhibition of PPAR- γ using either a dominant negative transgene (Pax-8) [20] or pharmacologic intervention with GW9662 *in vivo* (Glazer R.I. et al, submitted) induced expression of ER α in an otherwise ER α -negative mammary tumors and, furthermore, that addition of an ER α inhibitor completely inhibited the appearance of these lesions. Thus GW9662 together with ER α inhibitors may have translational significance in the treatment of ER α -negative breast cancer. In order to better evaluate the potential utility of GW9662 as a chemopreventive agent, we have conducted detailed bacterial mutagenesis and pharmacokinetics studies. Bacterial reverse mutagenicity assay using standard bacterial strains showed an increased number of revertants in the presence of GW9662. Recognizing that nitro groups can undergo bacterial reductive metabolism leading to positive results in the Ames assay [13, 21–24], the study was repeated using standard and nitro-reductase deficient strains of *Salmonella typhimurium*. Nitro-reductase activity in *Salmonella typhimurium* is known [25]. TA98 is reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frame shift mutagens and TA100 is reverted by base substitution mutagens. *S. typhimurium* tester strains TA98NR and TA100NR are lacking the “classical” nitroreductase and were isolated as niridazole resistant derivatives of *S. typhimurium* tester strains TA98 and TA100, respectively [23]. While standard strains, TA98 and TA100, caused a dose dependent increase in number of revertants, this was not the case when using nitroreductase deficient strains, TA98NR and TA100NR. Analogous observations were made using a positive nitro-containing reference compound 2,4,6-trinitrotoluene. 2,4,6-Trinitrotoluene is a known nitroreductase-dependent mutagen whose mutagenic activity was established in TA98 and TA100 tester strains by Einisto and Spanggord *et al.* [24, 26], respectively, and shown to be absent in TA98NR and TA100NR strains. Therefore as anticipated, the positive mutagenicity results were due to metabolism of the parent compound by bacterial nitroreductases and not due to its direct mutagenicity. The addition of oxidative metabolic activation system had no effect on the mutagenic potency of GW9662 in the frameshift strain TA98; however, metabolic activation decreased the mutagenic potency of GW9662 two-fold in the base-substitution strain TA100.

Oral administration of GW9662 to rats and dogs yielded essentially undetectable plasma levels of the parent compound. On the other hand, the amine metabolite, ACPB, was the

predominant species after i.v. administration of GW9662 in either animal species, using a highly sensitive and specific assay. Plasma levels of GW9662 were detectable after its i.v. administration, albeit concentrations were relatively low, especially in dogs. Pharmacokinetic profile in rats showed rapid clearance and extensive tissue distribution. The apparent clearance of GW9662 (51 L/hr/kg) was much greater than the hepatic blood flow (4 L/hr/kg) in rats and the apparent volume of distribution (84 L/kg) was much greater than the total body of water (0.7 L/kg) in rats. Both of these pharmacokinetic parameters may have accounted for low plasma levels of the parent drug after i.v. administration.

Virtual absence of the parent compound in the plasma by oral dosing in both rats and dogs with concomitantly very high levels of the amine metabolite, and the absence of the metabolite after i.v. dosing suggest that GW9662 undergoes extensive presystemic metabolism. Enteric bacteria are likely the most commonly responsible for metabolic reduction of drugs [27]. Rat and human intestinal bacteria have been shown to possess nitroreductase activity responsible for reduction of nitro containing drugs [28, 29]. Presystemic nitroreduction of GW9662 to ACPB by intestinal microflora would be consistent with this known bacterial nitroreductase activity and the above observations in the bacterial mutagenesis assay. However, presystemic metabolism of drugs can occur in the gut wall as well as lumen [30] and a potential metabolic contribution of enterocytes can't presently be discounted.

Drug metabolism capability of intestinal microflora is known [31]. Frequently, anaerobic reduction of nitro containing drugs by bacterial nitroreductases takes place in gut lumen. Nitro-reductases can have significant effects, beneficial and adverse, on drug and nutrient metabolism thus clinical implications [32]. Nitroreduction by intestinal microflora was implicated in teratogenicity of nitro containing drugs in rats [33].

We have shown here that GW9662 was not mutagenic in the absence of bacterial nitroreductases. However, nitroreductase-containing bacterial strains metabolized GW9662 to a mutagen that exerted its mutagenic activity predominantly via base-substitution. The main compound detected in plasma from rats and dogs following oral dosing with GW9662 was the amine metabolite, ACPB, consistent with the nitro reduction of the parent compound in the gut, most likely by bacterial nitroreductases. In view of the bacterial mutagenicity resulting from bacteria mediated reduction of nitro to amino moiety of GW9662 and the observation of the major metabolite resulting from nitroreduction of the parent compound following oral dosing, further toxicological studies of GW9662 are needed to better assess its utility as a potential anticancer agent

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A. GW9662 (2-chloro-5-nitro-N-phenylbenzamide)

B. ACPB (5-amino-2-chloro-N-phenyl benzamide)

Figure 1. Chemical structure of GW9662 (A) and its nitro reduced metabolite ACPB (B)

Figure 2. Plasma drug concentration of GW9662 after i.v. administration of 2.5 mg/kg to male Sprague-Dawley rats

Values represent mean $\pm$ standard deviation for $n = 3$ rats for the first 3 time points and $n = 1$ rat for 1 and 2 hr because the replicate samples were below the lower limit of quantitation (< 3 ng/ml). In the dog pharmacokinetic study, plasma samples were collected at multiple time points up to 24 hr after iv administration of GW9662; however parent drug was detected in samples collected at 5, 15 or 30 min after administration only, in concentrations ranging from 4.3 to 762 ng/ml. All other plasma samples collected from dogs treated iv with GW9662 had concentrations $< \text{LLOQ}$.

Figure 3. Plasma concentrations of the putative metabolite of GW9662, ACPB in fasted dogs after a single oral dose of 300 mg/kg GW9662. Individual data for four animals are presented. Numbers in the parentheses refer to a specific dog being studied.

Table 1

Bacterial reverse mutation assay in the absence of metabolic activation system

Agent (dose, µg/plate)	TA98	TA98NR	TA100	TA100NR
Untreated negative control	16 ± 4	15 ± 2	114 ± 14	242 ± 21
Vehicle	16 ± 3	16 ± 4	118 ± 16	230 ± 13
GW9662 (15)	29 ± 2	14 ± 2	388 ± 32 [#]	218 ± 11
GW9662 (50)	55 ± 14 [#]	14 ± 4	969 ± 22 [#]	243 ± 5
GW9662 (150)	130 ± 13 [#]	18 ± 2	1560 ± 50 [#]	247 ± 24
GW9662 (500)	272 ± 3 [#]	12 ± 3	2044 ± 46 [#]	250 ± 7
GW9662 (1500)	309 ± 23 [#]	16 ± 5	2186 ± 30 [#]	228 ± 20
Positive control *	262 ± 12 [@]	164 ± 17 [@]	1106 ± 27 [@]	1097 ± 84 [@]
Reference 2,4,6-trinitrotoluene, TNT (150)	181 ± 7 [#]	24 ± 4	845 ± 40 [#]	245 ± 6
Mutagenic Potency (revertants/µg)	0.5	0	17.0	0.0

Values represent average revertants per plate ± standard deviation.

* 2-Nitrofluorene (20 µg/plate) was used for TA98 and TA98NR strains.

* Sodium azide (2.5 µg/plate) was used for TA100 and TA100NR strains.

[#] Response 2X or greater

[@] Response 3X or greater

Table 2

Bacterial reverse mutation assay in the presence of metabolic activation system

Agent (dose, µg/plate)	TA98	TA98NR	TA100	TA100NR
Untreated negative control	25 ± 6	21 ± 5	113 ± 19	238 ± 6
Vehicle	23 ± 3	24 ± 4	112 ± 7	226 ± 16
GW9662 (15)	24 ± 3	15 ± 2	237 ± 2 [#]	249 ± 11
GW9662 (50)	48 ± 4 [#]	22 ± 6	595 ± 61 [#]	265 ± 4
GW9662 (150)	104 ± 15 [#]	26 ± 2	1213 ± 115 [#]	241 ± 12
GW9662 (500)	158 ± 24 [#]	16 ± 4	1519 ± 27 [#]	263 ± 4
GW9662 (1500)	197 ± 28 [#]	20 ± 8	1599 ± 14 [#]	266 ± 10
Positive control [*]	803 ± 36 [@]	770 ± 18 [@]	1553 ± 56 [@]	692 ± 34 [@]
Reference 2,4,6-trinitrotoluene , TNT (1000)	213 ± 4 [#]	38 ± 2	1056 ± 96 [#]	325 ± 42
Mutagenic Potency (revertants/µg)	0.6	0.0	7.3	0.0

Values represent average revertants per plate ± standard deviation.

Metabolic activation system included Aroclor 1254-induced rat liver post mitochondrial supernatant, S9 fraction.

^{*} 2-aminoanthracene (2.5 µg/plate) was used for TA98 and TA100 strains.

^{*} 2-Aminoflourene (10 µg/plate) was used for TA98NR and TA100NR strains.

[#] Response 2X or greater

[@] Response 3X or greater

Table 3

Pharmacokinetic parameters for GW9662 in Sprague-Dawley rats following i.v. administration of 2.5 mg/kg

$t_{1/2}$ (hr)	1.1
T_{max} (hr)	0.083
C_{max} (ng/mL)	127.0
C_0 (ng/ml)	232.4
AUC_{last} (hr $\times$ ng/mL)	42.8
AUC_{inf} (hr $\times$ ng/mL)	49.0
V (L/kg)	83.8
Cl (L/hr/Kg)	51.0