Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1709 (2005) 191 – 194
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Rapid report

Remarkable substituent effect: β-aminosquamocin, a potent dual inhibitor of mitochondrial complexes I and III☆

Romain A. Duval[a], Erwan Poupon[a,*], Ulrich Brandt[b,*], Reynald Hocquemiller[a]

[a]Laboratoire de Pharmacognosie associé au CNRS (UMR 8076, BioCIS), Centre d'Études Pharmaceutiques, Université Paris-Sud 11, 5, rue Jean-Baptiste Clément, 92296 Châtenay-Malabry CEDEX, France
[b]Zentrum der Biologischen Chemie, Fachbereich Medizin, Universität Frankfurt, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany

Received 1 July 2005; received in revised form 22 July 2005; accepted 29 July 2005
Available online 19 August 2005

Abstract

The introduction of a primary amine function on the terminal α,β-unsaturated lactone of squamocin **1**, a common structural hallmark of annonaceous acetogenins, shifted this specific inhibitor of mitochondrial complex I into a potent dual inhibitor of complexes I and III. The mechanism of action of β-aminosquamocin **2**, against these two respiratory targets, is studied and discussed in view of current structure–activity relationship knowledge in the acetogenin series.

Keywords: Annonaceous acetogenin; Squamocin; Cytotoxicity; Dual inhibitor; Complex I; Complex III

Acetogenins from the Annonaceae form a large group of bioactive lactonic polyketides [1,2] known in particular for their often outstanding level of cytotoxic activity [3]. Natural or semisynthetic acetogenins are also the most potent and specific inhibitors of the mitochondrial NADH: ubiquinone oxidoreductase (complex I) described so far [3–6], and their wide spectrum of biological activities is assumed to rely mainly on the inhibition of this ubiquitous target. However, strong discrepancies between the cytotoxicity and the potency of acetogenins as complex I inhibitors suggest the existence of secondary modes of action. A number of putative biological targets have been identified up to now [4,7–9]. In our ongoing search for novel acetogenin-derived agents devoted to fundamental or therapeutical purposes, here we present the unique biological properties of β-aminosquamocin **2**, a semisynthetic acetogenin found to be a potent dual inhibitor of mitochondrial complexes I and III.

Looking for adequate reactions to selectively modify complex natural products, we were recently attracted to the rich chemistry of azides [10]. Concomitantly, our interest in elucidating the mechanism(s) of action of annonaceous acetogenins prompted us to thoroughly investigate their chemical reactivity and structure–activity relationships. As their most common structural feature, all natural acetogenins possess a terminal lactone moiety which crucially influences their binding mode to complex I, but which role as an inhibitory pharmacophore remains unclear [4,5,11–14].

We discovered that heating dispersed squamocin **1** in a saturated aqueous solution of sodium azide containing one equivalent of zinc bromide yielded β-aminosquamocin **2** in a clean reaction with a satisfactory yield (Fig. 1). This original amination reaction[1] proceeded readily in boiling

☆ Supporting information for this article (complete experimental protocols, characterization data, and expanded bibliographic section) is available on the WWW under http://www.bba-direct.com or from the authors.

* Corresponding authors. E. Poupon is to be contacted for the chemistry, biochemistry and biology of annonaceous acetogenins, France. Fax: +33 1 46 83 53 99. U. Brandt for the biochemistry and molecular biology of mitochondrial complexes I and III. Fax: +49 69 6301 6970.
E-mail addresses: erwan.poupon@cep.u-psud.fr (E. Poupon), brandt@zbc.kgu.de (U. Brandt).

0005-2728/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2005.07.011

[1] Scope and limitations of this original reaction in neutral aqueous conditions will be disclosed elsewhere in due course. The closest example involved highly toxic HN_3, see Ref. [15].

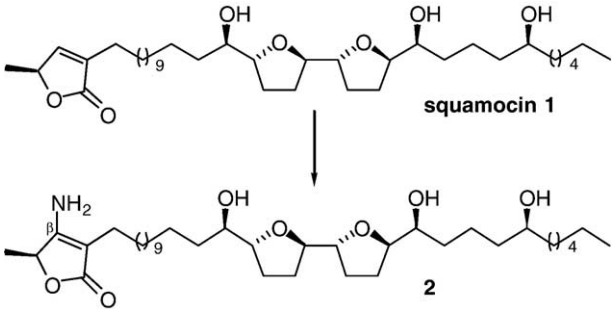

Fig. 1. Semisynthesis of β-aminosquamocin **2**. Reagents and conditions: NaN_3 (excess), $ZnBr_2$ (1 equiv.), H_2O, reflux 20 h (60–70%).

water despite the complete insolubility of squamocin **1**, and did not require the use of protecting groups [15]. With analogue **2** in hand, which displays strongly modified electronic distribution at the level of the crucial lactone ring, we turned our attention to its biological profile.

β-Aminosquamocin **2** was first identified as a very powerful cytotoxic agent, exhibiting even superior activity than squamocin **1** despite a four times weaker inhibitory activity against complex I from bovine heart mitochondria (Table 1). This observation led us to postulate that **2** should also target another cellular component in addition to complex I. Ubiquinol:cytochrome *c* oxidoreductase (complex III) appeared as a likely candidate considering the isoelectronicity of the formed β-aminobutenolide nucleus with the β-(*E*)-methoxyacrylate pharmacophore of classical complex III inhibitors (Fig. 2) [16,17]. β-Aminosquamocin **2** was indeed found to be a complex III inhibitor of high potency, exhibiting similar levels of activity against the two respiratory chain enzymes (Table 1, supplementary data). Natural molvizarin is the only acetogenin known to also inhibit complex III, although with a much lower potency (I_{50}=15 μM) [4]. Moreover, β-aminosquamocin **2** is the first dual inhibitor to exhibit high affinity for both complexes [4,18–20].

Annonaceous acetogenins as complex I inhibitors can be categorized into three functional groups following their competition profiles towards the classical agents rotenone and piericidin A [4,5] (Fig. 2). Squamocin **1** is a rotenone-type inhibitor (ubiquinone/semiquinone antagonist), acting at the level of the less specific binding site (rotenone/B site) of the enzyme. On the other hand, highly selective complex III inhibitors such as myxothiazol can also behave as atypical complex I inhibitors (ubiquinol antagonists) [18,19], acting at the level of an affinity site (capsaicin/C site) which has not been found to be targeted by annonaceous acetogenins up to now. To investigate whether the isoelectronicity of the β-aminobutenolide pharmacophore with the one of myxothiazol could be the basis for such an exceptional binding behaviour of β-aminosquamocin **2**, its competition profile was studied kinetically against both squamocin **1** and capsaicin [4]. Compound **2** was found to be exclusive with squamocin **1**, but behaved non-competitively towards capsaicin (Fig. 3). Assuming that β-aminosquamocin **2** does not act at the level of the site A of complex I, specifically occupied by rare inhibitors such as rolliniastatin-2 [4,5], it appears that **2** behaves in a manner superimposable to that of natural squamocin **1**.

As far as complex III inhibition is concerned, β-aminosquamocin **2** was found to act at the level of the Q_o site by cytochrome *b* reduction experiments [16] (Fig. 3). Together with the Q_i site inhibitor antimycin compound **2** affected but did not abolish cytochrome *b* reduction. This behaviour in a "double-kill" situation is usually observed with stigmatellins that require a reduced "Rieske" iron–sulfur cluster for binding. This interpretation was supported by the surprising observation that the spectral distortion induced by compound **2** in the α-region of the spectrum of reduced cytochrome *b* exhibited features characteristic for stigmatellins rather than β-(*E*)-methoxyacrylates [17]. Hence, judged from the specific site and mode of action within the Q_o pocket, β-aminosquamocin **2** has to be classified as a stigmatellin-type inhibitor [21].

Thus, despite a remarkable inhibitory potency against mitochondrial complexes I and III and the presence of a related Q_o pharmacophore, β-aminosquamocin **2** was found not to bind to the comparably hydrophilic capsaicin/C site of complex I targeted by ubiquinol antagonists. This suggests that natural acetogenins as well as acetogenin-derived inhibitors probably cannot act at this particular level within the large binding pocket of complex I, presumably because of their excessive lipophilicity. It remains to be established, however, whether the two main components of **2** (i.e., the polyoxygenated core and the terminal β-aminated lactone) both account for the dual-acting mode of this inhibitor, or if the β-aminobutenolide nucleus alone has to be considered a new Q_o pharmacophore of complex III inhibitors. Nevertheless, the overall behaviour of β-aminosquamocin **2** seems to confirm that the common butenolide of natural acetogenins mimics ubiquinone substrates when binding to respiratory chain complexes [11].

In conclusion, changing the electronic distribution in a crucial moiety enabled the preparation of a new acetogenin

Table 1
Inhibitory activities of squamocin **1** and β-aminosquamocin **2**

Compounds	Cytotoxicity (KB 3–1)[a]	Complex I inhibition[b]	Complex III inhibition[c]
1	1.6×10^{-13} M	2 nM	Inactive
2	$<10^{-14}$ M	8 nM	40 nM
Doxorubicin[d]	5.2×10^{-9} M	–	–
Rotenone[d]	–	30 nM	–
Capsaicin[d]	–	45 μM	–
Myxothiazol[d]	–	–	1 nM
Antimycin[d]	–	–	4 nM

[a] IC_{50} for human nasopharyngeal epithelioid carcinoma cells [24].
[b] IC_{50} for NADH:*n*-decylubiquinone oxidoreductase (bovine submitochondrial particles).
[c] IC_{50} for *n*-decylubiquinol:cytochrome *c* oxidoreductase (liposomal reconstitution of bovine enzyme).
[d] Reference compounds.

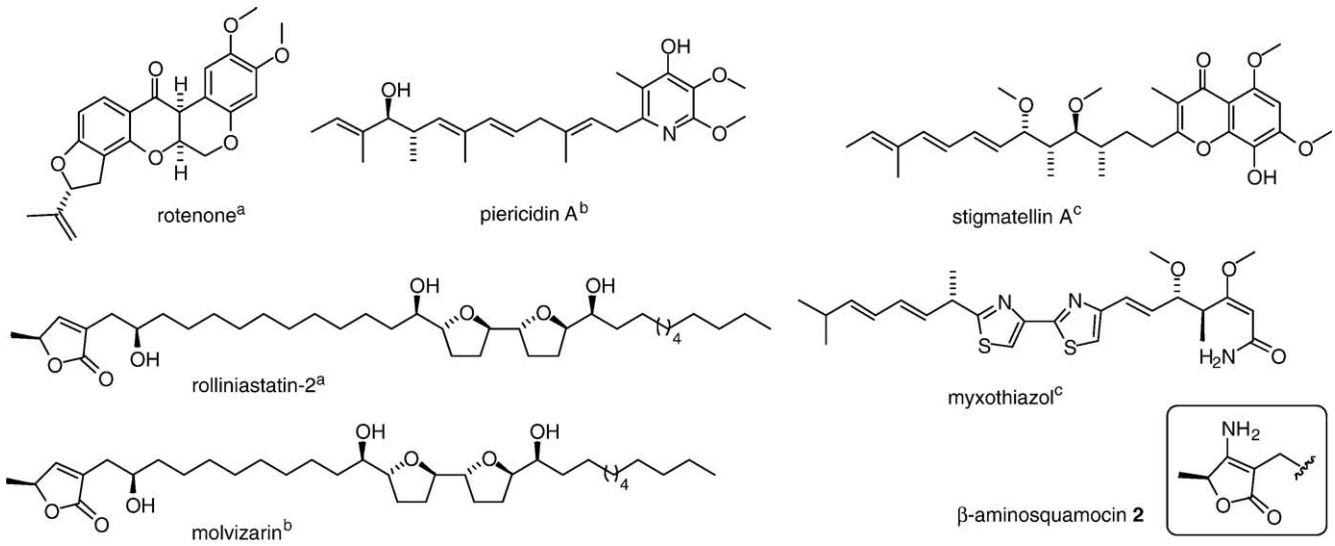

Fig. 2. Reference mitochondrial inhibitors. [a]specific inhibitors of complex I; [b]selective inhibitors of complex I; [c]selective inhibitors of complex III.

analogue with highly integrated pharmacophores, possessing an expanded biochemical profile as well as increased antitumor potential. Considering that inhibitors of mitochondrial complexes I and III hold important positions among modern agrochemicals [22,23], a phytosanitary application of the present discovery would be the semisynthesis of dual-acting, β-aminated acetogenins from crude Annonaceae extracts, to be used as potent natural compound-derived pesticides.

Acknowledgements

We are thankful to Pr G. Lewin for advice and fruitful discussions. R. A. Duval gratefully acknowledges Dr. S. Dröse, F. Streb and I. Siebels for valuable experimental assistance. Jean-Christophe Jullian is acknowledged for recording NMR spectra.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2005.07.011.

References

[1] A. Bermejo, B. Figadère, M.-C. Zafra-Polo, I. Barrachina, E. Estornell, D. Cortes, Acetogenins from Annonaceae: recent progress in isolation, synthesis and mechanisms of action, Nat. Prod. Rep. 22 (2005) 269–303.

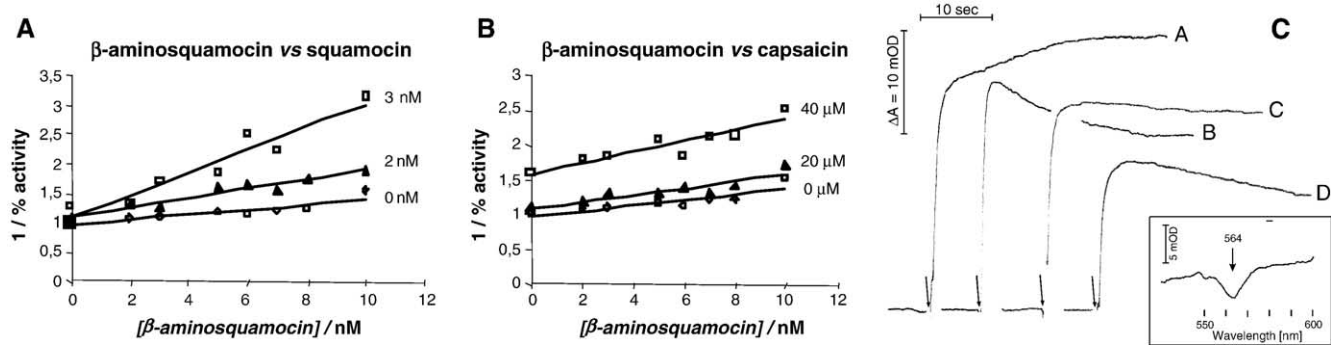

Fig. 3. Inhibitory profile of β-aminosquamocin **2** against mitochondrial complexes I and III. A and B: Competition kinetics of **2** against reference complex I inhibitors. NADH: *n*-decylubiquinone oxidoreductase activity of complex I in presence of competing inhibitors was measured in a Tris buffer (50 mM, pH 7.4) containing EDTA (1 mM), antimycin (2 μM), and KCN (2 mM). Inhibitors were incubated with submitochondrial particles (21 μg/mL) in presence of 2-*n*-decylubiquinone (60 μM) at 23 °C for 1 min, and reaction was started by adding NADH (final concentration of 100 μM). Enzymatic activity was measured following NADH oxidation in the dual wavelength mode (400 vs. 340 nm, $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) at 23 °C. C: Cytochrome *b* reduction kinetics and spectral change with **2**. The reduction of cytochrome bc_1 complex was followed at 562–575 nm after successive addition of inhibitors and 2-*n*-decylubiquinol (2 μM) (arrows). Experiments performed with myxothiazol are not shown. A: 50 μM antimycin; B: no inhibitor; C: 150 μM β-aminosquamocin **2**; D: 50 μM antimycin, 150 μM β-aminosquamocin **2**. The insert shows the cytochrome bc_1 complex difference spectrum following dithionite reduction with 80 μM β-aminosquamocin **2** *minus* without inhibitor.

[2] F.Q. Alali, X.-X. Liu, J.L. Mc Laughlin, Annonaceous acetogenins: recent progress, J. Nat. Prod. 62 (1999) 504–540.
[3] M.C. Zafra-Polo, M.C. González, E. Estornell, S. Sahpaz, D. Cortes, Acetogenins from Annonaceae, inhibitors of mitochondrial complex I, Phytochemistry 42 (1996) 253–271.
[4] M. Degli Esposti, A. Ghelli, M. Ratta, D. Cortes, E. Estornell, Natural substances (acetogenins) from the family Annonaceae are powerful inhibitors of mitochondrial NADH dehydrogenase (Complex I), Biochem. J. 301 (1994) 161–167.
[5] J.R. Tormo, M.C. González, D. Cortes, E. Estornell, Kinetic characterization of mitochondrial Complex I inhibitors using annonaceous acetogenins, Arch. Biochem. Biophys. 369 (1999) 119–126.
[6] J.R. Tormo, E. Estornell, T. Gallardo, M.C. González, A. Cavé, S. Granell, D. Cortes, M.C. Zafra-Polo, γ-Lactone-functionalized antitumoral acetogenins are the most potent inhibitors of mitochondrial complex I, Bioorg. Med. Chem. Lett. 11 (2001) 681–684.
[7] D.J. Morré, R. De Cabo, C. Farley, N.H. Oberlies, J.L. Mc Laughlin, Mode of action of bullatacin, a potent antitumor acetogenin: inhibition of NADH oxidase activity of HeLa and HL-60, but not liver, plasma membranes, Life Sci. 56 (1995) 343–348.
[8] J.C. Rowlands, J.E. Casida, NADH: ubiquinone oxidoreductase inhibitors block induction of ornithine decarboxylase activity in MCF-7 human breast cancer cells, Pharmacol. Toxicol. 83 (1998) 217–219.
[9] S.-N. Wu, H.-T. Chiang, F.-R. Chang, C.-C. Liaw, Y.-C. Wu, Stimulatory effects of squamocin, an annonaceous acetogenin, on Ca^{2+}-activated K^{+} current in cultured smooth muscle cells of human coronary artery, Chem. Res. Toxicol. 16 (2003) 15–22.
[10] C.H. Kolb, M.G. Finn, K.B. Sharpless, Click chemistry: diverse chemical functions from a few good reactions, Angew. Chem., Int. Ed. 40 (2001) 2004–2021.
[11] S. Arndt, U. Emde, S. Bäurle, T. Friedrich, L. Grubert, U. Koert, Quinone-Annonaceous acetogenins: synthesis and complex I inhibition studies of a new class of natural product hybrids, Chem. Eur. J. 7 (2001) 993–1005.
[12] H. Yabunaka, M. Abe, A. Kenmochi, T. Hamada, T. Nishioka, H. Miyoshi, Synthesis and inhibitory activity of ubiquinone-acetogenin hybrid inhibitor with bovine mitochondrial complex I, Bioorg. Med. Chem. Lett. 13 (2003) 2385–2388.
[13] T. Hamada, N. Ichimaru, M. Abe, D. Fujita, A. Kenmochi, T. Nishioka, K. Zwicker, U. Brandt, H. Miyoshi, Synthesis and inhibitory action of novel acetogenin mimics with bovine heart mitochondrial complex I, Biochemistry 43 (2004) 3651–3658.
[14] N. Ichimaru, M. Murai, M. Abe, T. Hamada, Y. Yamada, S. Makino, T. Nishioka, H. Makabe, A. Makino, T. Kobayashi, H. Miyoshi, Synthesis and inhibition mechanism of Δ-lac-acetogenins, a novel type of inhibitor of bovine heart mitochondrial complex I, Biochemistry 44 (2005) 816–825.
[15] E.A. Couladouros, Z.F. Plyta, S.A. Haroutounian, Efficient synthesis of aminonaphtoquinones and azidobenzohydroquinones: mechanistic considerations of the reaction of hydrazoic acid with quinones. An overview, J. Org. Chem. 62 (1997) 6–10.
[16] U. Brandt, H. Schägger, G. Von Jagow, Characterisation of binding of the methoxyacrylate inhibitors to mitochondrial cytochrome *c* reductase, Eur. J. Biochem. 173 (1988) 499–506.
[17] U. Brandt, U. Haase, H. Schägger, G.J. Von Jagow, Significance of the "Rieske" iron–sulfur protein for formation and function of the ubiquinol-oxidation pocket of mitochondrial cytochrome *c* reductase (*bc1* complex), J. Biol. Chem. 266 (1991) 19958–19964.
[18] M. Degli Esposti, A. Ghelli, M. Crimi, E. Estornell, R. Fato, G. Lenaz, Complex I and complex III of mitochondria have common inhibitors acting as ubiquinone antagonists, Biochim. Biophys. Res. Commun. 190 (1993) 1090–1096.
[19] M. Degli Esposti, M. Crimi, A. Ghelli, Natural variation in the potency and binding sites of mitochondrial quinone-like inhibitors, Biochem. Soc. Trans. 7 (1994) 209–213.
[20] W. Oettmeier, K. Masson, M. Soll, E. Reil, Acridones and quinolones as inhibitors of ubiquinone functions in the mitochondrial respiratory chain, Biochem. Soc. Trans. 7 (1994) 213–216.
[21] G. Von Jagow, T.A. Link, Use of specific inhibitors on the mitochondrial *bc1* complex, Methods Enzymol. 126 (1986) 253–271.
[22] M. Degli Esposti, Inhibitors of NADH-ubiquinone reductase: an overview, Biochim. Biophys. Acta 1364 (1998) 222–235.
[23] P. Lümmen, Complex I inhibitors as insecticides and acaricides, Biochim. Biophys. Acta 1364 (1998) 287–296.
[24] R. Duval, G. Lewin, R. Hocquemiller, Semisynthesis of heterocyclic analogues of squamocin, a cytotoxic annonaceous Acetogenin, by an unusual oxidative decarboxylation reaction, Bioorg. Med. Chem. 11 (2003) 3439–3446.