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INVESTIGATION OF SIDE-CHAIN SAR, FORMULATION AND INJECTION SITE TOLERATION OF PYRAZOLO[3,4-*f*]QUINOLINE DERIVATIVES: A POTENT SERIES OF IN VIVO ACTIVE IMMUNOSTIMULANTS

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Abstract: Pyrazolo[3,4-*f*]quinoline derivatives represent a novel class of immunostimulant with potent in vivo effects in a murine infection model. Side-chain SAR, formulation issues and injection site toleration studies have been addressed and compounds suitable for extensive in vivo evaluation have been identified. The results of these investigations are presented in this report.

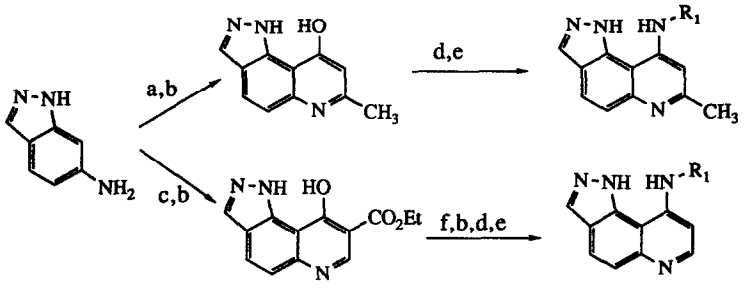
Infections are a leading cause of morbidity and mortality among cancer chemotherapy patients, severe burn victims, surgery patients and AIDS patients due to the often severe immunosuppression accompanying the underlying disease. These infections may be caused by any number of pathogens including bacteria, fungi, protozoa and mycoplasma. Stimulation of the non-specific immune response is expected to significantly assist these patients in avoiding such infections as well as to control those already established.[1-3]

In addition to these applications in human medicine, an immunostimulant could play a role in livestock production. Periods of stress (e.g. shipping, weaning, handling, dietary changes, co-mingling) have been associated with increased incidences of bacterial disease in cattle and swine. This is consistent with studies demonstrating stress to have a deleterious effect on the immune system.[4] It is expected that an immunostimulant, alone or in conjunction with a conventional antibiotic, would provide an improved clinical outcome relative to antibiotic alone resulting in considerable economic benefit to the food producer.[4]

A recent study[5] of partial structures and analogs of imidazo[4,5-*f*]quinoline based immunostimulants[6] identified a novel, potent class of in vivo active immunostimulants containing a pyrazolo[3,4-*f*]quinoline nucleus. An effort to optimize activity within this series was undertaken with emphasis placed on the R_1 position and to a lesser extent R_2 (Table 1). The promising activity of these compounds in a murine model prompted us to consider progression of selected analogs to large animal models and forced consideration of solubility and injection site problems associated with these compounds. The results of the SAR studies at R_1 and R_2 as well as a discussion of solubility and injection site issues are the subjects of this report.

Chemistry: The synthesis of the target compounds was accomplished using previously published chemistry[5, 7] illustrated in Scheme 1.

Scheme 1



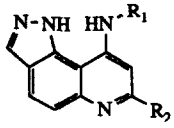
(a) ethyl acetoacetate, $CaSO_4$, HOAc (b) Dowtherm A, reflux (c) diethyl ethoxymethylenemalonate, toluene (d) $POCl_3$, DMF (e) RNH_2, ethanol (f) KOH, methanol

Biology: Immunostimulant activity was evaluated in a mouse protection assay in which increased survival over non-drug treated controls was the endpoint. Briefly, mice were dosed at -24 h with drug and at time 0 h with a lethal inoculum of *E. coli* and a subtherapeutic dose of antibiotic (gentamicin). The mice were monitored for 96 h and survivors in the drug treated groups compared with survivors in the control groups (no drug treatment).[8] The results of these evaluations are shown in Table 1.

Results and Discussion: Several trends are apparent upon examination of the immunostimulant activity of the analogs in Table 1.[9] (1) Homologous R_1 substituents produced compounds of comparable potency (cf. **1, 2, 3** and **4**). (2) Generally, R_2 can be methyl or hydrogen with no loss in potency (cf. **7** and **8**; **9** and **10**); however, exceptions to this generalization exist (cf. **14** and **15**). Previous work with another sub-series of immunostimulants[10] has shown this position to be tolerant of a phenyl substituent as well. Thus, this site is relatively amenable to modification and could, therefore, be useful in modulating the physicochemical properties of this series of compounds. (3) Incorporation of hydrophilic substituents at R_1 significantly reduced biological activity. (4) The most potent analogs compare favorably to related compounds previously disclosed by Norwich-Eaton (i.e. **19**; MED = 6 mg/kg)[5,6] and Pfizer (i.e. **18**; MED = 6 mg/kg).[5] Interestingly, the meta methoxy substituted phenyl of **18** appears superior to the corresponding para substituted analog **12**. Further exploration of meta substituted analogs would seem warranted.

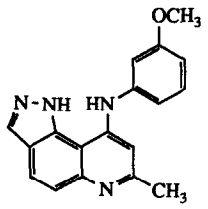
The mechanism of action of these compounds is unknown, however a simple antibacterial mode of action has been ruled out.[5] The lack of an in vitro correlate to the in vivo immunostimulant activity makes precise discussion of SAR difficult. Many factors could be responsible for the absence of in vivo activity in addition to lack of inherent immunostimulant activity.

Table 1. Immunostimulant Activity of Pyrazolo[3,4-*f*]quinoline Derivatives

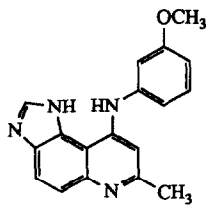


Compound	R_1	R_2	MED* (mg/kg)	Solubility†
1	4-Me-C_6H_4	CH_3	6.25	C
2	4-Et-C_6H_4	CH_3	12.5	B
3	4-Pr-C_6H_4	CH_3	6.25	B
4	4-Bu-C_6H_4	H	6.25	B
5	4-Cl-C_6H_4	CH_3	1.85	C#
6	4-Cl-C_6H_4	H	12.5	C#
7	4-BuO-C_6H_4	CH_3	1.85	C#
8	4-BuO-C_6H_4	H	3.13	B
9	4-($C_6H_5CH_2O$)-C_6H_4	CH_3	0.62	C
10	4-($C_6H_{11}CH_2O$)-C_6H_4	H	0.62	C
11	4-($C_6H_{11}CH_2O$)-C_6H_4	CH_3	0.62	C
12	4-CH_3O-C_6H_4	CH_3	16.7	ND
13	4-HO-C_6H_4	CH_3	16.7	ND
14	C_6H_{11}	CH_3	1.85	A
15	C_6H_{11}	H	TOXIC	A
16	3-pyridyl	CH_3	>50	ND
17	4-(HO_2CCH_2S)-C_6H_4	CH_3	>50	ND

*Minimum effective dose (MED) is the dose (given s.c.) which provides statistically significant ($p < 0.05$) protection as compared to control animals. †Solubility classifications of mesylate salts : A signifies ≥ 25 mg/ml; B signifies > 6 but < 25 mg/ml; C signifies < 6 mg/ml. #Hydrochloride salt. ND = not determined



18



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The potent immunostimulant activity of these compounds in our murine model prompted consideration of selected analogs for evaluation in economically important species. The feasibility of such an evaluation was impacted by the poor water solubility of most of these compounds. The ability of selected compounds to be formulated was assessed and is shown in Table 1. Dosage forms in which the compound was dissolved at ca. 25 mg/ml in distilled water (no buffering) were considered to have satisfied this objective. Mesylate salts were found to be routinely more soluble than their corresponding hydrochlorides.

Three compounds (**14**, **18**, and **19**) were chosen for possible advancement to large animal efficacy studies based on a combination of their potency and efficacy in the murine model as well as their formulation properties. Injection site toleration is an important feature of a compound targeted for animal health applications where dosing is anticipated to be by intramuscular or subcutaneous routes. This parameter was assessed for these three compounds using goats as a compound sparing model of cattle toleration.[11]

Compound **19** induced the most severe lesions of the compounds tested. It induced severe necrosis and edema at the injection site. The lesions were devoid of intact vascular tissue suggesting they would probably not resolve on their own. In addition, significant amounts of compound remained at or near the injection site. Intramuscular administration of **18** produced a significantly less extensive lesion consisting primarily of localized areas of inflammation and necrosis. The presence of intact vascular tissue suggested these lesions would most likely resolve themselves. Gross examination of injection sites from animals treated with **14** indicated this compound induced lesions similar to those observed with **18**.

Based upon the potency and efficacy in the murine infection model and their acceptable formulation and injection site toleration profiles, **14** and **18** were chosen for extensive evaluation in cattle efficacy trials. The results of these investigations will be published in due course.

Acknowledgment

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8. *Escherichia coli* N63 (serotype O18) was maintained as a frozen stock in bovine blood at - 70 °C. On the day before challenge, stock culture was thawed and streaked for isolation on brain-heart infusion agar (BHI). After overnight incubation, three 1-mL Prompt-innoc® (Becton-Dickinson Microbiology Systems, Cockeysville, MD) vials were prepared according to the manufacturers directions and used to inoculate 100 mL of L broth (Difco). The broth culture was incubated at 37 °C with shaking for approximately 3 hours, at which time the optical density (600 nm) reached 0.3-0.4, corresponding to approximately 8 x 10^8 CFU/mL as determined by viable count. This culture was then diluted in cold L broth to achieve a concentration near 4 x 10^7 CFU/ml.

   Female NSA (CF-1) mice weighing 11-16 grams (Harlan Sprague Dawley, Indianapolis, IN) were infected by intraperitoneal (i.p.) injection of 0.5 mL of bacterial culture as described above. Gentamicin was administered subcutaneously (s.c.) at 0.5, 4, and 24 h post-infection. Immunomodulators were prepared in pyrogen-free saline or water for injection (USP) and administered 24 h prior to infection unless otherwise noted. A hand-held glass or electric

(Omni 1000) tissue homogenizer was used to prepare suspensions of drugs in saline. Ten mice were used per treatment group.

9. All final compounds were characterized by ^1H NMR, combustion analysis and/or mass spectral data which were fully in accord with expected structures. Data for a representative compound is shown. **5**: ^1H NMR (DMSO-d_6) ∂ 2.68 (s, 3 H), 7.06 (s, 1 H), 7.62 (m, 4 H), 7.68 (d, 1 H, J = 9 Hz), 8.28 (d, 1 H, J = 9 Hz), 8.80 (s, 1 H); mass spectrum m/z 309 (M^+, 100); Anal. calcd for $C_{17}H_{13}N_4Cl \cdot HCl \cdot H_2O$: C, 56.21; H, 4.44; N, 15.42 . Found: C, 56.21; H, 4.40; N, 15.24.

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11. To standardize the injection site toleration determinations, experimental compounds were suspended in sterile water at a concentration of 20 mg/mL and 2.5 mL were injected into the semitendinosus muscle. This dose is equivalent to approximately 5 mg/kg. Injections sites were examined for gross pathology five days post-injection. Two compounds were evaluated simultaneously in each animal (i.e. one compound per leg). Each compound is compared to a control treatment consisting of 2.5 mL of sterile water. In addition, within a single study, each compound was evaluated in a single animal against each other compound being examined. This experimental design allowed replicate evaluations of each compound and the effects of animal to animal variation were minimized.