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SYNTHESIS AND BIOLOGICAL CHARACTERIZATION OF SQBAZIDE, A NOVEL BIOTINYLATED PHOTOAFFINITY PROBE FOR THE STUDY OF THE HUMAN PLATELET THROMBOXANE A_2 RECEPTOR

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Abstract: SQBAzide, a biotinylated, azido derivative of the TXA_2 receptor antagonist, SQ31,491, was synthesized and characterized. The compound specifically inhibited human platelet aggregation mediated by TXA_2 receptor activation and irreversibly labeled platelet TXA_2 receptors upon exposure to ultraviolet light. This probe should prove to be of significant value for the study of the receptor-ligand binding domain.

Keywords: Receptors; Labelling; Prostaglandins; Antagonists.

Introduction

It is well known that the arachidonic acid metabolite, thromboxane A_2 (TXA_2), interacts with a G-protein-coupled seven-transmembrane receptor to cause platelet aggregation and vascular smooth muscle contraction.[1,2] Furthermore, evidence has accumulated that this biological activity is linked to both hemostasis and the genesis of certain thromboembolic disorders, including myocardial infarction and stroke.[3-5] Based on these considerations, substantial interest has focused on the development of specific TXA_2 receptor antagonists to modulate TXA_2-mediated biological effects. However, despite these research efforts, attempts at rational drug design have been hampered by a lack of information concerning the TXA_2 receptor ligand-binding domain. In this regard, knowledge of the TXA_2 receptor ligand-binding domain has, for the most part, been limited to radioligand binding studies performed on receptor protein altered through site-directed mutagenesis[6,7] or by chimeric construction[8]. While frequently insightful, this approach has certain limitations concerning potential shifts in the tertiary structure of the protein. Thus, site mutations in the IV, V, VI and VII transmembrane TXA_2 receptor regions have resulted in less than definitive results concerning the location of the ligand binding pocket.

As an alternative approach to altering receptor structure by amino acid substitution, photoaffinity labeling is commonly employed.[9-13] This procedure for labeling the ligand-binding domain of a receptor relies on the use of a specific receptor agonist or antagonist, coupled to a photoactivatable group (often an azide). Upon exposure to ultraviolet light, the azide forms covalent linkages with proteins which are in closest proximity. Proteins specifically labeled by the photolabel are then digested and purified, with the labeled fragments identified as putative constituents of the binding pocket.

We report here the synthesis and biological evaluation of SQBAzide, a novel photoreactive derivative of

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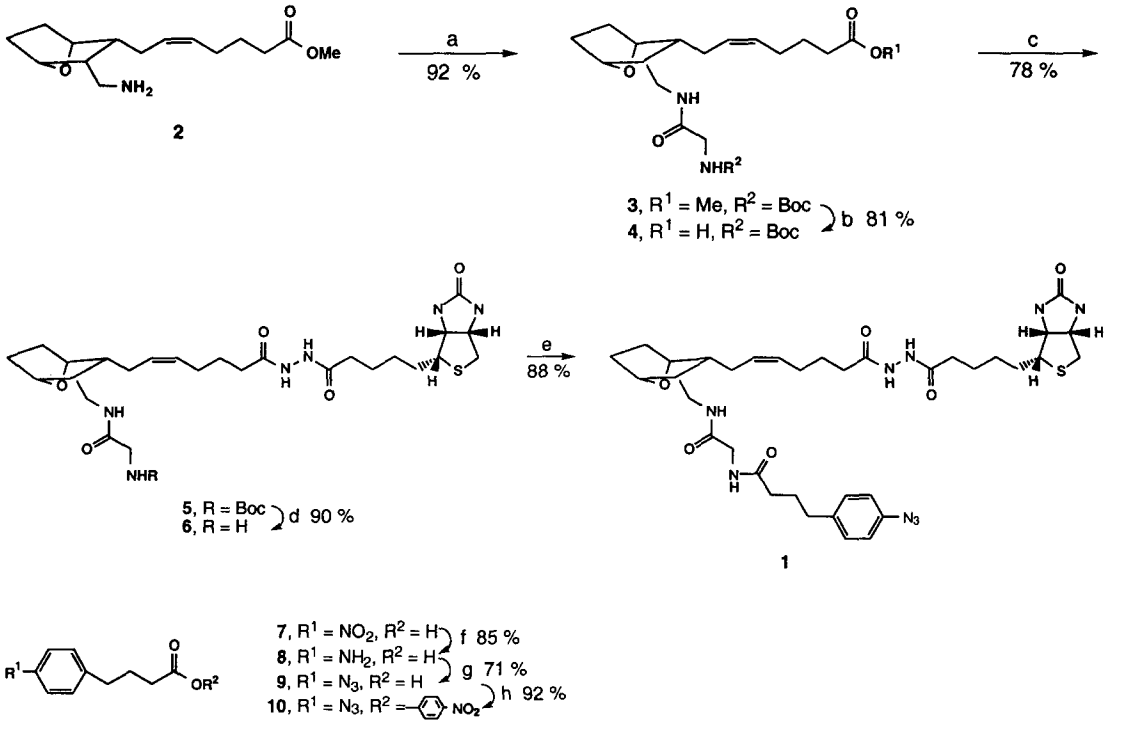
the TXA_2 receptor antagonist, SQ31,491. This bifunctional probe has the unique advantage of containing a biotin group in addition to the photoactivatable azide moiety. This modification provides significant benefits in the purification and tracking of the photoactivity-labeled fragments.

Synthesis

Scheme 1 illustrates the pathway used for the synthesis of the photoactivatable compound, SQBAzide **1**.

Scheme 1



Reagents and conditions: (a) N-Boc glycine, i-Bu chloroformate, N-Me piperidine, CH_2Cl_2, -15° C, 2 h. (b) NaOH, 1N MeOH, r.t., 36 h. (c) Biotin hydrazide, i-Bu chloroformate, N-Me piperidine, CH_2Cl_2, -25° C, 1 h, r.t. 2 h. (d) TFA, r.t., 15 min. (e) Compound **10**, Et_3N, DMF, r.t., 2 h. (f) H_2, 10 % Pd/C, MeOH, r.t., 6 h. (g) 1. $NaNO_2$, H_2SO_4, H_2O, 0°, 45 min. 2. NaN_3, H_2O, 0° 40 min. (h) 4-Nitrophenol, DCC, CH_2Cl_2, r.t. 1 h.

We opted for a strategy in which the key compoud **6** allows the introduction of a variety of photoactivatable substituents in the lower chain and also the modification of the lenght of the spacer arm between the core of the compound and the photolabel. The starting compound, methyl [1*S*-[1α,2α(*Z*),3α,4α]]-7-[3-aminomethyl)-7-oxabicyclo[2.2.1] hept-2-yl]-5-heptenoate (**2**) was obtained as described by Nakane et al.[14] The reaction between compound **2** and N-protected glycine gave compound **3**,[15] which was hydrolyzed with a solution of sodium hydroxide to form the free acid **4**. Reaction of compound **4** with biotin hydrazide in the presence of *i*-butyl

chloroformate and N-methyl morpholine[16] provided the biotinylated compound **5**. N-deprotection with trifluoroacetic acid gave the amine **6**, which was reacted with the active ester **10** to produce the photoactivatable compound, biotinylhydrazido[1S-[1α,2α(Z),3α,4α]]-7-[3-[[[[[(4-azidophenyl)propylcarbonyl]amino]acetyl]amino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoate (**1**).

The 4-azidophenyl butyric acid 4-nitrophenyl ester **10** was obtained from the commercially available 4-nitrophenyl butyric acid (**7**). Catalytic reduction to the amine **8**, followed by nitrosation and reaction with sodium azide gave the azidophenyl compound **9**. Treatment of **9** with p-nitrophenol and N-dicyclohexyl carbodiimide supplied the active ester **10**.

Biological Evaluation

The pharmacological effect of SQBAzide was tested in aggregation studies performed on platelet-rich plasma (PRP) isolated from CPD (citrate-phosphate-dextrose)-anticoagulated human blood as previously described.[17] In order to block endogenous production of TXA_2, the PRP was incubated with indomethacin (20 μM). Various concentrations of SQBAzide were then incubated with PRP 5 min prior to the addition of submaximal concentrations (eliciting approximately 80% maximal aggregation) of the TXA_2 receptor agonist, U46619 (1-3 μM), ADP (10 μM) or the divalent cation ionophore, A23187 (2 μM). The aggregatory response was measured on a model 400 Lumi-aggregometer (Chronolog Corp., Havertown, PA) using the turbidometric method.[18] Figure 1A depicts the antagonistic effects of SQBAzide on U46619-induced platelet aggregation. Preincubation with azide concentrations greater than 1 μM resulted in an almost complete inhibition of platelet aggregation, while concentrations around 500 nM represented 50% inhibition (IC_{50}) of aggregation. The specificity of this inhibition was tested by measuring aggregation stimulated by two agents (ADP and A23187) which activate platelets through pathways independent of the TXA_2 receptor. It was found that SQBAzide (2 μM) was unable to inhibit aggregation induced by either ADP (Fig 1B) or A23187 (Fig 1C). These results demonstrate that SQBAzide exerts its inhibitory effect on platelet aggregation specifically through the TXA_2 receptor-signal transduction system.

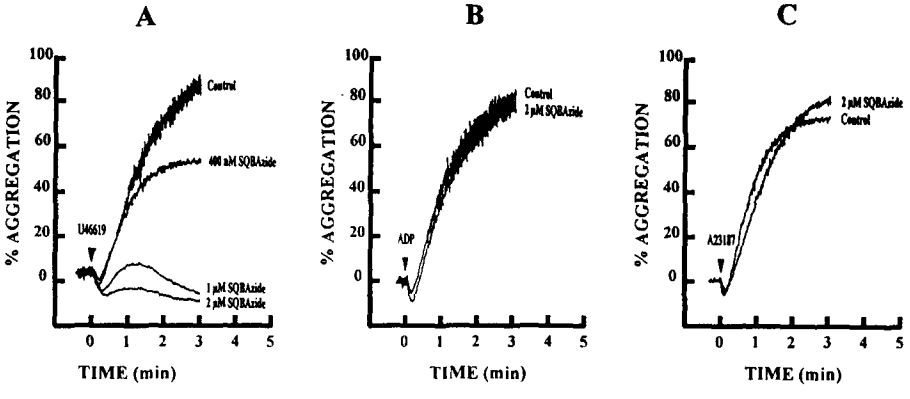


Figure 1. Effect of SQBAzide on human platelet aggregation induced by U46619, ADP or A23187. Platelet-rich plasma was incubated with various concentrations of SQBAzide or vehicle for 5 min prior to the addition of 1-3 μM U46619 (**A**), 10 μM ADP (**B**) or 2 μM A23187 (**C**). Each aggregation curve is representative of multiple traces obtained from three separate blood donors.

Experiments were next conducted to examine whether the observed inhibition was mediated through SQBAzide interaction with the TXA_2 receptor ligand-binding domain. In these studies, SQBAzide binding to platelet TXA_2 receptors was determined by measuring its ability to displace binding of the potent, radiolabeled TXA_2 antagonist, [^{3}H]SQ29,548. Briefly, 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)-solubilized platelet membranes were incubated with [^{3}H]SQ29,548 (2 nM) for total binding samples. [^{3}H]SQ29,548 (2 nM) plus unlabeled SQ29,548 (2 μM) were co-incubated in the nonspecific binding samples, and [^{3}H]SQ29,548 (2 nM) plus various amounts of SQBAzide were co-incubated in competition binding samples. After a 30 min incubation at room temperature, the protein samples were immobilized on GF/B glass fiber filters (presoaked in 0.3% polyethyleneimine) by vacuum filtration and immediately washed twice with 5 ml of buffer (25 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) at 4 °C. The filters were assayed for radioactivity by liquid scintillation spectroscopy using a Beckman LS 6800. The average [^{3}H]SQ29,548 specific binding achieved in these experiments was 92%. Fig 2 illustrates the resultant SQBAzide competition binding curve. In this figure, nonlinear regression analysis revealed a sigmoidal-shaped inhibition curve characteristic of direct receptor antagonism. Application of the Cheng-Prusoff relationship[19] to this curve yielded a K_i of 625 nM for SQBAzide. This value correlates well with the 500 nM IC_{50} obtained from aggregation studies described above. Collectively, these findings provide evidence that SQBAzide specifically interacts with the TXA_2 receptor ligand-binding domain and that this receptor interaction is the mechanism by which SQBAzide inhibits TXA_2-mediated platelet aggregation.

To assess the ability of SQBAzide to specifically and irreversibly label TXA_2 receptors, competition photoaffinity labeling studies were undertaken. CHAPS-solubilized platelet membranes were incubated with SQBAzide (2 μM) for 15 min in the dark and the samples were then subjected to photolysis with ultraviolet light for 1 min at a distance of 5 cm using a 100W Olympus mercury lamp. Photolysis was terminated by the addition of dithiothreitol (4 mM) to the samples, followed by ten times concentration of the product by centrifugal filtration through 0.1 μM low-binding Durapore membranes (Millipore Corp., Bedford, MA). Proteins within the concentrated samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE),[20] and the entire gel was transferred to a nitrocellulose membrane. The receptor protein was then blocked for 1 hr with 3% gelatin in Tris-buffered saline (30 mM Tris base, 120 mM NaCl, pH 7.4) and probed for irreversibly bound SQBAzide by incubating the membrane for 2 hr with 1% gelatin in Tris-buffered saline containing 37.5 nM ^{125}I-streptavidin, which binds the biotin functional groups of SQBAzide. After washing with 0.1% TWEEN in Tris-buffered saline for 1 hr, autoradiography of the membrane was performed, revealing intense labeling of TXA_2 receptor protein (Fig 3, lane 1). In parallel studies, the specificity of this receptor labeling was examined by competing for ligand binding sites with a structurally unrelated TXA_2 receptor antagonist, BM13.505 (K_d = 1 μM). Briefly, CHAPS-solubilized platelet membranes were incubated for 15 min with BM13.505 (1 mM) prior to SQBAzide (2 μM) addition. Photolysis, SDS-PAGE and ^{125}I-streptavidin labeling were carried out as described above. It can be seen (Fig 3, lane 2) that competition with BM13.505 effectively prevented photolabeling of TXA_2 receptor protein by SQBAzide. Similar results were obtained in competition photolabeling experiments using a

different TXA_2 receptor antagonist, SQ29.548 (data not shown). These findings therefore provide evidence that SQBAzide specifically and irreversibly labels the TXA_2 receptor ligand-binding region.

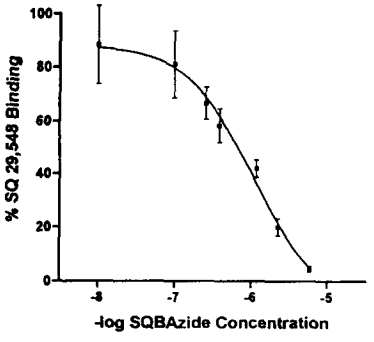


Figure 2. Competition binding curve of SQBAzide for CHAPS-solubilized platelet TXA_2 receptors. Binding of various concentrations of SQBAzide to platelet TXA_2 receptors was determined by measuring the ability of SQBAzide to displace binding of the radiolabeled TXA_2 receptor antagonist, [^{3}H]SQ29,548. Each point represents the mean ± S.E.M. of triplicate values obtained from three separate blood donors.

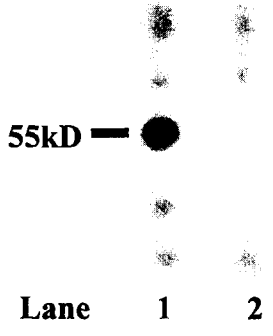


Figure 3. Competition photoaffinity labeling of platelet TXA_2 receptors. CHAPS-solubilized platelet membranes were incubated with SQBAzide (2 μM) in the absence (lane 1) or presence (lane 2) of the competing antagonist, BM13.505 (1 mM).

Conclusion

In this communication we have reported the synthesis and biological characterization of SQBAzide, a novel, biotinylated, azido antagonist of the TXA_2 receptor. This compound was shown to inhibit human platelet aggregation through direct interaction with the TXA_2 receptor ligand-binding domain. Furthermore, photolabeling competition experiments demonstrated that SQBAzide covalently labels elements of this ligand-binding domain. It is believed that this new generation of bifunctional TXA_2 receptor probes will be of significant benefit to studying ligand interactions with the TXA_2 receptor protein.

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