Use of "N-in-One" Dosing to Create an in Vivo Pharmacokinetics Database for Use in Developing Structure–Pharmacokinetic Relationships

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Abstract □ The purpose of this work was (1) to determine if useful in vivo pharmacokinetic data could be obtained after simultaneous administration of 5–22 compounds of a chemically congeneric series to dogs and (2) to determine if structure–pharmacokinetic relationships could be derived from such studies. Mixtures of structurally related α-1 antagonist compounds (**5**–**22**) were administered intravenously to conscious dogs. Blood samples were taken over the next 24 h and analyzed by LC/MS to determine plasma levels and pharmacokinetics of each compound. The pharmacokinetics of 17 of these compounds were also determined after individual administration. Results obtained in the N-in-One format for 17 compounds correlated well with results obtained when these same compounds were administered individually. The N-in-One method is a useful method for obtaining pharmacokinetic data on 5–20 molecules in a single animal at one time. The increased throughput in obtaining important pharmacokinetic information should enhance the drug discovery process. In addition, it was possible to determine the extent to which various chemical substitutions did or did not affect pharmacokinetic parameters.

Introduction

Success in drug discovery increasingly relies on the ability to rapidly identify quality molecules that possess the desired attributes of bioavailability, chemical tractability, selectivity, and potency. Combinatorial chemistry and high throughput biochemical screening techniques should have a major impact on the process of generating and identifying potent and selective compounds in drug discovery.[1] The identification of compounds that possess the desired pharmacokinetic (oral bioavailability, clearance, volume of distribution, and half-life) and pharmacodynamic profile has become a time-consuming process in drug discovery programs. While in vivo studies are expensive to conduct, they are also extremely informative. Thus, substantial enhancements in the capacity to characterize molecules in vivo could help relieve a bottleneck in the current drug discovery process.

Increased throughput in in vivo pharmacokinetic screening has recently been reported by (a) dosing mixtures of compounds to a single animal and (b) by pooling samples from singularly administered compounds prior to analysis.[2−8] Both methods capitalize on tandem liquid chromatography/mass spectrometry (LC/MS) as a sensitive and specific method for analysis. We have referred to the former method as N-in-One dosing, where N is the number of compounds coadministered. The N-in-One approach provides an opportunity to study the pharmacokinetics of several compounds under identical conditions while minimizing sample processing time, analysis time, and the number of animals required. Initial reports have shown the method applicable to (a) a wide range of structural classes of compounds, (b) iv and oral dosing, (c) compounds that are renally and/or metabolically eliminated, and (d) a wide variety of species including mice, rats, dogs, and monkeys.

This contribution expands on a previous report of increased capacity of in vivo pharmacokinetic evaluations.[2] This paper reports results of N-in-One studies of 5−22 α-1a antagonist compounds that provided sufficiently good estimates of pharmacokinetic such that compound progression decisions could be made. In addition, structure-pharmacokinetics relationships were developed to help define molecular changes that do and that do not alter pharmacokinetics.

Methods

Compounds—All compounds were synthesized at Glaxo Wellcome and gave satisfactory analysis by TLC, ^{1}H NMR, mass spectrometry, and elemental analysis.[11]

Pharmacokinetic Study—*N-in-One Dosing*—Male mongrel dogs (weight range 15−20 kg) were fasted overnight and fitted with two temporary cephalic vein cannulae on the day of study. Each dog received 5−22 compounds per dose session. Dose solutions involving mixtures of five or less compounds were prepared by dissolving compounds in 50 mM sodium acetate buffer (pH 4.5). Dose solutions with more than five compounds were prepared by dissolving compounds in 100 mM sodium acetate buffer containing 30% propylene glycol and 1% Tween 80 at a concentration of 0.3 mg each compound/mL. The dose solution was infused over 5 min into the right cephalic vein cannula. Each compound was given at a dose level of 0.25 mg/kg body weight (5-in-One) or 0.3 mg/kg body weight (>5-in-One). Blood samples (4 mL) were obtained from the left cephalic vein cannula in heparinized syringes at 0 (predose), 5, 15, 30, 45 min, 1, 1.5, 2, 4, 6, 8, and 24 h after the infusion began. The plasma was separated from the red blood cells by centrifugation and frozen until APCI LC/MS/MS or APCI LC/MS analysis.

Individual Dosing—Male mongrel dogs (weight range 15−21 kg) were fasted overnight and then fitted with two temporary cephalic vein cannulae on the day of study. Each compound was dissolved in 50 mM sodium acetate buffer (pH 4.5) at a concentration of 1.0 mg/mL and administered at a dose of 1 mg(base)/kg body weight via a 5 min intravenous infusion into the right cephalic vein. Blood was sampled, and plasma was obtained and stored as described above until analysis by HPLC with fluorescence detection. Each compound was studied in one dog, except for compounds **19** and **59** which were studied in four and two dogs, respectively. This

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Table 1—Pharmacokinetics of Compound 19 in Individual and N-in-One Studies[a]

	n	half-life (min)	clearance (mL/min/kg)	volume of distribution (mL/kg)
individual studies	4	248 ± 37	0.8 ± 0.3	202 ± 101
N-in-One studies	9	326 ± 96	0.9 ± 0.3	272 ± 99

[a] Data are mean ± standard deviation for the number of observation shown. $p > 0.05$ using Student's *t*-test.

research complied with national legislation and with company policy on the Care and Use of Animals.

Sample Analysis—*N-in-One Dosing*—Calibration standards were prepared at each calibration level for each compound by spiking blank dog plasma over the range of 1 to 2300 ng/mL. Aliquots of the standards and samples (200 μL) were precipitated with 400 μL of acetonitrile that contained an internal standard (compound **18** or **9**) at a concentration of 400 ng/mL. After vortexing and centrifugation, the resulting supernatant was transferred to a clean tube and evaporated to dryness under nitrogen at 35 °C.

The analysis was performed on a Finnigan TSQ-700 mass spectrometer with Atmospheric Pressure Chemical Ionization (APCI). The residues were reconstituted in mobile phase (200 μL), centrifuged, and injected onto either a 3 μm, 50 × 4.6 mm (5-in-One study) or a 5 μm, 250 × 4.6 BDS Hypersil C18 column (>5-in-One studies). The mobile phase consisted of 5 mM ammonium acetate (pH 4.0) and acetonitrile at a flow rate of 1 mL/min. The percentage of acetonitrile was adjusted in each study to retain the mixture from the column void volume and elute all compounds within a k' range of about 1−10. The first 2 min of the HPLC effluent were diverted to waste to minimize contamination of the APCI source. Detection was by selected reaction monitoring (SRM) in positive ion mode for the 5-in-One study. A more generic approach using MS rather than MS/MS was used for quantitation of larger mixtures by scanning in selected ion monitoring mode (SIM). A longer column was necessary for mixtures >5 compounds to increase selectivity and afford more specificity because SIM was used. The run time per sample was 6 min for the 5-in-One study and a maximum of (approximately) 30 min for the other N-in-One studies.

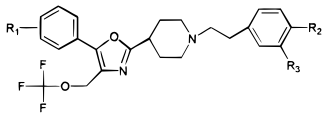
Individual Dosing—Plasma calibration standards were constructed from 5 to 200 ng/mL for each compound. Aliquots of standards and samples (50−100 μL) were precipitated with two volumes of acetonitrile (no internal standard) and prepared as described above. Samples above 200 ng/mL were diluted prior to protein precipitation. The plasma extracts were reconstituted in mobile phase (100 mL) and injected onto a Keystone BDS Hypersil C18 (150 × 3 mm, 3 μm) HPLC column at a temperature of 30 °C. The compounds were eluted with a 25 mM ammonium acetate buffer (pH = 4.5) and acetonitrile mobile phase at a flow rate of 0.5 mL/min. The compounds were detected using a fluorescence detector. The mobile phase composition and wavelength settings were varied to optimize conditions for each compound.

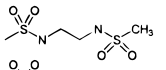
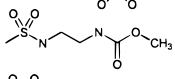
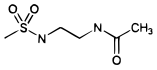
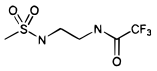
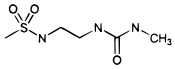
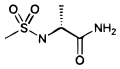
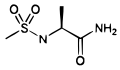
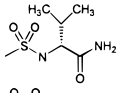
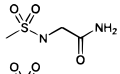
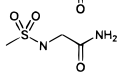
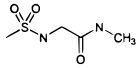
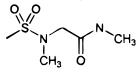
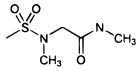
Pharmacokinetic and Statistical Analysis—The clearance (CL), steady-state volume of distribution (V_{ss}), and elimination phase half-life ($t_{1/2}$) of each compound were derived from the respective plasma concentration versus time curve using noncompartmental methods. Statistical comparisons between groups were done by an unpaired *t* test. Results in Tables 2−6 reflect pharmacokinetic data from 1 to 4 dogs in individual or N-in-One format.

Results

Comparison of Pharmacokinetics after Individual and N-in-One Dosing—Table 1 compares the pharmacokinetic parameters of compound **19** when dosed individually to those obtained when coadministered with 4−21 other compounds. There was no statistical difference for any parameter between the two methods. Figure 1 shows the linear regression analyses for the plasma elimination half-life, clearance and steady-state volume of distribution of 17 compounds when measured by both individual and N-in-One methods. There was good agreement in the pharmacokinetic parameters, particularly clearance, obtained from N-in-One dosing and individual dosing.

Table 2—Pharmacokinetic Data for Compounds 1–15[a]



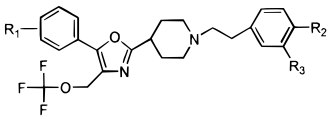
Cmpd	R1	R2	R3	CL (mL/min/kg)	t1/2 (min)	Vss (mL/kg)
1	4H	OMe	H	14	59	785
2	4H	OMe		2.0	144	151
3	4H	OMe		9.1	144	647
4	4H	Ome		10	156	600
5	4H	Ome		26	72	2035
6	4H	Ome		6.8	72	210
7	4H	OMe		11	120	1102
8	4H	OMe		11	114	930
9	4H	OMe		22	107	1241
10	4H	OMe		28 ± 8	123 ±47	4133 ±2514
11	4H	OMe		18	135	3175
12	4F	OMe		11	432	4778
13	4H	OMe		22	144	3450
14	4H	OMe		25 ±4	101 ±34	3004 ±651
15	4F	OMe		12	204	2907

[a] Data are present as the mean ± the standard deviation. Compound 19 data is mean of $n = 4$ individual dogs, compounds 26, 27, 31, 33, 48, and 59 are $n = 3$ observations from either individual dogs or from an N-in-One study; the remaining means are $n = 2$ (if standard deviation is shown) otherwise $n = 1$.

Pharmacokinetic data from all studies (individual and N-in-One dosed) are compiled in Tables 2−6. Mean data are presented in cases where the compound was dosed in more than one dog or was studied by both N-in-One and individual dosing. The range of pharmacokinetic values obtained from this chemical series was fairly large. For example, CL ranged from 0.3 to 44 mL/min/kg (146-fold); $t_{1/2}$ ranged from 45 to 1072 min (24-fold); and V_{ss} ranged from 41 to 14178 mL/kg (346−fold). The extent of protein binding was determined for 21 molecules. These were highly protein bound molecules which ranged from 92−99.94% bound (133-fold variation in unbound fraction).

Effect of Chemical Modifications on Pharmacokinetic Properties—*Effect of 4-F on Left-Hand Phenyl Ring*—There are several examples of pairs of molecules (**11, 12; 14, 15; 18, 19; 30, 31; 35, 36; 55, 56; 19, 21**) where the

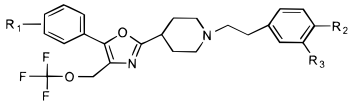
Table 3—Pharmacokinetic Data from Compounds 16–29[a]

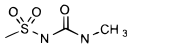
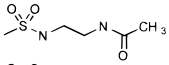
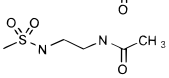
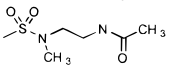
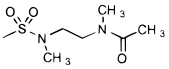
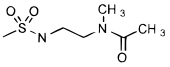


Cmpd	R1	R2	R3	CL (mL/min/kg)	t1/2 (min)	Vss (mL/kg)
16	4F	OMe	H	7.9	318	2677
17	4F	OMe	F	6.7	324	2233
18	4H	OMe		4.5 ±0.7	85 ±1	523 ±199
19	4F	OMe		0.8 ±0.3	248 ±37	202 ±101
20	3F	OMe		5.0	90	523
21	4Cl	OMe		0.9 ±0.3	336 ±64	417 ±218
22	3F,4Me	OMe		5.4	67	626
23	2F,3F	OMe		2.6	45	242
24	2F,5F	OMe		5.3 ±0.9	109 ±68	616 ±21
25	2F,4F	OMe	H	8.9 ±3.7	130 ±23	1595 ±812
26	3F,4F	OMe		2.0 ±0.8	333 ±118	643 ±374
27	2F,4F	OMe		1.0 ±0.7	287 ±86	334 ±110
28	2F,3F,4F	Ome		0.6 ±0.4	429 ±182	240 ±182
29	2F,4F,5F	OMe		2.3	138	372

[a] See footnote of Table 2.

Table 4—Pharmacokinetic Data from Compounds 30–39[a]



Cmpd	R1	R2	R3	CL (mL/min/kg)	t1/2 (min)	Vss (mL/Kg)
30	4H	OEt		2.8 ±0.9	129 ±72	375 ±82
31	4F	OEt		0.8 ±0.5	264 ±86	186 ±35
32	2F,4F	OEt		1.1 ±0.8	176 ±57	172 ±95
33	4F	OEt		0.3 ±.2	286 ±103	2017 ±3314
34	4F	OEt		11	144	1950
35	4H	OEt		16	126	1992
36	4F	OEt		5.5	126	675
37	4F	OEt		14	66	1539
38	4F	OEt		18	54	1806
39	4F	OEt		9	71	516

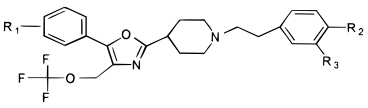
[a] See footnote of Table 2.

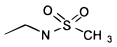
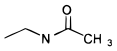
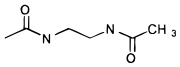
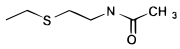
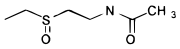
only structural difference was the presence or absence of a F-substitution in the 4-position of the phenyl ring at the 5-position of the oxazole ring. In each case, the effect of the 4-F substitution was to reduce CL, although the magnitude varied from a 36% decrease (**55, 56**) to greater than 5-fold decrease (**18, 19**). CL was reduced in all cases despite the fact that CL of the non-F molecules varied from 2.8 to 44 mL/min/kg. Similar effects on CL were obtained when a chlorine was placed in the 4-position. The CL of **21,** the 4-chloro compound, was similar to **19**, the 4-F analogue (0.9 vs 0.8). In contrast, a 4-methyl substitution did not reduce clearance. The CL of compound **22** increased with 4-methyl substitution compared to its di-F analogue (**26**) (2.0 vs 5.4 mL/min/kg). The position of the halogen appeared to be important in determining the CL of the compound. The presence of F in the 3-position (**20**) did not decrease CL as much as a 4-position F- or Cl-substitution. The CL for **19** was 6 times less than its 3-F homologue (**20**). The importance of 4-position halogenation in lowering CL was also evident in a series of compounds with multiple halogenations on the left-hand side phenyl. The CL of the 2,4-F compound (**27**) was one-half that of the 2,3-F (**23**) and one-quarter that of the 2,5-F compound (**24**). Compounds **19**−**21**, **23**−**24**, and **26**−**29** contain one or more halogen substitutions on the left-hand phenyl ring (either F or Cl) with R_2 = methoxy and R_3 = SO_2NH_2. Of these nine compounds, three compounds (**20**, **23**, and **24)** have H in the 4-position with 0−3 fluorines in other positions on the ring. The remaining six compounds are all 4-halogen compounds. The CL values for **20, 23**, and **24** are 5.0, 2.5, and 5.3 mL/min/kg, respectively, whereas the CL of the other six compounds are lower, ranging from 0.7 to 2.3 mL/min/kg. On the basis of metabolites identified from bile samples of dogs dosed with compound **11**, it is likely that the halogen-substitution on the 4-position blocked a common hydroxylation pathway of metabolism on the left-hand side phenyl ring resulting in a decrease in metabolic clearance.

The effect on V_{ss} of F-substitution on the left-hand phenyl varied. V_{ss} decreased in five pairs of examples of 4-position halogenation from 16 to 66%; however, V_{ss} increased 50% for **12** compared to **11**. The net effect of 4-F substitution on $t_{1/2}$ was mixed depending on the magnitude of change in CL and volume. For example, compounds **11** and **19** experienced a 200% increase in their $t_{1/2}$ whereas **36** and **56** saw essentially no change. 4-Chloro or 4-methyl substitution did not change V_{ss}. No trends were observed in the modest changes in V_{ss} for multiply halogenated compounds. Changes in $t_{1/2}$ thus followed from the changes in CL for these compounds.

*Effect of F-Substitution on Right-Hand Phenyl Ring (**16, 17**; **40, 41**; **41, 42**)*—The placement of F on the right-hand phenyl ring at R_2 or R_3 led to changes in pharmacokinetic parameters that were inconsistent with what was observed with left-hand phenyl ring substitution. Comparisons of **16, 17** as well as **40, 41** and **41, 42** show that putting F in both the R_3 (meta) position with R_2 = methoxy (**16, 17**) or

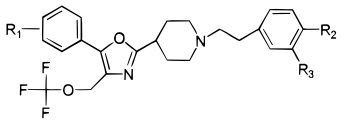
Table 5—Pharmacokinetic Data from Compounds 40–54[a]

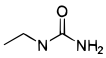
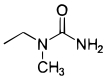
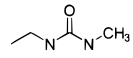
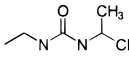
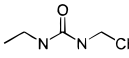
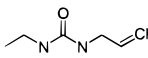
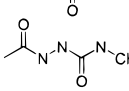


Cmpd	R1	R2	R3	CL (mL/min/kg)	t1/2 (min)	Vss (mL/kg)
40	4F	H	H	4.1	720	2743
41	4F	F	H	7.4	107	884
42	4F	F	F	3.3	89	246
43	4F		H	6.2 ±1.7	653 ±112	5101 ±757
44	4F		H	5.7	486	3350
45	4F		H	17	78	2419
46	4F		H	6.4	846	6192
47	4F		H	34 ±1	324 ±51	14178 ±6375
48	4F	NH_2	H	6.3 ±1.5	1072 ±502	8052 ±1493
49	4F	NMe	H	6.6	894	5584
50	4F		H	24	66	1725
51	4F	NO_2	H	10	126	838
52	4F		H	18	240	5298
53	4F		H	11	78	828
54	4F		H	14 ±10	211 ±7	3632 ±2819

[a] See footnote of Table 2.

Table 6—Pharmacokinetic Data from Compounds 55–66[a]



Cmpd	R1	R2	R3	CL (mL/min/kg)	t1/2 (min)	Vss (mL/kg)
55	H		H	44	108	4967
56	4F		H	28	114	3655
57	4F		H	33	126	5085
58	4F		H	8.4	252	2102
59	4F		H	2.5 ±1.4	982 ±110	3128 ±1376
60	4F		H	5.9	468	2795
61	4F		H	26 ±0	95 ±10	3375 ±1053
62	4F		H	6.1	270	1808
63	4F		H	12	282	3024
64	4F		H	19 ±3	244 ±122	4173 ±330
65	4F		H	9.3	648	7754
66	4F		H	8.0 ±0.6	570 ±187	4308 ±1852

[a] See footnote of Table 2.

at R_3 with R_2 = H (**40, 41**) had either no effect on or increased CL (CL = 7.9 vs 6.7 mL/min/kg; 4.1 vs 7.4 mL/min/kg, respectively). However, putting F in both the R_3- and R_2-position (**41, 42**) reduced CL by 55% (7.4 vs 3.3 mL/min/kg). The addition of F to R_2 (**40, 41**) or addition of F to both R_2 and R_3 (**40**, **42**) decreased V_{ss}. A modest decrease in V_{ss} was also observed when F was substituted at R_3 in the R_2 = methoxy series (**16**, **17**).

*Effect of Adding SO_2NH_2 to R_3 or R_2 (**1, 18; 25, 27; 19, 47**).* Placing a sulfonamide in the R_3 position decreased CL from 14 to 4.5 mL/min/kg (comparing **1** and **18**, respectively). The V_{ss} also decreased (51%), and $t_{1/2}$ was increased from 59 to 85 min. When this same chemical modification was made to **25**, a larger decrease in CL was observed (from 8.9 to 1.0 mL/min/kg for compounds **25** and **27**, respectively). V_{ss} also decreased from 1595 to 344 mL/kg and there was little effect on $t_{1/2}$. In sharp contrast, when the sulfonamide was placed at the R2 position (**47**) CL increased from 0.8 to 34 mL/min/kg, a 42-fold increase compared to **19**. V_{ss} was also dramatically increased from 202 (**19**) to 14178 mL/kg (**47**), a 70-fold increase. However, the dramatic alterations in CL and V_{ss} led to a relatively modest 31% decrease in $t_{1/2}$ from 248 to 324 min.

Effect of Substituted SO_2NH_2 in R_3-position—Substitution on the sulfonamide in the R_3-position when R_2 = methoxy (**2**−**11**, **13**, **14**, **18**) resulted in higher CL values for all compounds compared to the unsubstituted **18** with the exception of **2** which had a CL about one-half that of **18**. The V_{ss} also tended to be higher for substituted compounds (523 mL/kg for **18** and ranging from 600 to 3464 mL/kg for all others) except for compounds **2** and **6** which had lower V_{ss} values of 151 and 210 mL/kg, respectively. Similar effects (increased CL and V_{ss}) were observed with substituted sulfonamides in the 4-F series (**12, 15** compared to **19**) when R_2 = methoxy. For example, compound **15** had a larger CL (12 mL/min/kg) and a larger V_{ss} (2407 mL/kg) than its unsubstituted counterpart **19** (0.8 mL/min/kg, 202 mL/kg). Similarly, substitution on the sulfonamide in the R_2-position in the R_3 = ethoxy series **(30, 35; 31, 35, 36**−**39)** usually led to increases in CL (0.8−2 mL/min/kg compared to 5.5−18 mL/min/kg) and an increase in V_{ss} (1992 compared 375 mL/kg; or 516−1806 compared to 186 mL/kg). The notable exception in this case was **33,** an *N*-methyl sulfonylurea, which had the lowest CL (0.3 mL/min/kg) of not only all of the substituted sulfonamides, but also of all the 66 compounds presented in this paper, and a very low V_{ss} of 123 mL/kg. Protein binding was determined on five of the compounds in this N-substituted sulfonamide series and free fraction was greater for all the substituted molecules compared to that of the unsubstituted analogue. This may partly explain the larger volumes and the higher clearances. The higher clearances may also arise from increased metabolism (N-dealkylation) of the sulfonamide substitution, a metabolic pathway that was

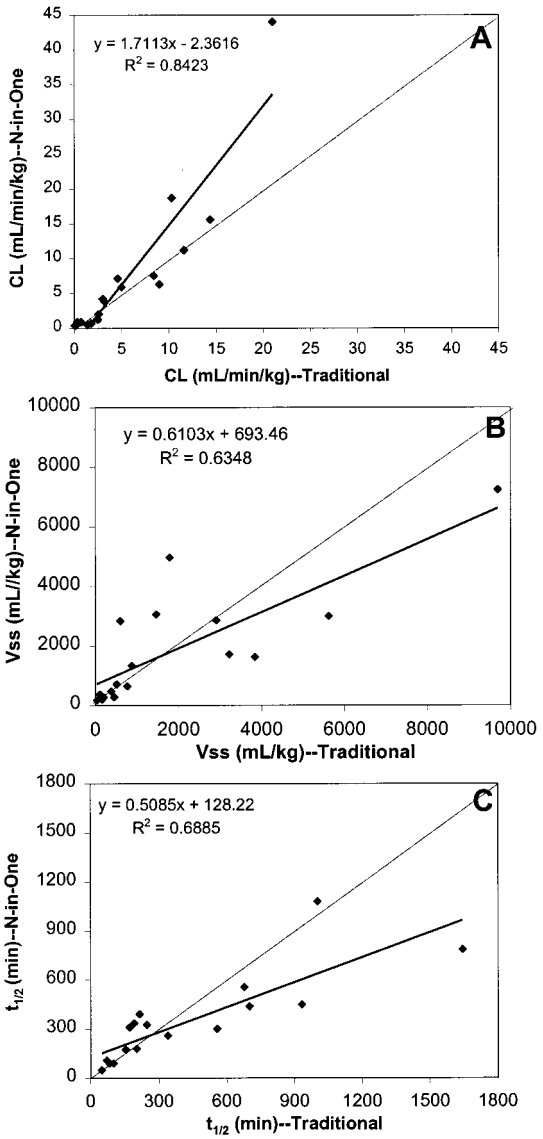


Figure 1—Linear regression (bold line) of CL, V_{ss}, and $t_{1/2}$ data for 17 compounds by both individual and N-in-One methods.

observed in in vitro dog microsomal metabolism studies of several compounds including compound **11**. Finally, one pair of enantiomers was synthesized by substituting *R*- or *S*-alanine on the R_3 sulfonamide. One enantiomer (**8**) had 50% lower CL compared to its opposite enantiomer (**9**). V_{ss} was 27% lower and $t_{1/2}$ was little changed (114 vs 107 min for **8** vs **9**, respectively).

Effect of Chemical Modifications to R3-Sulfonamide Substitutions—Methylation of the sulfonamide nitrogen (**37**) or the acetamide nitrogen (**39**) or both (**38**) increased the CL of the compound compared to that of **36**. When both nitrogens were methylated (**39**), CL was increased more than that of a single methylation (18 vs 9.0 or 14 mL/min/kg). V_{ss} was also increased when the sulfonamide nitrogen is methylated (**37** or **38**), but not when the acetamide alone was methylated (**39**). The net effect on $t_{1/2}$ was a consistent decrease of about 50% comparing **36** to **37**–**39**.

When the amide nitrogen was methylated (**13**), CL was not changed much (22%) compared to the unmethylated compound (**11**). When both the amide and sulfonamide nitrogens were methylated (**14**), the CL was still similar to when one nitrogen was methylated (**11**). However, comparing the 4-F compounds **12** and **15,** CL was unaffected when both nitrogens were methylated (11 to 12 mL/min/kg). No consistent changes were noted in V_{ss}, and no major differences in $t_{1/2}$ were observed in these series. Finally**,** replacement of a methyl group with a trifluromethyl group on the R_3 sulfonamide (**4, 5**) resulted in a 160% increase in CL, 239% increase in V_{ss}, and a 50% reduction in $t_{1/2}$.

*Comparison of Ethoxy- vs Methoxy-Substitution at R_2 (**4, 35; 18, 30; 19, 31; 25, 32**)*—No consistent change in pharmacokinetic properties was observed when the size of the alkyl group on R_2 was increased from methyl to ethyl. The notable structural difference between these compounds is that **30**–**32** are unsubstituted sulfonamides, whereas **35** has a ethylene acetamide moiety on its sulfonamide nitrogen. The direction and magnitude of change in pharmacokinetic parameters may be related to the size of the R_3 substituent. For the pair of substituted sulfonamides (**4**, **35**) the CL increased by 60% and the V_{ss} increased by 232% when the methoxy was changed to ethoxy. For the unsubstituted sulfonamides **(18, 30; 19, 31)**, the CL and V_{ss} decreased or were unchanged. The net effect of these changes on $t_{1/2}$ was minimal, except in the case of **30**.

Effect of Adding an N-Methyl Group to R_2 Amide[56–58]—In this example of *N*-methylation, addition of a methyl group to the amide of **56** to generate **57** had little effect on CL, V_{ss}, or on $t_{1/2}$. In contrast to the earlier two examples of methylation at the R_3-substituent, adding a second methyl group (**58**) dramatically reduced the CL from 33 mL/min/kg (**57**) to 8.4 mL/min/kg (about 4-fold). V_{ss} also drops by more than 50% (from 5085 mL/kg to 2102 mL/kg) which attenuates the increase in $t_{1/2}$ to 138% (from 126 to 252 min).

Effect of Adding an N-Methyl Group to Right-Hand Aniline: Comparing NH_2 to NO_2—When the aniline nitrogen was methylated (**49**), CL was not greatly altered compared to **48** (6.3 to 6.6 mL/min/kg). V_{ss} and $t_{1/2}$ were unaffected as well. Adding a second methyl group (**50**) caused CL to increase by almost 4-fold (from 6.3 to 24 mL/min/kg) with a further decrease in V_{ss} (from 8052 to 1725 mL/kg) and a decrease in $t_{1/2}$ by 94% to only 66 min. Oxidation of the amine to a nitro group (**51**) also increased CL by 59% and decreased V_{ss} by 90% compared to **48** and hence also resulted in a large decrease in $t_{1/2}$ of 84%.

*Effect of Alkyl Substitution on the Terminal Nitrogen of the Phenylmethyleneurea on R_2 (**59**–**64**)*—Single methylation of the proximal nitrogen resulted in an increase in CL of 136% (**59** compared to **60**), a modest decrease in V_{ss}, and a large decrease in $t_{1/2}$ of 52% (from 982 to 468 min). When the terminal nitrogen was methylated, the effect on CL was even greater going from 2.5 to 26 mL/min/kg (**59** compared to **61**), and there was little effect on V_{ss} (from 3128 to 3375 mL/kg). The net effect on $t_{1/2}$ was a dramatic reduction from 982 to 95 min (**59**, **61**). When larger alkyl groups were added to the terminal nitrogen of **59**, the effect was still a net increase in CL and decrease in $t_{1/2}$, but the magnitude of the changes were not as great compared to the effect of adding a single methyl group.

*Comparing Methylene Acetamide to Methylene Sulfonamide or Acetal at R_2 (**40**, **43**–**45**)*—Placing an acetal group at R_2 (**45**) increased CL from 4.1 to 17 mL/min/kg relative to no substitution (**40**). V_{ss} was not greatly affected (2743 vs 2419 mL/kg) and $t_{1/2}$ was, therefore, greatly reduced (78 vs 720 min). Extending the acetal to a methylene acetamide (**44**) resulted in a lower CL value of 5.7 mL/min/kg and a higher V_{ss} value of 3350 mL/kg leading to an intermediate $t_{1/2}$ of 486 min. When the methylene acetamide was replaced with a bulkier methylene sulfonamide (**43**), CL (6.2 mL/min/kg) remained essentially the same as **44** and V_{ss} increased still further to 5101 mL/kg. The $t_{1/2}$ is 653

min, which is the longest $t_{1/2}$ of this group of compounds, yet still less than the unsubstituted compound (**40**).

Effect of Other R_2 Substitutions—Comparing the pharmacokinetics of the sulfonamide to the sulfone in the R_2-position, the sulfonamide (**47**) had a 5-fold higher CL and 2-fold higher V_{ss} than the methyl sulfone (**46**). The $t_{1/2}$ was lowered by a factor of 1.6-fold, comparing **47** to **46**. Oxidation of the thioether (**53**) to its sulfoxide (**54**) resulted in a slight increase in CL (from 11 to 14 mL/min/kg) whereas V_{ss} quadrupled, going form 828 to 3632 mL/kg. The effect on $t_{1/2}$ was a 171% increase going from 78 to 211 min. Extension of the urea in the R_2-position (**65**) with a methylene group (**59**) led to a >3-fold increase in CL (2.5 to 9.3 mL/min/kg) and a 37% increase in V_{ss}. The $t_{1/2}$ was decreased by 42%.

Discussion

The original goal of this effort was to determine if N-in-One studies could be used to rapidly determine pharmacokinetic consequences of changes in chemical structure within a congeneric chemical series. In essence, that goal was achieved. This conclusion is supported by the linear regression analysis on the pharmacokinetic parameters for 17 compounds studied by the individual method of one compound in one dog or in various N-in-One studies. It is also supported by the work with **19** which was examined in all nine N-in-One studies and in four separate single drug studies (Table 1).

A second feature of this work was to permit the development of structure−pharmacokinetic relationships with the 66 compounds tested using this method. Of the 66 compounds, 22 were examined in at least two dogs as mixtures or individually. The general robustness of the data afforded the opportunity to make hypotheses about structure−pharmacokinetic relationships that would suggest further study or the resynthesis of compounds to investigate the hypothesis. There are some caveats and limitations to using this method. It is not clear if it applies uniquely to this set of compounds or if the method applies to all compound mixtures. More studies by us and others will be needed to answer this question. A number of limitations have been previously cited[2] and those apply here as well.

In the research program from which these compounds were derived, the desired criteria for the three PK variables were: CL < 5 mL/min/kg, $t_{1/2}$ > 240 min and V_{ss} < 1000 mL/kg. At the other extreme, compounds having either CL > 15 mL/min/kg, $t_{1/2}$ < 120 min, or V_{ss} > 3000 mL/kg were judged unacceptable. Of the 66 compounds studied, there were only five molecules which met all three pharmacokinetic criteria (four in the R_2 = methoxy series, and one in the R_2 = ethoxy series). On the other hand, only two compounds had unacceptable values for all three parameters and these were in the series where R_2 = H. This led to the conclusion that continued modification of compounds with R_2 = methoxy or ethoxy would lead to compounds with the best pharmacokinetic profile. In addition, halogen substitution at the 4-position of the left-hand-side phenyl and removal of substitutions on the R_3-sulfonamide consistently resulted in compounds with lower CL. However, the latter did not appreciably increase the half-life of the compound, since removal of the sulfonamide substitution also consistently led to compounds with reduced volumes of distribution. Compound **33** appears to be unique in pharmacokinetic terms since most N-substituted sulfonamides (**2**−**15; 35**−**39**) had CL values between 2 and 34 mL/min/kg, yet for **33** the CL was 0.3 mL/min/kg, the lowest value obtained for all of the molecules examined. Compound **33** was studied in three dogs with consistent results and therefore raises the question as to why this methylsulfonylurea had such a low (and desirable) CL.

Another example with unexpected results is compound **46** and **47**, which differ only in one being a methyl sulfone, whereas **47** is a sulfonamide in that position. Despite this similarity, there is a 5-fold difference in CL. The explanation for this difference is not clear. Neither the clogP values (3.4 and 3.2, respectively), nor different electron-withdrawing effects (σ values of 0.68 vs 0.62 via Hammett equation) seem likely explanations. One possibility is that glucuronidation may be an additional elimination pathway for the sulfonamide thus contributing the higher clearance. This example highlights the value in doing the N-in-One approach to generate data that can lead in directions that would not have been anticipated from first principles.

Both in vitro drug metabolism and cell permeation studies and in vivo pharmacokinetic experimentation will be needed in order to more fully expand our current understanding of the various factors governing pharmacokinetics.[9,10] It is envisioned that the enhanced capacity to generate qualitative and quantitative pharmacokinetics and drug metabolism data will provide information that will enable the iterative generation and refinement of predictive rules regarding the many factors that govern pharmacokinetics.

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