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The Search for Novel Human Pancreatic α-Amylase Inhibitors: High-Throughput Screening of Terrestrial and Marine Natural Product Extracts

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Specific inhibitors of human pancreatic α-amylase (HPA) have potential as oral agents for the control of blood glucose levels in the treatment of diabetes and obesity. In a search for novel inhibitors, a library of 30000 crude biological extracts of terrestrial and marine origin has been screened. A number of inhibitory extracts were identified, of which the most potent was subjected to bioassay-guided purification. A family of three glycosylated acyl flavonols, montbretins A–C, was thereby identified and characterized as competitive amylase inhibitors, with K_i values ranging from 8.1–6100 nM. Competitive inhibition by myricetin, which corresponds to the flavone core, and noncompetitive inhibition by a second fragment, ethyl caffeiate, suggest a binding mode for these inhibitors.

Introduction

The incidence of diabetes and obesity is increasing at an alarming rate, with diabetes alone currently afflicting 6% of the population throughout the western world, and ranking as the third most prevalent disease. The number of sufferers worldwide is expected to rise from 177 million to 300 million individuals by 2030.[1] Both of these chronic diseases exact a high toll in terms of human health outcomes and in the cost of health care. Beyond the reduced quality of life and life expectancy that is experienced by people who are afflicted with diabetes, ~40% are likely to develop long-term complications such as hypertension, hyperlipidemia, cardiovascular disease, cancer, stroke, kidney failure and blindness.[2–4] A significant risk factor for the development of diabetes is obesity, which in itself has become a serious health issue.[5]

Human pancreatic α-amylase (HPA) is a key enzyme in the digestive system and catalyzes the initial step in the hydrolysis of starch, which is a principal source of glucose in the diet. Detailed structural and mechanistic studies have been performed on HPA, including analyses of HPA–inhibitor binary complexes.[6–10] It has been previously demonstrated that the activity of HPA in the small intestine correlates to post-prandial glucose levels, the control of which is an important factor in diabetes and obesity.[11] Modulation of HPA activity through the therapeutic use of inhibitors would therefore be of considerable medical relevance in the treatment of these debilitating diseases. Although two α-glucosidase inhibitors, acarbose (Precose™ or Glucobay™) and miglitol (Glyset™) in current medical use, their effectiveness is limited by deleterious side effects.[12–14] These two drugs target both HPA and other digestive α-glucosidases, however some of these undesired side effects might arise from the nonspecific inhibition of nondigestive α-glycosidases. These side effects are perhaps compounded by the systemic absorption of these drugs, or their metabolites in the case of acarbose, and hence their distribution throughout the body. Unlike most oral drugs, poor absorption is a desirable quality for an HPA inhibitor because the effect is only required in the gut, and low systemic availability would reduce unwanted side effects. New and more effective human drugs that can target HPA specifically, and can operate at lower dosages with longer-term effects and lower systemic availability would be valuable tools to combat the rapidly increasing incidences of these conditions.

Natural products have long proved to be valuable sources of enzyme inhibitors; indeed the HPA inhibitor acarbose was isolated originally from *Streptomyces* extracts.[15] The advantage of choosing to examine crude biological extracts is that each extract likely contains countless primary and secondary metabolites, many of which will not have been previously characterized. This therefore allows us to dramatically increase the chemical space being sampled. The library that was investigated in this study consisted of 30000 extracts from the NCI (National Cancer Institute, U.S.), which are derived from both terrestrial and marine sources. Natural product extract libraries have already proven to be a highly valuable resource and have

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resulted in the discovery of FDA-approved anticancer drugs. It is also worth noting that many plants are used as traditional medicines for the treatment of diabetes, and as such, can be expected to contain potential compounds of interest.[16]

Results and Discussion

Development of a HTS assay for HPA inhibitors

The most versatile and robust assay for HPA is based upon the enzymatic cleavage of a synthetic aryl glycoside substrate to yield a chromophoric product, the release of which can be monitored in a continuous fashion. The advantages of a continuous assay over an end-point assay are many-fold, particularly in that the assay is less affected by the high background readings that arise from the extract composition. Additionally, a UV-visible assay has benefits over a fluorescence-based assay in that the assay is considerably less sensitive to "quenchers", which often give false positives under fluorescence-based assay conditions. The commercially available amylase substrate 2-chloro-4-nitrophenyl α-D-maltotrioside (CNP-G3) proved ideal for this assay because the pK_a of the chloronitrophenyl leaving group (pK_a = 5.45)[17] is considerably lower than the pH value of the assay (pH 7.0), hence a high extinction coefficient (ε = 11 mM^{-1} cm^{-1}) for the chromophore is obtained under these conditions. The assay solution also included Triton X-100 (0.01%) to minimize the detection of promiscuous inhibitors.[18,19] The extract samples, which were DMSO solutions that contained 5 mg mL^{-1} of dried methanolic extract, were tested at a dilution of 60 nL in a final assay volume of 60 μL (5 μg mL^{-1} final extract concentration). The enzymatic activity of HPA was found to be completely unaffected by the addition of this small amount of DMSO (0.1%) and Triton X-100 (0.01%). Each sample was run in duplicate, and the replicate was run on a separate plate that additionally contained 4′-*O*-methyl-α-D-maltosyl fluoride (1 mM final concentration). This was done in the hope of identifying inhibitors that are capable of undergoing in situ elongation in a manner similar to that reported by us previously.[20] Unfortunately, no compounds gave enhanced inhibition in the presence of the glycosyl fluoride. Although disappointing, the replicates nonetheless served their normal role. Two test plates that contained a serial dilution of the known HPA inhibitor acarbose were run as the first and last plate of each batch in order to ensure the robustness and integrity of the assay for each given batch that was analyzed.

The data for each plate were normalized relative to the high controls that were contained therein, and the method of Zhang et al. was employed to evaluate the data quality.[21] The average Z' statistic, which represents the quality of the control samples and provides an indication of assay suitability, was determined to be 0.86. This number (which approaches a value of 1 in a "perfect" screen) indicates that the obtained data were of high quality and ideally suited to HTS studies. Additionally, the average Z value of 0.82, which represents how well the library is tolerated, demonstrated that the assay was remarkably robust and the data therein were very reliable. The hit threshold was set at 3 standard deviations from the mean of the sample set (corresponding to 81% residual activity) and samples for which both replicates fell within the hit boundary were selected for further investigation.

HPA Screening Results

Figure 1 shows the data set that was obtained from the screening of 30000 NCI extracts, the hit boundaries are marked as a dotted line.

The majority of the samples are clustered around 100% residual activity for both replicates, as would be expected for a sample set where the extracts are predominantly noninhibitory. A few outliers show 100% residual activity for one replicate only, with the second replicate showing apparent inhibition. Visual inspection of the data for these samples showed that the apparent lower activity was due to a downward step in the UV absorption, which most likely arose from a bubble in the assay well; the observed rate on either side of the step was close to 100% residual activity. Because this effect is merely an artifact of the assay conditions, and arose from the inclusion of a detergent, these samples are not identified as hits. As such, only samples in which both replicates fall within the hit window were selected for further validation. To validate the 30 extracts that were identified as hits from the primary screen, each hit was re-evaluated manually on a standard UV/Vis spectrophotometer. In this secondary screen, 25 of the extracts gave reproducible inhibition and were confirmed to be "true hits", whereas 5 extracts showed no significant HPA inhibition, thus, they identified themselves as false positives. This corresponds to a very high hit validation rate of 83% and an overall hit-rate of 0.08%.

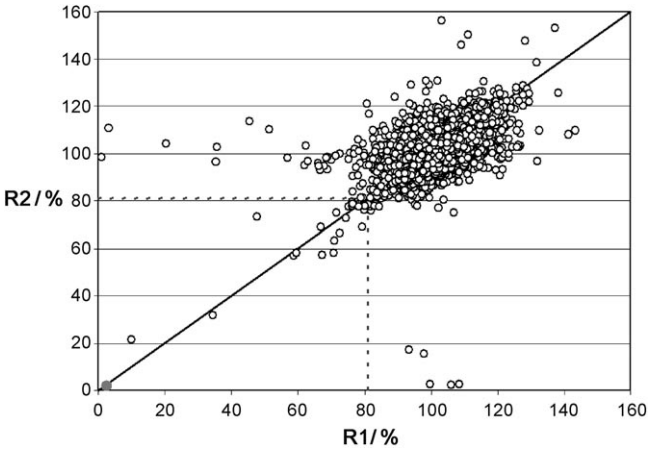


Figure 1. Data set from HTS of 30000 NCI extracts with the sample selected for further study (close to origins) highlighted in bold (●). Data points expressed as residual activity (%); **R1**: replicate **1**, **R2**: replicate **2**.

Bio-assay guided isolation of HPA inhibitors

The extract with the most significant inhibitory activity (2% residual activity) was selected for further study. Prior to detailed

investigation of the active components, a preliminary kinetic analysis of the HPA inhibition was performed on the crude extract. A dilution series showed a semilogarithmic sigmoidal dose–response curve that is typical of a "well-behaved" inhibitor (Figure 2A), and revealed a very low IC_{50} value of

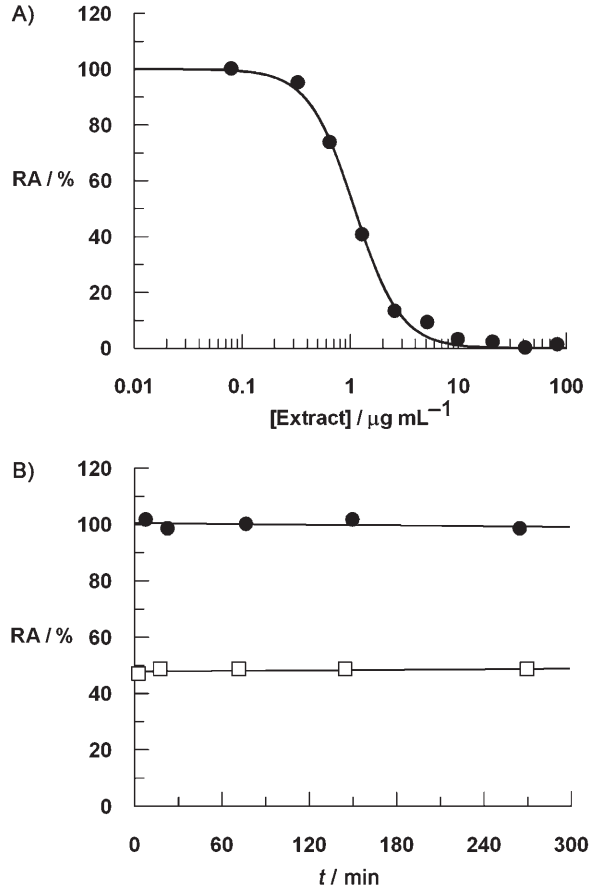
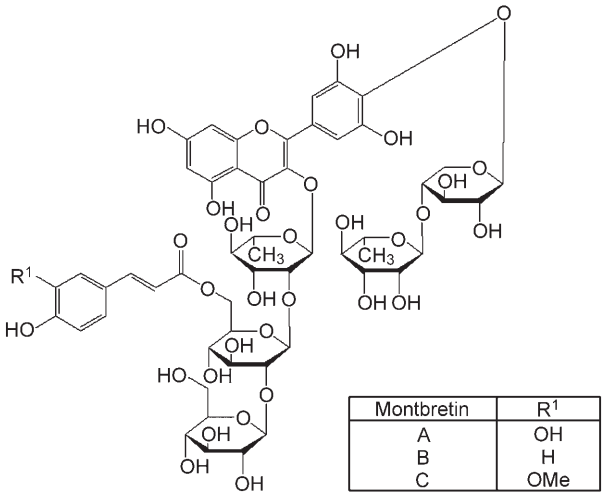


Figure 2. A) IC_{50} determination of the crude extract; B) Time-dependent inhibition (●) Control, no inhibitor, (□) HPA plus crude extract. RA: residual activity (%).

2.7 μg mL^{-1}. Additionally, the crude extract showed no time-dependent inactivation of HPA; the level of inhibition remained constant for over 4 h (Figure 2B). With these tests we hoped to exclude the possibility of analyzing extracts that contained compounds with undesirable modes of action such as enzyme denaturation or covalent enzyme modification. The encouraging inhibitory profile and lack of time-dependent inactivation indicated that this was unlikely to be occurring. This extract was therefore deemed to be suitable for further investigation and crude material was obtained in a larger quantity from the NCI.

The sample that was obtained was a dried methanolic extract of the bulbs from *Crocosmia* sp., a perennial plant of the *Iridaceae* family that is native to South Africa. In order to isolate the principal bioactive components from the complex mixture of the extract, a series of bioassay-guided purification steps were performed. At each step the column fractions were assayed for HPA inhibition, and the active fractions were taken forward.

After extensive purification, a family of three related compounds was obtained. Through a combination of 2D NMR spectroscopy and mass spectrometry, two of these compounds were identified as the known glycosylated acyl-flavonols montbretins A and B (Scheme 1). These compounds were originally



Scheme 1. Montbretins A–C that were isolated from *Crocosmia* sp.

isolated from an extract of *Crocosmia crocosmiiflora*, a garden hybrid of *C. aurea* and *C. Pottsii*;[22] a plant that has seen traditional use as an antitumour agent in Japanese folk medicine. The remaining family member was identified as a methyl ether of the cinnamic acid moiety and was named montbretin C (Scheme 1).

Bioflavonoids are secondary plant metabolites, and over 9000 structural variants have been identified to date.[23,24] They occur ubiquitously in nature, and are thought to play a variety of roles from growth regulation to self-defense. In humans, the health-promoting benefits of consumption of this class of compounds are well described and include reduced incidence of heart disease and certain types of cancer. These pharmacological activities are ascribed to the sequestering of reactive oxygen species (ROS), which is due to their natural anti-oxidant behavior and/or the modulation of enzymatic activities. Flavonols are a sub-family of the flavonoids that are distinguished by a 2,3-unsaturation, a 3-hydroxyl group and 4-oxo group on the C-ring of the flavan nucleus. In plants, these flavonols are frequently glycosylated to reduce reactivity and increase water solubility, and of these, myricetin is a commonly occurring aglycone.[25]

The montbretins, which contain the myricetin flavonol core, are glycosylated at the 3 and 4′ positions. The 3-hydroxyl carries an α-linked linear trisaccharide that consists of D-glucopyranosyl-(β1→2)-D-glucopyranosyl-(β1→2)-L-rhamnopyranose; the central D-glucosyl sugar bears a 6-*O*-cinnamic ester, which is differently substituted among the family members. The 4′ position bears a β-linked D-xylose unit, which is itself appended on its 4-hydroxyl with an α-linked L-rhamnopyranosyl moiety.

Kinetic analyses of the three family members that were isolated showed montbretin A to be a considerably more potent

inhibitor of HPA than montbretins B and C (Table 1). Clearly, the presence of the free *meta*-hydroxyl group of the cinnamic acid moiety is crucial to the tight binding of montbretin A because its removal or methylation (montbretins B and C respectively) lowers the inhibitory activity by almost 1000-fold.

Table 1. Inhibition of HPA by montbretins A–C.

Montbretin	R^1	K_i [nM]
A	OH	8.1
B	H	3600
C	OMe	6100

Because montbretin A was observed to be both the most inhibitory and the most abundant family member, it was therefore chosen for further study. Detailed kinetic analysis of montbretin A demonstrated it to be a tight-binding competitive inhibitor of HPA ($K_i = 8.1$ nM, Figure 3). Additionally, montbretin A showed a high level of selectivity towards HPA when tested against a series of glycosidases, including other enzymes from the GH13 family (Table 2), which is a highly desirable attribute for any potential HPA-targeted therapeutic.

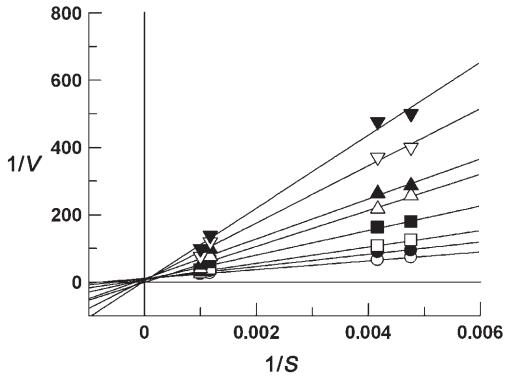


Figure 3. Double reciprocal plot showing Montbretin A to be a tight-binding, competitive inhibitor of HPA.

Interestingly, montbretin A appeared to show time-dependent inhibition towards the β-glucosidase from *Agrobacterium* sp. (Abg), and the extent of inhibition *decreases* with time (data not shown). Indeed, pre-incubation of montbretin A with Abg prior to the addition of the assay substrate resulted in no observable β-glucosidase inhibition. This *exo*-acting glycosidase has been shown to be promiscuous with regard to the substrate aglycone, hence it was deduced that montbretin A was actually acting as a substrate for Abg and the terminal β-linked glucose residue was being cleaved. The subsequent residue of montbretin A is also a β-linked glucose residue, however this residue bears a large 6-*O*-caffeic ester moiety and would therefore not be processed by Abg.[26] Furthermore, upon re-testing of this truncated montbretin A-derived compound, no significant change in the inhibitory prowess with respect to HPA was observed; this suggests that the terminal glucosyl residue is not required for HPA activity.

Given that flavonoids are well known to possess both antioxidant and prooxidative properties, an initial concern was

Table 2. Glycosidase specificity of montbretin A.

Glycosidase	RA [%]
α-amylase (HPA)	11
β-glucosidase (*Agrobacterium* sp.)	100
β-galactosidase (*E. coli*)	98
β-hexosaminidase (jack bean)	99
α-mannosidase (jack bean)	100
α-galactosidase (green coffee bean)	100
α-glucosidase (brewers' yeast)	97

[a] Residual enzyme activity at 0.1 μM montbretin A.

that montbretin A might be inactivating HPA through redox modifications.[23,27] This mode of action was deemed unlikely because of the competitive nature of the inhibition and the wide range of K_i values for the structurally similar family members. However, in order to refute this possibility, the inhibition of HPA by montbretin A was measured both in the presence and absence of 5 mM dithiothreitol (DTT). The addition of DTT maintained the enzyme and reagents in a reducing environment, thereby preventing the occurrence of any redox chemistry either in solution or within the enzyme active site. Because no effect on HPA inhibition was observed upon the inclusion of DTT, a redox-type mechanism of action was deemed unlikely. Additionally, flavonoids can also chelate metal cations, which raises the possibility that the inhibitor might be extracting the essential HPA calcium ion. In order to discount this mechanism of action, kinetic studies were performed in the presence of 1 mM calcium chloride, with the result that no change in inhibition was observed.

In order to investigate which structural motifs of montbretin A contribute to HPA inhibition, commercially available compounds that correspond to the two aromatic portions; the flavonol core (myricetin) and the 6-*O*-acyl group (caffeic acid) were examined independently as HPA inhibitors. Interestingly, myricetin was indeed an HPA inhibitor ($K_i = 110$ μM, Figure 4A), albeit several orders of magnitude less potent than montbretin A. Furthermore the inhibition was observed to be of a competitive nature; this indicates that inhibition arises from binding in the enzyme active site. Ethyl caffeiate, the ethyl ester of caffeic acid, was found to be a weak inhibitor of HPA ($K_i =$ 1.3 mM, Figure 4B). The inhibition mode in this case was observed to be noncompetitive, thus suggesting that the inhibition is arising through interactions that are remote from the active site. These findings suggest an attractive model for the observed inhibition by montbretin A in which the flavonol core occupies the active site, while the caffeic acid moiety binds to a second site. The sugar residues act as linkers, and quite possibly also provide additional binding interactions. As indicated by the differential binding strength of montbretin A versus B and C, the *meta*-hydroxyl group of the caffeic acid is likely playing a crucial role in correctly orienting the inhibitor into the most productive binding mode. Hopefully, future structural studies of enzyme–inhibitor complexes will provide insights into the exact binding mode.

Other glycosylated flavonols have previously been identified from plants that have been used as traditional treatments for

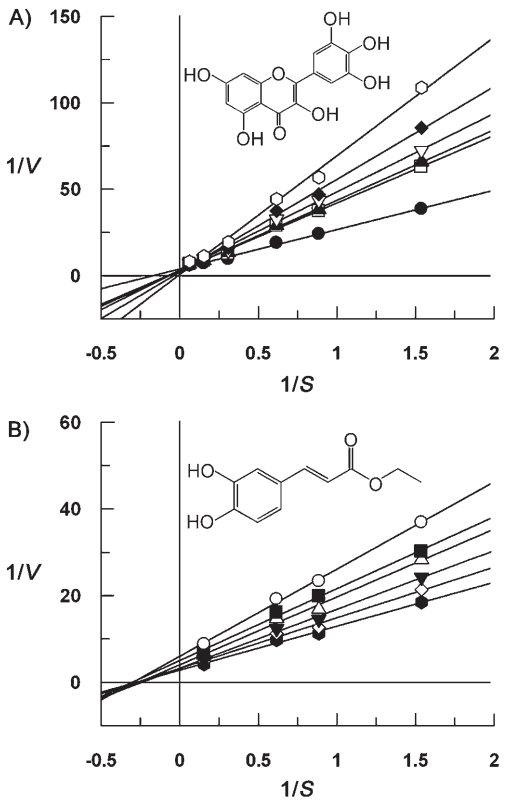


Figure 4. Double reciprocal plot of the HPA inhibitors; A) myricetin ($K_i = 110$ μM, competitive); B) ethyl caffeiate ($K_i = 1.3$ mM, uncompetitive).

diabetes.[28] While the mechanism of action for many of these compounds has not been determined,[29,30] some, such as the myrciacitrins,[31–33] have been demonstrated to be aldose reductase inhibitors,[34,35] while others are thought to be glycation inhibitors.[36] A few members have also shown α-glucosidase inhibition.[32,35,37–39] None however have been described as α-amylase inhibitors.

Conclusions

Through the screening protocol outlined above, we were able to examine 30000 biological extract samples in the search for inhibitors of human pancreatic α-amylase. From these data we have identified 25 inhibitory extracts, a hit rate of ~0.1%. The detailed investigation of the most inhibitory extract resulted in the isolation of a family of glycosylated acyl-flavonols, one of which was demonstrated to be a tight-binding and specific HPA inhibitor. By using the structure–activity relationship obtained from the kinetic analysis of these family members, along with that of two small-molecule fragments, we were able to speculate as to the binding mode that is employed by this family of compounds to achieve such potent inhibition. This extract, which resulted in both the isolation of a novel compound (montbretin C), and the discovery of novel inhibitory behavior for two previously described compounds (montbretins A and B), demonstrate the great potential for novel HPA inhibitor discovery from crude natural product extracts. There remain several other inhibitory extracts from this work that have yet to be evaluated further.

Experimental Section

Primary screening: The screen was performed on a Beckman Coulter Biomek FX Laboratory Automation Workstation (Fullerton, CA, USA) that was equipped with a 96-channel pipetting head and a low-volume 96-pin High Density Replicator. This workstation was integrated with a Beckman Coulter DTX880 plate reader with UV/Vis capability, which allowed for sequential assay plate processing and reading. The assay was run in 384-well plates that contained a 60 μL volume of 50 mM sodium phosphate buffer (pH 7.0), 100 mM sodium chloride, CNP-G3 (1 mM final concentration) and HPA (1 μg mL^{-1} final concentration). Triton X-100 (0.01%) was also present to minimize nonspecific inhibition.[18,19] The CNP-G3 substrate was employed at a sub-K_M concentration ($K_M = 3.6$ mM) both for economy of quantity, and also to promote the identification of competitive over noncompetitive inhibitors. The extracts were added to the assay plate by using three transfers of a High-Density Replicator pin tool (20 nL per transfer). Each sample was run in duplicate, with the second duplicate run on a separate plate that additionally contained 4′-*O*-methyl-maltosyl-α-luoride (1 mM final concentration). All of the assay plates contained 32 high controls (no inhibitor). The inhibitors and enzyme were allowed to incubate together at room temperature for 10 min to allow for detection of "slow-on" type inhibitors. The reaction was initiated by the addition of substrate, and the subsequent release of the chloronitrophenolate anion was monitored continuously at 405 nm for 7 min. The plate reader software (Beckman Coulter Multimode Detection) was then used to calculate the rate of the reaction in each well. The rate for each extract was normalized with respect to the high controls, and the data for each sample reported as % residual activity. For each sample the two replicates were plotted against one another (x, y), and those samples which fell within the hit window (set at 3 standard deviations from the mean of the sample set) were selected for validation. An aliquot (1 μL) of each of the extracts was retested in a half-area six-well plate (100 μL final volume), and those samples that gave reproducible inhibitory activity were identified as "true hits".

Preliminary evaluation: The extract with the most significant inhibitory activity was serially diluted (1:2 dilution factor) in a half-area 96-well plate, and the HPA inhibition was measured as described previously. The data that were thus obtained were fit to the IC_{50} model in the analysis program GraFit[40] and resulted in an IC_{50} value of 2.7 ± 0.1 μg mL^{-1}. In order to evaluate the time-dependent nature of the inhibition, the enzyme and inhibitor were incubated together at room temperature and aliquots were removed and tested for HPA activity over a period of 4 h.

Isolation and identification of the montbretins: A dried crude methanolic extract of the bulbs *Crocosmia* sp. was obtained from the NCI open plant repository. The crude material (2 g) was partitioned between ethyl acetate and water, and the aqueous fraction then partitioned against butanol. The butanolic fraction was applied, 150 mg at a time, to a column that was packed with Sephadex™ LH-20 that was pre-swollen in methanol for size exclusion chromatography. The resulting fractions were grouped based on biological activity. The active fraction was purified by HPLC by using 22% aqueous acetonitrile, to afford three fractions. Further purification of the main fraction by using a gradient from 30% to 40% aqueous acetonitrile over 30 min afforded montbretin A (15 min, 8.4 mg) as a yellow powder. A gradient from 30% to 70%

aqueous acetonitrile over 30 min on the second fraction afforded montbretin B as a yellow powder (16 min, 0.9 mg), and a gradient from 20% to 30% aqueous acetonitrile was used on the third fraction, which afforded montbretin C (20 min, 1.6 mg) as a yellow powder (for NMR data see the Supporting Information).

Column fractions from each step of the purification process were sub-sampled (100 μL) into 96-well plates, which were then allowed to evaporate to dryness. By using the Biomek FX, the fractions were redissolved in water and varying aliquots (1–10 μL) were transferred to a 384-well plate, and were analyzed for HPA inhibitory activity by using the same protocol that was described for the HTS study.

Kinetic analysis of the active compounds: The K_i values and mode of inhibition of montbretin A, myricetin and ethyl caffeiate were determined by measuring the rate of reaction at differing inhibitor concentrations for a series of substrate concentrations. Reactions were performed on either a Varian Cary300 or Cary4000 UV/Vis spectrophotometer at 400 nm. The substrate concentration (CNP-G3) was typically varied from $^1/_5$ of to five times the K_M value. A similar range of inhibitor concentrations was attempted, but some limitations were encountered. The lowest concentration of montbretin A that could be measured was 4 nM ($^1/_2$ the K_i value) due to the very low enzyme concentration that was required to remain significantly below the inhibitor concentration. Conversely the limited aqueous solubility of myricetin and ethyl caffeiate meant that the highest inhibitor concentration that was determined for these compounds was 1.5-times the K_i value. Double reciprocal plots of the data for montbretin A and myricetin indicated that these compounds were competitive inhibitors of HPA. A good fit of the data to the competitive inhibition model in the analysis program GraFit was achieved, and K_i values of 8.1 ± 0.5 nM and 110 ± 15 μM respectively were obtained. A double reciprocal plot of the data that was obtained with ethyl caffeiate demonstrated that this compound was a noncompetitive inhibitor of HPA. In this case, a good fit of the data to the noncompetitive inhibition model in Grafit was observed, and a K_i value of 1.3 ± 0.1 mM was obtained. The K_i values of montbretins B and C were determined by the range finder method. The rate of reaction for a series of varying inhibitor concentrations was measured at a fixed substrate concentration. From a Dixon plot of the data, the intercept of the line of best fit through these points with the $1/V_{max}$ line is equal to the $-K_i$ value. From these data, K_i values of 3.6 ± 0.1 μM and 6.1 ± 0.1 μM were obtained for montbretins B and C respectively.

Acknowledgements

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Keywords: amylase · diabetes · enzymes · glucosidase · high-throughput screening · montbretin · obesity

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