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Evaluation of nitrate-substituted pseudocholine esters of aspirin as potential nitro-aspirins

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Abstract—Herein we explore some designs for nitro-aspirins, compounds potentially capable of releasing both aspirin and nitric oxide in vivo. A series of nitrate-bearing alkyl esters of aspirin were prepared based on the choline ester template preferred by human plasma butyrylcholinesterase. The degradation kinetics of the compounds were followed in human plasma solution. All compounds underwent hydrolysis rapidly ($t_{1/2} \sim 1$ min) but generating exclusively the corresponding nitro-salicylate. The one exception, an *N*-propyl, *N*-nitroxyethyl aminoethanol ester produced 9.2% aspirin in molar terms indicating that the nitro-aspirin objective is probably achievable if due cognisance can be paid to the demands of the activating enzyme. Even at this low level of aspirin release, this compound is the most successful nitro-aspirin reported to date in the key human plasma model.

NO-NSAIDs are compounds in which a non-steroidal anti-inflammatory drug is coupled to a nitric oxide donating moiety through a spacer group, usually an ester.[1] They are also referred to as cyclo-oxygenase (COX) inhibitory NO donors (CINODs).[2] The original pharmacological rationale for these compounds was that nitric oxide has gastro-protective effects which would ameliorate the gastrointestinal toxicity of the NSAID. A general observation is that the nitric oxide and COX-inhibitory partners exhibit synergy especially in models of carcinogenesis.[3,4]

Aspirin is an obvious candidate for the NO-hybrid approach. It is gastro-toxic even at the low doses used in cardio-protection while its antiplatelet effects are complementary to those of NO.[5] NCX-4016, the prototypical NO-aspirin, has shown promising effects in a variety of cardiovascular disease models and in cancer.[6,7] Alternative NO-aspirin hybrid designs include 1-(pyrrolidin-1-yl) diazen-1-ium-1,2-diolates[8] (e.g., **2**), furoxans[9], and aspirin derivatives of isosorbide mononitrate.[10] The actions of these compounds appear to be complex and not easily explicable in simple terms of aspirin and nitric oxide release. Indeed there is no evidence that any of the compounds reported so far are capable of significant aspirin release in human tissue.[9,11] In the case of NCX-4016 irreversible cyclooxygenase inhibition is reported to be mediated by the intact drug.[12]

The difficulty in designing aspirin ester prodrugs (with or without an NO-donating capability) is that aspirin already has an ester, its acetate, which becomes highly labile in plasma when the carboxylic acid is converted to an ester.[13] The dominant esterase in human plasma, butyrylcholinesterase (BuChE), is not efficient in the hydrolysis of negatively charged substrates, but processes neutral phenylacetates extremely rapidly.[14] In order to release aspirin in vivo, the carrier group (bearing the NO-donor) must be detached at a faster rate than the acetyl ester, whose hydrolysis the carrier ester promotes. This conundrum was the focus of numerous investigations at the simple prodrug level, long before the prospect of an NO-aspirin emerged. One of the very few successful solutions was the design of glycolamide esters, which act as pseudocholine substrates for BuChE.[13] The *N*,*N*-diethylglycolamide of aspirin and its hydroxy-analogue (**3**) were reported to undergo hydrolysis in dilute human plasma releasing around 50% aspirin on a molar basis. There have been reports of glycolamide esters of NSAIDs,[15,16] including one recent nitrate-bearing glycolamide of naproxen,[17] but the obvious promise of this approach to the design of *NO-aspirins* does not appear to have been evaluated.

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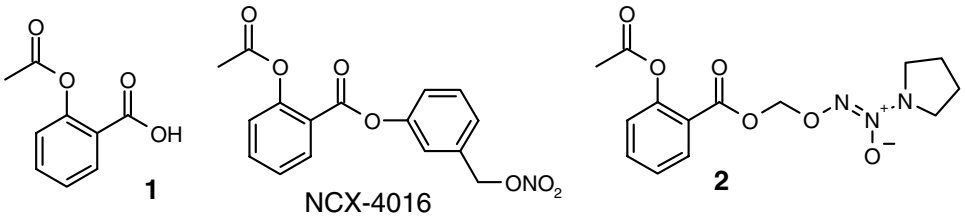
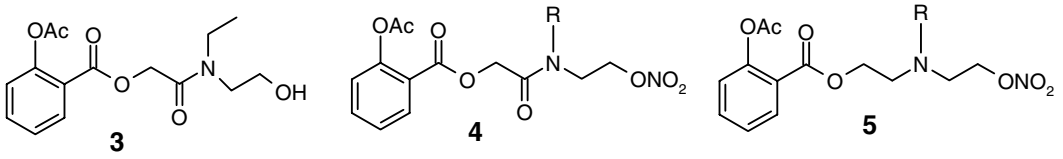


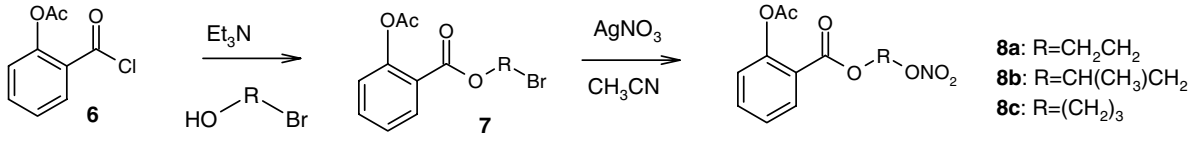
The purpose of this study was to determine if the productive hydrolysis characteristics of the aspirin glycolamides could be maintained when the carrier group is further substituted with an NO-donor, for example, **4**. Some related ethanolamine esters (**5**) as well as simple nitrooxyalkyl esters were also tested as true aspirin prodrugs, also potentially capable of releasing nitric oxide.

Four ethanolamine carrier groups were prepared (Scheme 3). The appropriate *N*-alkyl amino ethanol was nitrated using fuming nitric acid in DCM.[19] The resulting nitroxyalkylammonium salts were dissolved in 7 M NaOH and alkylated using bromoethanol.[20] Esterification of **10** with commercially available acetylsalicoyl chloride in the presence of triethylamine afforded the mono- or di-nitrate esters (**5a**–**d**).

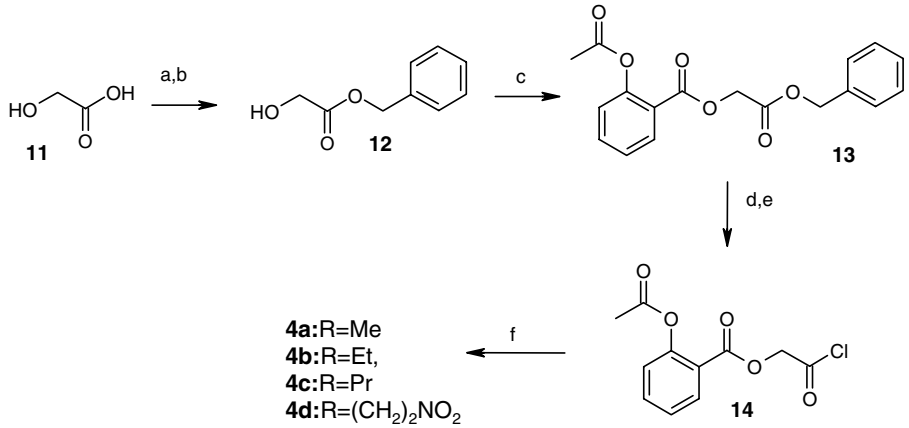


Simple nitroxyalkyl esters (**8a**–**c**) were prepared by stirring acetylsalicoylchloride **6** with the appropriate bromoalcohol in the presence of triethylamine followed by Br-displacement using $AgNO_3$ in acetonitrile.[18] In preparing the nitroglycolamide esters, the linking glycolic acid unit was introduced by esterification with benzyl protected glycolic acid (**12**). The resulting ester **13** was deprotected and treated with oxalyl chloride to give the acid chloride. The glycolamides **4a**–**d** were generated in the presence of the appropriate alkylamino nitrate. The *N*-ethyl, *N*-hydroxyethylglycolamide ester of aspirin (**3**), reported to release aspirin in human plasma solution,[13] was prepared as a reference (Schemes 1 and 2).

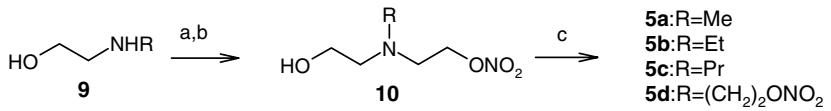
The various nitrate-esters were incubated in dilute human or rat plasma solution (pH 7.4, 37 °C) or in the presence of horse serum BuChE in order to predict their potential to act as prodrugs for aspirin in vivo. The product profile was determined using an RPHPLC/PDA method capable of discriminating between aspirin, salicylic acid, the parent ester and its deacetylated analogue-salicylate esters elute after aspirin esters in RPHPLC and possess a distinctive absorbance at 296 nm. The disappearance of test compounds followed pseudo first-order kinetics with half-lives ($0.693/k_{obs}$) in the range 20 s–5 min (Table 1). V_{max} and K_m values were obtained by non-linear regression of the disappearance



Scheme 1. Synthesis of simple nitroxyalkyl aspirin esters.

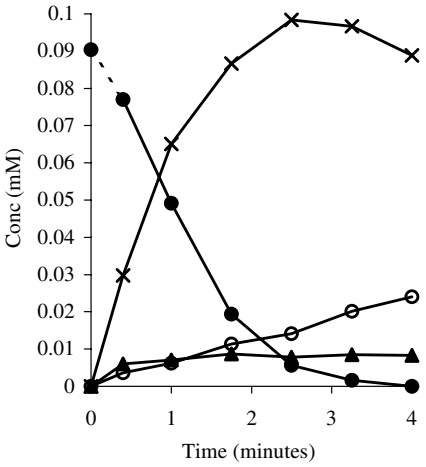


Scheme 2. Synthesis of nitroxyglycolamides. Reagents: (a) $CsCO_3$, $MeOH/H_2O$; (b) BnBr, DMF; (c) **6**, Et_3N, DCM; (d) H_2, Pd/C; (e) $(COCl)_2$, DCM; (f) $RN(CH_2)_2ONO_2$, Et_3N.



Scheme 3. Synthesis of ethanolamine nitrate substituted esters. Reagents and conditions: (a) HNO_3, DCM, 0 °C then acetic anhydride; (b) bromoethanol, 7 M NaOH, rt; (c) **6**, Et_3N, DCM.

Table 1. Kinetic parameters for nitroaspirin hydrolysis in plasma solution



Progress curve for the hydrolysis of ester **5c** *in 10% buffered human plasma (pH 7.4) at 37ºC:* **5c** *(●), salicylate ester (x), aspirin (▲) and salicylic acid (o).*

Compound	K_m, $\times 10^4$ M	V_{max}, $\times 10^4$ M	k_{obs}, min	$t_{1/2}$, s	Aspirin release (%)
8a	4.62	2.060	0.44	96	<0.5
8b	7.17	4.24	0.47	88	<0.5
8c	2.904	1.26	0.34	134	<0.5
4a	5.13	3.68	0.6	70	<0.5
4b	3.06	2.59	0.68	62	<0.5
4c	4.83	3.57	1.13	69	<0.5
4d	5.405	2.23	6.12	367	<0.5
5a	1.235	2.64	2.62	21	<0.5
5b	3.352	2.51	0.66	63	<0.5
5c	3.073	4.62	1.33	39	9.2
5d	12.82	7.8	0.63	67	<0.5

data to the integrated Michaelis–Menten equation.[21] In the cases of all but one of the esters, deacetylation predominated over carrier group detachment with no aspirin production. The hydrolysis was most rapid in the case of the ethanolamine esters (**5**) with half-lives of a minute or less. The most promising compound, the *N*-propyl, *N*-nitroxy-ethyl aminoethyl ester **5c** liberated 9–10% aspirin on a molar basis. Interestingly, this was not the most rapidly hydrolysed ester, underlining the fact that the *relative* rates of aspirin ester and acetyl ester hydrolysis is what determines the extent of aspirin release. The experiment was repeated with similar results when human plasma was replaced by purified horse serum butyrylcholinesterase at about the same concentration as the enzyme is found in plasma. The hydrolysis rate was also found to be dependent on esterase concentration without any change in hydrolysis direction.

The hydrolysis pathways of the glycolamide esters (**4a**–**d**) were both complex and unproductive. None of the compounds produced more than 0.5% aspirin on a molar basis in any of the solutions tested-aqueous pH 7.4 (37 °C), human or rat plasma solution or in solutions containing purified horse BuChE. Meanwhile, the *unsubstituted N*,*N*-diethylglycolamide ester liberated around 60% aspirin in agreement with the report of Bundgaard et al. Nitroxy-substitution of the *N*-alkyl group therefore has a deleterious effect on hydrolysis characteristics, promoting the usual acetyl group hydrolysis over aspirin liberation. This seems likely to be due to differences in interaction orientation between the carrier group and the BuChE active site. In human plasma solution and in the presence of horse BuChE, initial acetyl group hydrolysis was unexpectedly followed by cleavage at the glycolamide bond, generating the glycolic acid ester of salicylic acid. In aqueous solution (pH 7.4, 37 °C) the glycolamides underwent quite rapid hydrolysis at the amide bond and acetyl groups in parallel ($t_{1/2} \sim 90$ min). Since the unsubstituted glycolamides were reported to enjoy reasonable aqueous stability by Bundgaard (and do not look especially unstable), we are forced to conclude that the nitrate group promotes hydrolysis of the neighbouring glycolamide bond. Hydrolysis in rat plasma solution occurred rapidly and exclusively at the acetyl site generating the glycolamide esters of salicylic acid, with no evidence of glycolamide hydrolysis over the time course of the experiment, indicating that BuChE might have contributed to the glycolamide hydrolysis in the other experiments.

The design of a NO-aspirin mutual prodrug or hybrid is challenging. Our experiments indicate that ethanolamine esters hold some promise as a platform. The design process needs to take account of plasma BuChE because even if it that is not the intended vector for drug release, it will make a very effective and destructive competitor. A structure-based design approach employing one of the excellent models of human BuChE[22,23] might be useful in that regard.

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