Biochemical Pharmacology Vol. 16, pp. 753–760. Pergamon Press Ltd. 1967. Printed in Great Britain

BIOCHEMICAL PROPERTIES OF ANTI-INFLAMMATORY DRUGS—XI

STRUCTURE–ACTION RELATIONSHIP FOR THE UNCOUPLING OF OXIDATIVE PHOSPHORYLATION AND INHIBITION OF CHYMOTRYPSIN BY N-SUBSTITUTED ANTHRANILATES AND RELATED COMPOUNDS

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(*Received* 31 *August* 1966; *accepted* 20 *October* 1966)

Abstract—The uncoupling activity of anthranilic acids depends upon each of the following factors: (a) an unsubstituted carboxylic group, (b) an *ortho*-imino group and (c) aromatic character. Uncoupling activity could be correlated with the affinity of the drug for albumin amino groups, provided that the compound was not so acidic that it would bind to albumin yet fail to partition into mitochondrial lipid.

Requirements for chymotrypsin inhibition were less stringent than (but generally followed) those for uncoupling activity.

Some novel aniline derivatives are described (Table 1).

SEVERAL different types of non-steroidal anti-inflammatory (antirheumatic) drugs share the property of uncoupling oxidative phosphorylation in liver mitochondria.[1–3] These drugs also act upon connective tissue both *in vitro* and *in vivo* to suppress the incorporation of inorganic phosphate into organic phosphates and the biosynthesis of mucopolysaccharide sulphates by these tissues.[2,4,5] These findings suggest that these particular drugs may therefore uncouple the phosphorylation of adenine nucleotides from cellular oxidation in connective tissues, thereby suppressing ATP†-linked endergonic reactions which are part of the inflammatory response in these tissues.

Certain N-substituted anthranilic acids (so called 'fenamic' acids) exhibit anti-inflammatory activity in laboratory animals[6–9] and are powerful uncoupling agents.[2,4,10] This report describes some studies of the relationship between chemical structure, uncoupling activity and the anti-chymotrypsin (esterase) activity[11] for a number of substituted aminobenzoic acids.

The possible role of non-enteric chymotryptic enzymes as mediators of the inflammatory response is discussed in another communication.[11]

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† Abbreviations used: ATP—adenosine-5′-triphosphate; CI-583—N-(2,6-dichloro-m-tolyl)-anthranilic acid; Mefenamic acid—N-(2,3-xylyl)anthranilic acid; Flufenamic acid—N-(α′α′α′-trifluoro-m-tolyl)anthranilic acid; AA—anthranilic acid; 3-AB,4-AB—isomeric aminobenzoic acids; DNP—2,4-dinitrophenyl-; TNP—2,4,6-trinitrophenyl-; Bz—benzoyl-; TNBal—2,4,6-trinitrobenzaldehyde.

EXPERIMENTAL

A. *Chemicals*

Mefenamic acid, flufenamic acid and CI-583 were provided by Parke, Davis & Co., Hounslow. N-Cholyl-anthranilic acid was obtained from the Maybridge Chemical Co., Tintagel, Cornwall. Other compounds were synthesized by procedures given in Beilstein's Handbuch except those described elsewhere[10] or noted below. N-2, 4-Dinitrophenylanilines (and other amines) were prepared by the method described for AA and PAS.[12] N-2,4,6-trinitrophenyl derivatives were prepared by reaction between the amine and picrylsulphonic acid.[13] Hitherto uncharacterized nitro (and other) compounds, crystallized from aqueous ethanol or aqueous acetic acid, are listed in Table 1. These were all free of contaminating nitrophenols, as demonstrated by paper ionophoresis at several pH's and thin layer chromatography on water-sprayed silica plates irrigated with 1% (v/v) butanol in chloroform.

(*cis*) Hexahydroanthranilic acid was prepared from hexahydrophthalic anhydride (Fluka, A. G., Buchs, Switzerland) both via the N-hydroxyimide[16] and also by the standard procedure for preparing anthranilic acid from phthalimide[17] but using hexahydrophthalimide (m.p. 138°). The product in each case (m.p. 240°, found N 9·8%) reacted with ninhydrin and yielded a N-benzoyl derivative, m.p. 178° (from aqueous ethanol) not raised by repeated crystallizations ($C_{14}H_7O_3N$ requires C, 68·0%; H, 6·9%; N, 5·7%; found C 68·2%; H, 6·7%, N, 5·8%). The N-benzoyl derivative of *cis* hexahydroanthranilic acid has a reported m.p. 177°; the *trans* isomer, m.p. 226°.[18]

Anthranilic acid amides were prepared from isatoic anhydride (Maumee Chemical Co., Toledo 8, Ohio) as described.[19]

N-Methyl-N-substituted anthranilic acids prepared by standard procedures were purified by recrystallization until they gave a negative reaction to the chlorine-KI-starch reagent.[20]

Bromination of aniline derivatives dissolved in acetic acid was carried out with 1·2 × calculated amount of bromine (20% v/v in acetic acid). Attempts to brominate aniline-2-sulphonic acid[21] only yielded 2,4,6-tribromaniline, m.p. 120° (from acetic acid).

B. *Biochemical*

Drug action on oxidative phosphorylation was studied using rat liver mitochondria with succinate (30 mM) as substrate.[22] Relative drug binding to amino groups of bovine plasma albumin was determined colorimetrically using 2,4,6-trinitrobenzaldehyde.[23, 24]

Inhibition of α-chymotrypsin (0·8 μg/ml) was measured using N-acetyl-L-tyrosine ethyl ester as substrate (0·25 mM) at room temperature and titrating liberated protons with 10 mM sodium hydroxide using a pH-stat (Radiometer, Copenhagen) as described.[11]

RESULTS AND DISCUSSION

In general, potent anthranilates had little or no effect on respiration at concentrations at which they effectively lowered the P/O ratio to 20–40 per cent that of the controls (absolute P/O values were 1·3–1·7 in these drug-free controls[22]). Exceptions to this generalization are noted in the appropriate table.

TABLE 1. CHARACTERIZATION OF SOME N-SUBSTITUTED ANILINES

	Melting points (uncorrected)		Molecular formula	% N	
	Found	Reported*		Calc.	Found
N-DNP derivatives of:					
β-Alanine	145–6°	145° (155–6°)			
Anthranilic acid	265°	270° (262°)			
3-Aminobenzoic acid	156°	—	$C_{13}H_9N_3O_6$	13·8	13·7
4-Aminobenzoic acid	307°	—	$C_{13}H_9N_3O_6$	13·8	14·0
cis 2-Aminocyclohexane-carboxylic acid	204°	—	$C_{13}H_{15}N_3O_6$	13·6	13·4
Aniline-2-sulphonic acid	242°	—	$C_{12}H_9N_3O_7S$	12·4	11·9
Sulphanilic acid	129°	130°			
3-Amino-2-naphthoic acid	281–284°	—	$C_{17}H_{11}N_3O_6$	11·9	12·1
3-Aminophthalic acid	340° (d)	—	$C_{14}H_9N_3O_8$	12·1	12·0
o Arsanilic acid	260° (d)	—	$C_{12}H_{10}N_3O_7As$	11·2	10·8
p Arsanilic acid	298°	—	$C_{12}H_{10}N_3O_7As$	11·2	11·2
N-TNP derivatives of:					
β-Alanine	184–5°		$C_9H_8N_4O_8$	18·6	18·4
Anthranilic acid	275–279° (d)	272°			
cis 2-Aminocyclohexane-carboxylic acid	211–213°	—	$C_{13}H_{14}N_4O_9$	15·8	15·4
3,5-Dibromo derivatives of:					
Anthranilamide	209–212°	196–7°	$C_7H_6N_2OBr_2$	9·5	9·7
Methyl anthranilate	178°	84°	$C_8H_7NO_2Br_2$	4·5	4·5
N-Methyl anthranilic acid	184°	—		4·5	4·5
N-Benzoyl derivative of:					
3-Amino-2-naphthoic acid	220°	—	$C_{18}H_{14}NO_4$	4·8	4·7

DNP—2,4-dinitrophenyl, TNP—2,4,6-trinitrophenyl.
* Ref. 12, 14, 15 and Beilstein's Handbuch.

There was a reasonably good correlation between the relative potency of these compounds as uncoupling agents and their ability to suppress the colour formation when 2,4,6-trinitrobenzaldehyde reacts with bovine plasma albumen (Tables 2–4). This colour reaction primarily involves interaction of the aldehyde with (lysyl ϵ-)amino

TABLE 2. (i) UNCOUPLING OF OXIDATIVE PHOSPHORYLATION AND (ii) RELATIVE BINDING TO ALBUMIN (ϵ-LYSYL) AMINO GROUPS BY SOME N-ACYL AMINOBENZOIC (AB) ACIDS AND RELATED COMPOUNDS

Acid	Conc. $\times 10^{-4}$M	P/O (% control)	TNBal-Albumen (% inhibition*)
None	—	100	0
N-Acetyl-AA	10	84	13
N-Cholyl-AA	5	30	80
N-*p* Tosyl-AA	20·0	84	0
N-3,5-Dinitrobenzoyl-AA	0·4	38	82
N-Benzoyl-AA	0·8	30	75
N-Benzoyl-N-methyl-AA	2·5	100	17
N-Benzoyl-3-AB	2·5	100	24
N-Benzoyl-4-AB	2·5	94	37
N-Benzoyl-HHAA	2·5	100	9
N-Benzoyl-*o*-arsanilic	2·5	85	10
N-Benzoyl-2-anilinesulphonic	5·0	72	60
N-Benzoyl-3-amino-2-naphthoic	0·4	38	58
N-Benzoyl-3-aminophthalic	5·0†	100	57
N-Benzoyl-β-alanine	2·5	94	8
N-Salicyl-AA	0·2	54	80
N-Salicyl-4-AB	1·5	100	68
γ-Resorcyl-AA			63
γ-Resorcyl-4-AB			56

* With 1 mM drug.

† Too insoluble for adequate testing.

(i) P/O ratios expressed as percentages of P/O ratios in drug-free controls.

(ii) Quenching by 1 mM drug of the coloration formed by 0·1 mM 2,4,6-trinitrobenzaldehyde with 0·1 mM bovine plasma albumin in 0·1 M phosphate, pH 7·4 measured at 525 mμ after 30 min = % inhibition. AA = anthranilic acid; HHAA = hexahydro AA (2-aminocyclohexane-carboxylic acid).

groups of the albumen molecule.[23] An acidic drug which inhibits colour formation probably does so by binding to some of these reactive albumen groups (or otherwise rendering them less accessible to the aldehyde). By extrapolation, it might be inferred that these anthranilates uncouple oxidative phosphorylation because they may also block certain hydrophobic amino groups participating in mitochondrial energy conservation. No evidence was obtained that the most potent anthranilates (fenamic acids) uncouple mitochondrial phosphorylation by interaction with protein thiols.[24]

Relationship between chemical structure and the uncoupling and albumin (amino)-binding activities in vitro. These studies showed that optimum drug activity *in vitro*, as exemplified by CI-583, flufenamic acid and N-2,4-dinitrophenylanthranilic acid, is obtained when the following structural requirements are fulfilled:

(i) Requirement for a carboxyl group. Esters and amides, in the few cases examined where these compounds were sufficiently soluble for adequate testing, failed to display the activity of the parent acid (see Table 4). An acetophenone analogue of dibromo-anthranilic acid was much less potent than this acid (Table 4). The corresponding phenylarsonic or benzenesulphonic acids were very much less active uncoupling

agents. The *o* sulphonic acids did bind to the lysyl amino groups of albumin but were much less potent in uncoupling phosphorylation than might have been predicted from their activity in suppressing the albumen-trinitrobenzaldehyde colour reaction. This anomaly is indubitedly due to the low p*Ka*'s of these sulphonates which effectively prevent their concentration within the lipid-rich phase of mitochondria (where uncoupling drugs must act). The effect of reducing the lipophilic character of the molecule is also seen when the activity of 3-aminophthalates is compared with that of the corresponding anthranilates.

TABLE 3. (i) UNCOUPLING OF OXIDATIVE PHOSPHORYLATION AND (ii) RELATIVE BINDING TO ALBUMIN AMINO GROUPS BY N-ARYL ANILINE ACIDS (INCLUDING THE FENAMIC ACIDS) AND RELATED COMPOUNDS

Acid	Conc. × 10^{-4}M	P/O (% control)	TNBal-Albumin % inhibition
N-Methyl-AA	25	60	20
N-Methyl,N-nitroso-AA	50	98	2
N-Benzyl-AA	4	62	45
N-Cyclohexyl-AA	4	82	65
N-Cyclohexyl-AA amide	7·5†	100	0
N-Phenyl-AA	1·2	60	76
Diphenylamine-2,2′-dicarboxylic acid	10*	70	64
N-*o* Tolyl-AA	1·2	48	69
N-*m* Tolyl-AA	1·2	38	75
N-*p* Tolyl-AA	1·2	32	64
Mefenamic acid	1·2	17	60
	0·5	72	22‡
Flufenamic acid	0·5	15	50‡
	0·2	76	
CI-583	0·5	42	60‡
	0·2	92	
N-DNP-AA	0·5	40	65‡
N-DNP,N-methyl-AA	2·5	90	0‡
N-DNP-3-AB	2·5	70	37‡
N-DNP-4-AB	5	70	11‡
N-DNP-HHAA	2·5	52	37‡
N-DNP-β-alanine	2·5	97	30‡
N-DNP-3-aminonaphthoic	0·25†	60	§
N-DNP-3-aminophthalic	2·5	90	65‡
N-DNP-*o* arsanilic	2	72	34‡
N-DNP-*p* arsanilic	2	100	15‡
N-DNP-2-anilinesulphonic	2	15	43‡
N-DNP-sulphanilic	2	100	14‡
N-TNP-AA	0·4	50	41
N-TNP-*o* arsanilic	2·5	95	57
N-TNP-2-anilinesulphonic	5	83	42
N-TNP-HHAA	1	58	74
N-TNP-β-alanine	2	65	47
N-TNP-6-aminohexoic	2	84	36

Abbreviations and details as described in Table 2. Inhibition TNBal-albumin reaction by 1 mM drug except when noted (‡ = with 0·5 mM drug).

* Also inhibted respiration at this concentration.

† Very limited solubility.

§ With 0·5 mM drug only.

(ii) Requirement for an *ortho* secondary amino group. An N-substituent was not an absolute requirement for uncoupling activity if sufficient lipophilic character was obtained by nuclear substitution e.g. dibromo-anthranilic acid (Table 4). N-Substituted

3- and 4-aminobenzoates were either much less active than the corresponding anthranilates, or completely inactive. The only notable exception to this generalization was dibromo-4-aminobenzoic acid (Table 4). Likewise when a derivative of aniline-2-sulphonic acid uncoupled phosphorylation the corresponding sulphanilic acid (i.e. 4-amino isomer) did not.

TABLE 4. (i) UNCOUPLING OF OXIDATIVE PHOSPHORYLATION AND (ii) RELATIVE BINDING TO ALBUMIN AMINO GROUPS BY SOME BROMANILINE ACIDS AND OTHER ANTHRANILATE DERIVATIVES

Compound	Conc. × 10^{-4}M	P/O (% control)	TNBal-Albumin % inhibition
AA	25	100	3
5-Bromo-AA	5	60	28
3,5-Dibromo-AA	2	0	72
	1	54	
Methyl-3,5-dibromo-AA(ester)	2·5	83	0
3,5-Dibromo-AA amide	5	88	0
3,5-Dibromo-N-methyl-AA	2·5	10	57
	1	82	
3,5-Dibromo-N-acetyl-AA	5	46	38
3,5-Dibromo-*o* arsanilic	10*	64	*
3,5-Dibromo-4-AB	5	40	50
3,5-Dibromo-2-aminoacetophenone	5	60	*
AA-anilide	7·5	96	0
Salicylanilide	1·5	0	26

Details as given in Table 2.
* Too insoluble for adequate testing.

Derivatives of anthranilic acid with 2-substituents on the amino group were devoid of activity. This is strikingly shown when the (in)activity of N-2,4-dinitrophenyl- and N-benzoyl-, N-methylanthranilic acids is compared with that of the corresponding (N-desmethyl) anthranilic acids. Such compounds are also much weaker acids than the mono-N-substituted anthranilates.[25]

(iii) Requirement for an aromatic N-substituent. Table 3 shows that the nature of an N-acyl substituent was not critical provided that it did not confer too much hydrophilic character (e.g. diphenylamine-dicarboxylic acid). It is probable that phenolic metabolites of the fenamic acids would also be less potent than the parent acids. Acidic N-trinitrophenylaniline compounds were less active in vitro than the corresponding N-dinitrophenyl derivatives (Table 3) which suggests that steric hindrance by the third nitro group diminishes the interaction of the acidic group with the mitochondrial (uncoupling) receptor and with plasma albumin amino groups. Steric hindrance in the vicinity of an essential (enolic)group diminishes the uncoupling activity and albumin-binding affinity of certain indan-1,3-diones.[24] The apparent requirement for a secondary amino group (discussed above) might be explained similarly.

An N-aryl substituent was not essential for optimum activity *in vitro*. N-Benzoyl-derivatives were also active. N-benzoyl-anthranilic acid was actually more potent in uncoupling phosphorylation than either N-benzyl- or N-phenyl- anthranilic acids. However, N-toluenesulphonyl-anthranilic acid was virtually inactive and may again reflect steric hindrance, by the sulphono group, of the (essential) carboxyl group.

N-Cyclohexyl- and N-methyl-anthranilic acids were only weak uncoupling agents indicating that a planar N-substituent contributes to the overall drug activity.

(iv) Requirement for an aromatic nucleus. Derivatives of hexahydroanthranilic acid were either inactive or much less potent than the corresponding anthranilic acids. Derivatives of β-alanine had virtually no uncoupling activity, although N-trinitrophenyl-β-alanine (and TNP-6-aminohexoic acid) did bind to albumin amino groups. This loss of drug activity on saturating the benzene nucleus (carrying the pharmacophores) parallels the loss of activity when 2-hydroxy and 2-mercaptobenzoic acids are hydrogenated: the corresponding cyclohexane-carboxylic acids are almost devoid of uncoupling[4, 26] (and albumin-binding[23]) activity.

In other respects((i)–(iii)), there is also a close parallel between the structural requirements for the uncoupling activity of 2-mercaptobenzoic,[26] salicylic[4] and lipophilic anthranilic acids.

Studies with α-chymotrypsin

Table 5 indicates that a somewhat simliar structure–action relationship probably governs the potency of mefenamic acid as an inhibitor of chymotrypsin (esterase

TABLE 5. INHIBITION OF CHYMOTRYPSIN BY SOME ANTHRANILATES

Compound	Initial rate hydrolysis
None	1·0
Mefenamic acid	0·33
N-Phenyl-AA	0·27*
N-Cyclohexyl-AA	0·87
N-DNP-AA	0·12
N-DNP-HHAA	0·80*
(N-DNP-β-alanine	0·90)
N-DNP,N-Methyl-AA	0·76
N-Bz-AA	0·60*
N-Bz-HHAA	0·94
N-Benzyl-AA	0·52
N-Bz-4AB	0

Enzyme activity measured at 22° with N-acetyl-L-tyrosine ethyl ester (0·25mM) as substrate and 0·5mM drugs (added in 50 μl dimethylsulphoxide) in final volume of 6 ml. Initial rate of proton liberation on adding 5 μg enzyme titrimetrically.

Abbreviations as in previous tables.

* Followed by a rather rapid decline in reaction rate.

activity). Analogues with either a tertiary amino group or N-alkyl substituent and derivatives of 2-aminocyclohexane-carboxylic acid were very much less potent inhibitors. However, an amino group *ortho* to the carboxyl group was not essential for chymotrypsin inhibition (*vide* powerful inhibition by N-benzoyl-4-aminobenzoic acid). These findings support some previous conclusions concerning the structural requirements for a compound to be an inhibitor of the esterase activity of α-chymotrypsin(with N-acetyl-L-valine methyl ester as substrate).[27]

Acknowledgements—I am grateful to Messrs. A. B. L. Binks and I. F. Skidmore for assistance at various times with these studies and to Drs. J. A. L. Gorringe and H. O. J. Collier for supplying the fenamic acids. Financial support was provided by the Nuffield Foundation, London and the U.S. Public Health Service (National Heart Institute, Bethesda).

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