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Biotinylated quercetin as an intrinsic photoaffinity proteomics probe for the identification of quercetin target proteins

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ABSTRACT

Quercetin is a flavonoid natural product, that is, found in many foods and has been found to have a wide range of medicinal effects. Though a number of quercetin binding proteins have been identified, there has been no systematic approach to identifying all potential targets of quercetin. We describe an O7-biotinylated derivative of quercetin (BioQ) that can act as a photoaffinity proteomics reagent for capturing quercetin binding proteins, which can then be identified by LC–MS/MS. BioQ was shown to inhibit heat induction of HSP70 with almost the same efficiency as quercetin, and to both inhibit and photocrosslink to CK2 kinase, a known target of quercetin involved in activation of the heat shock transcription factor. BioQ was also able to pull down a number of proteins from unheated and heated Jurkat cells following UV irradiation that could be detected by both silver staining and Western blot analysis with an anti-biotin antibody. Analysis of the protein bands by trypsinization and LC–MS/MS led to the identification of heat shock proteins HSP70 and HSP90 as possible quercetin target proteins, along with ubiquitin-activating enzyme, a spliceosomal protein, RuvB-like 2 ATPases, and eukaryotic translation initiation factor 3. In addition, a mitochondrial ATPase was identified that has been previously shown to be a target of quercetin. Most of the proteins identified have also been previously suggested to be potential anticancer targets, suggesting that quercetin's antitumor activity may be due to its ability to inhibit multiple target proteins.

1. Introduction

Quercetin (Fig. 1A) is a natural product with multiple medicinal properties which belongs to the flavonoid family.[1] Initially extracted from red wine, quercetin was subsequently found to exist widely in leaves, fruits, and vegetables,[2] and is available as a dietary supplement. Quercetin has antioxidant,[3,4] anti-inflammatory,[5] and anticancer,[6–8] activities with almost no human toxicity.[9] At the molecular level, quercetin has been found to inhibit many ATP binding enzymes and in particular kinases,[10] suggesting that some of its biological effects may be due to inhibition of signaling pathways. Thus quercetin is an interesting lead compound for further pharmaceutical development,[7] and has been discussed in over 6000 journal publications. In spite of this, very little is known about the full range of proteins that it targets, and which targets are predominantly responsible for a particular biological effect.

Our interest in quercetin initially arose from its well known ability to inhibit the heat induction of heat shock protein 70 (HSP70), one of many proteins induced by heat,[11] that is, also known as HSP70-1a, HSP70-1 and HSP72.[12,13] One of us had previously found that HSP70 deficient mouse embryonic fibroblasts are more sensitive to radiation following heat treatment,[14] suggesting that agents that suppress heat induction of HSP70 might also function as radiosensitizers. Despite the classification as a heat responsive protein, HSP70 is also induced, along with other heat shock proteins, in response to wide range of chemical and physiological stresses, and is overexpressed in many cancers.[15–17] Thus small molecule inhibitors of HSP70 and its induction may have potential

Abbreviations: BSA, bovine serum albumin; CaMK2, Ca^{2+}/calmodulin-dependent protein kinase II; CK2, casein kinase II; COSY, correlation spectroscopy; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; HMBC, heteronuclear multiple bond coherence spectroscopy; HMQC, heteronuclear multiple quantum coherence spectroscopy; HRESI, high resolution electrospray ionization mass spectrometry; HSF1, heat shock factor 1; HSP, heat shock protein; IC_{50}, 50% inhibitory concentration; LC–MS/MS, combined high performance liquid chromatography tandem mass spectrometry; LRESI, low resolution electrospray ionization mass spectrometry; MES, morpholinoethanesulfonic acid; NMP, *N*-methyl-2-pyrrolidone; TFA, trifluoroacetic acid; TLC, thin layer chromatography; Tris, tris(hydroxymethyl)aminomethane; UVA, ultraviolet light of 290–320 nm.

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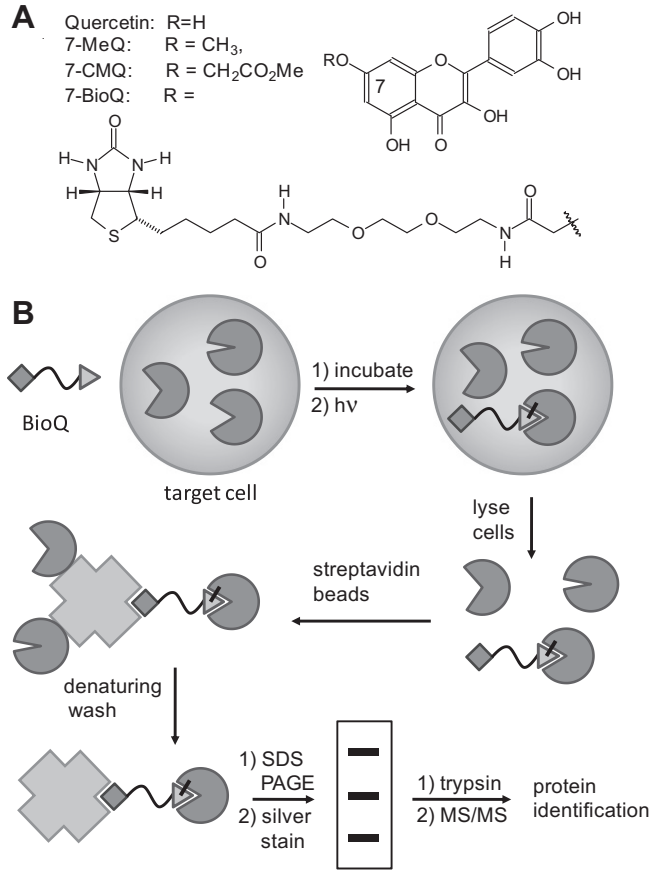

Figure 1. Strategy for pull down and identification of quercetin target proteins. (A) Structure of quercetin and derivatives. BioQ was designed for pulling down protein targets of quercetin. (B) Steps involved in pulling down and identifying quercetin target proteins with BioQ.

therapeutic value for treating cancer. Unfortunately, quercetin's multiple biological effects and the high concentration required to inhibit HSP70 induction diminish its potential as a therapeutic agent or adjuvant.

Identifying the protein target(s) responsible for quercetin's inhibition of HSP70 induction, however, could aid in the development of more selective agents. One systematic approach to identifying potential targets has involved screening chromatographic fractions of cells extracts for quenching of protein fluorescence in the presence of quercetin, followed by SDS–PAGE of the active fractions, and MASCOT analysis of the trypsinized fragments.[18] Unfortunately, without authentic protein samples, it is difficult to establish which of the protein bands detected in the SDS–PAGE gel are the quercetin binding proteins.

We decided to adopt a more promising approach for identifying protein targets of a bioactive compound that involves derivatizing the compound with biotin which serves as an affinity tag allowing the bound proteins to be isolated with streptavidin.[19,20] The isolated proteins are then separated and identified by bottom up mass spectrometry. There have been several reports in which compounds with K_d's of around 1 nM can be used to pull down target proteins in this manner from cell lysates.[21–23] To identify more weakly binding proteins, phage display libraries have been used in place of cell lysates to afford a higher concentration of protein,[24] although this approach is limited by the contents of the library. Another approach is to increase the affinity of the compound by attaching a photocrosslinking agent,[19,20,25] or by making use of intrinsic photoaffinity properties of the compound. In all cases, biotin must be attached to the compound at a position that does not interfere with target protein binding.

To guide the synthesis of biotinylated quercetin derivatives, we recently synthesized all of the mono-methyl and selected carbomethoxymethyl derivatives of quercetin (Fig. 1A) and determined their ability to inhibit heat shock induction of HSP70.[26] We found that the C7 and C3′ hydroxyls on quercetin can be derivatized with a bulky carbomethoxymethyl group without affecting its ability to inhibit HSP70 induction, and could therefore be used to attach biotin. We also found that all quercetin derivatives capable of inhibiting heat induction of HSP70, were also able to inhibit two protein kinases known to activate heat shock transcription factor 1 (HSF1), casein kinase II (CK2) and Ca^{2+}/calmodulin-dependent protein kinase II (CAMK2), suggesting that these may be targets of quercetin's action. The K_i's for these enzymes, and for most known enzyme targets of quercetin are in the micromolar range, indicating that a photoaffinity approach would be required to pull down the quercetin binding proteins. Quercetin is intrinsically photoactive,[27] however, and has previously been shown to photocrosslink malate dehydrogenase to which it binds,[28] suggesting that it could serve as its own photoaffinity reagent. Herein, we report the synthesis of the C7-biotinylated quercetin derivative, 7-BioQ (Fig. 1A), and its use for pulling down potential quercetin target proteins by the strategy shown in Figure 1B. We show that BioQ can be photocrosslinked to CK2 in vitro, and to various proteins in vivo following heat shock. Mass spectrometric analysis of trypsinized fragments of the pulled down proteins led to the identification of heat shock protein 70 and 90, two ATPases, an ubiquitin-activating protein, and a translation initiation factor as possible quercetin targets. One of the ATPases, mitochondrial ATP synthase, has already been shown to be a quercetin binding protein,[29] while the others remain to be validated. Interestingly, most of these proteins have been previously identified as potential therapeutic targets for cancer, which could potentially explain some of the anticancer effects of quercetin.

2. Chemistry

2.1. Synthesis of the biotin quercetin conjugate, BioQ

The selective coupling of biotin to the 7-OH of quercetin was carried out by two synthetic routes (Fig. 2) based on a previously developed method for the selective methylation and benzylation of the 7-OH.[30] The first route was to alkylate the 7-OH with *tert*-butylchloroacetate after which the ester would be converted to an acid and coupled to an amine derivative of biotin. Thus, quercetin penta-acetate was refluxed in anhydrous acetone with excess *tert*-butyl chloroacetate in the presence of potassium carbonate and catalytic potassium iodide with TLC monitoring to minimize over-alkylation. The *tert*-butyl ester **2** was converted to the acid **3** by treatment with 20% trifluoroacetic acid in methylene chloride under anhydrous conditions to limit competing hydrolysis of the remaining acetate protecting groups. Coupling of the amino biotin derivative **6**[31] with the tetra-acetate acid **3** was problematic. Both reactants are very polar and only dissolved well in dimethyl formamide or water–acetonitrile mixtures. When the reaction was carried out in the presence of *N*,*N*-diisopropylethylamine with HATU, DCC, or ByBOP, extensive decomposition of the quercetin tetra-acetate occurred, which may have been the result of deacetylation. Similar results were observed with DCC or EDC in dimethyl formamide in the absence of base, suggesting the possible involvement of the amino group of amino biotin **6** and hence the necessity of a buffered solution in neutral to acidic pH. Final treatment with EDC and *N*-hydroxysuccinamide at a slightly acidic pH of 6, in the presence of MES buffer, afforded the desired coupled product **5**

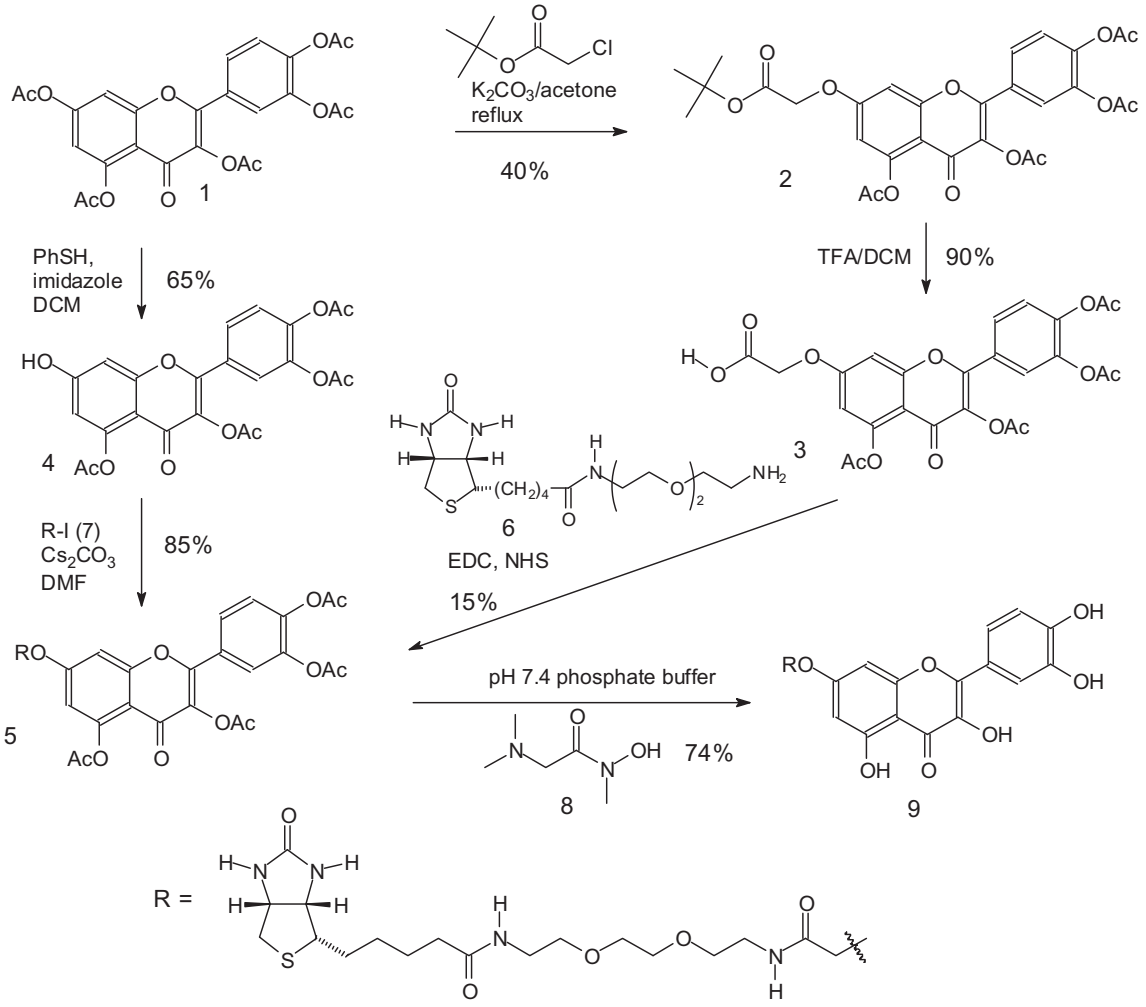

Figure 2. Two synthetic routes to BioQ.

in 10–15% yield. Attempts to improve the yield by further lowering the pH, however, failed to give desired product. Complete deacetylation was attempted under basic conditions, such as aqueous sodium hydroxide or sodium methoxide in methanol, but only afforded the desired biotinylated quercetin **9** in low yield. Biotinylated quercetin could be obtained, however, in 74% yield upon treatment with the hydroxamic acid **8** under near neutral conditions in pH 7.4 phosphate buffer.

Because direct coupling of the quercetin acid **3** with biotin amine **6** was so problematic we investigated whether we could directly alkylate the 7-OH of quercetin with the commercially available biotin alkylator, biotin PEO iodoacetamide **7**. In principle, one could simply use the method described above in which quercetin penta-acetate is alkylated selectively at O7 by refluxing with the alkylating agent and potassium carbonate in acetone. We were, however, concerned that the iodoacetamide **7** might self-react under these conditions, so we decided instead to alkylate quercetin tetra-acetate **4** under milder conditions. Quercetin tetra-acetate **4** has been previously prepared by treatment of the penta-acetate **1** with lipase,[32] or more recently, with imidazole and thiophenol in *N*-methyl-2-pyrrolidone (NMP).[33] We found that the latter method using dichloromethane in place of NMP afforded quercetin tetra-acetate **4** in 65% yield (Fig. 2). Treatment of **4** with the iodoacetamide **7** in anhydrous dimethyl formamide with cesium carbonate in the dark afforded the desired biotinylated quercetin tetra-acetate **5** in 85% yield. Attempts to use potassium carbonate, in place of cesium carbonate, failed to give the desired product. Deacetylation of compound **5** with *N*-methyl-2-dimethylamino-acetohydroxamic acid again afforded biotinylated quercetin **9**.

2.2. Structural characterization of BioQ 9

To verify that the biotin was indeed coupled to the hydroxyl at position 7 of quercetin, and had not been coupled to another hydroxyl that might have been produced by transesterification of the acetate groups, we characterized the product by COSY, HMQC, and HMBC NMR (Figs. 3, S1–S3). Aromatic protons H15 and 16 could be readily identified by their large *ortho*-coupling in the 1D ^{1}H NMR and a strong crossspeak B in the COSY spectrum (Fig. S1). This allowed identification of proton 12 through a crosspeak A with H16, leaving aromatic protons H6 and H8 which showed a crosspeak C between them. The assignment of protons H6 and H8 allowed identification of C7 in the HMBC spectrum through crosspeaks J and H (Fig. S3). C7 in turn showed a crosspeak F to the H17 protons of the acetamide group verifying the connection of the biotin derivative to O7. The assignment of the H17 protons was confirmed by a crosspeak E with C18 via HMBC, which in turn showed a correlation K with the amide NH (d). The biotin group could be easily identified by the crosspeak (D) between H19 and H20. The rest of structure could be confirmed by key proton–carbon crosspeaks in the HMBC spectrum, such as

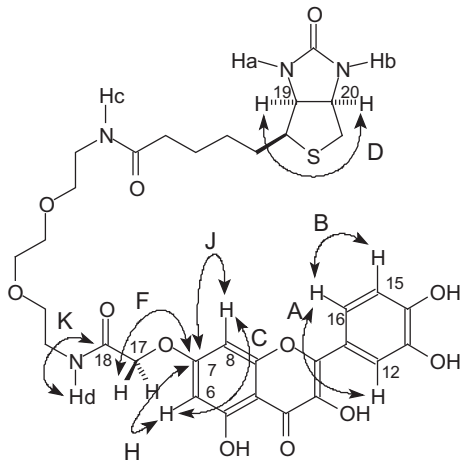

Figure 3. Structural characterization of BioQ by COSY, HMQC, and HMBC. Through bond correlations A, B, C, and D were observed in ^{1}H–^{1}H COSY, one bond ^{1}H–^{13}C correlations were made by HMQC, and multiple through bond correlations F, H, J, and K were by HMBC. See Figs. S1–S3 for the 2D spectra.

crosspeaks G (Hb–C21), Q (Ha–C21), I (H6–C5), L (H8–C9), M (H15–C14), N (H16–C11), O (H12–C11) and P (H12–C14).

3. Results

3.1. Substrate activities of BioQ

Previously, we had shown that quercetin and its 7-*O*-methyl and carbomethoxymethyl derivatives could inhibit CK2 and CAMK2, and that this inhibition was correlated to its ability to inhibit heat induction of HSP70.[26] To determine whether derivatization of quercetin at O7 with a much larger biotin-containing group would interfere with binding to these enzymes, the ability of BioQ to inhibit these enzymes was assayed by the same in vitro protein kinase inhibition assay we used previously for the simple quercetin derivatives (Table 1). The IC_{50}s of both quercetin and BioQ for CK2 were similar and about 5 μM, while the IC_{50} of BioQ for CAMK2 was about eightfold higher than for quercetin (26 μM compared to 3 μM).

To determine whether BioQ could inhibit heat induction of HSP70, Jurkat cells, which have a very low basal expression of HSP70, were heat shocked in the presence of 145 μM quercetin or BioQ, and assayed by western blotting.[26] BioQ showed substantial inhibition of HSP70 induction compared to the controls, though not as complete as quercetin (Fig. 4). The lower inhibition of HSP70 induction by BioQ is consistent with a higher in vitro IC_{50} for CAMK2 than observed for quercetin, and our previous conclusion that effective inhibitors had to be good inhibitors of both CK2 and CAMK2.[26] It may also be that BioQ is not as permeable as quercetin and unable to achieve a high enough concentration in the cell to be completely effective against CAMK2, for which BioQ has an in vitro IC_{50} of 26 μM compared to 3.0 for quercetin (Table 1). None-the-less, the ability of BioQ to cause substantial inhibition of heat shock induction of HSP70 validated its use as an affinity

Table 1
In vitro IC_{50} values for casein kinase II (CK2) and calmodulin-dependent kinase II (CAMK2) inhibition using the PKLight protein kinase assay

	Quercetin (μM)	BioQ (μM)
CK2	5.6 ± 1.2	3.2 ± 0.7
CAMK2	3.0 ± 0.6	25.9 ± 0.8

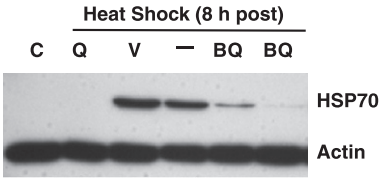

Figure 4. Ability of BioQ to inhibit heat shock induction of HSP70 in Jurkat cells. Jurkat cells were treated with 145 μM quercetin (Q), DMSO vehicle (V) or 145 μM BioQ (BQ) for 2 h prior to a 30 min 43 °C heat shock (HS) and then allowed to recover at 37 °C for 8 h before Western blot analysis of HSP70 and actin levels. Unheated Jurkat cells (C) and heated Jurkat cells in the absence of any additive (–) were used as controls. The results were reproducible.

probe for proteins involved in this and possibly other biological process. In this regard, other derivatives of the 7-hydroxy group of quercetin have been shown to retain antitumor activity.[34]

3.2. In vitro pull down of casein kinase 2 with BioQ

Our attention then focused on demonstrating that we could use streptavidin to pull down CK2 with the BioQ probe. Initial experiments indicated, however, that the streptavidin agarose beads would also non-specifically bind and pull down proteins when using the lysis buffer that would eventually be used for the in vivo pull down experiments. Despite many attempts, we were unsuccessful at finding a wash buffer that would remove non-specifically bound proteins from the streptavidin beads without also removing BioQ bound proteins.

Because quercetin has been reported to be photoreactive and capable of photocrosslinking to malate dehydrogenase,[28] we thought that UV irradiation could be used to photocrosslink BioQ to CK2 and other target proteins. Photocrosslinking would prevent CK2 or other target proteins from dissociating during affinity purification with the streptavidin and allow more stringent washing steps. Quercetin and BioQ show two absorption maxima at about 270 and 380 nm of about equal absorptivity (Fig. S4), the latter of which is photoactive and ideal for in vivo photocrosslinking experiments due to the lower toxicity of UVA light. Irradiation of quercetin and BioQ in 10 mM pH 7.2 PBS buffer with a medium pressure mercury arc lamp filtered with wood's glass which transmits light from 320–400 nm, with a peak intensity at 365 nm, led to significant irreversible bleaching of the longer wavelength absorption maximum within 30 min (Fig. S4). No bleaching was observed, however, in Tris buffer, possibly due to quenching by the buffer.[35]

In a model photocrosslinking study, an equimolar mixture of two readily available peptides containing 13 of the 20 amino acids, DRVYIHPFHL (angiotensin I) and RPKPQFFGLM (substance P), was irradiated with 4 mM of BioQ. Analysis by LC–MS/MS (Figs. S5 and S6) showed the formation of an adduct between angiotensin I and a photofragment of BioQ, demonstrating BioQ's ability to photocrosslink to a protein target. The molecular weight of the BioQ photofragment detected is consistent with the known photochemistry of quercetin.[27,28]

To verify the ability of BioQ to photocrosslink to a target protein, we incubated increasing concentrations of CK2 (from 1 μg to 4 μg) with BioQ in 400 μL 10 mM PBS buffer (pH 7.2) with and without irradiation for 30 min with Wood's glass filtered medium pressure mercury arc lamp. The mixture was then incubated with streptavidin beads, followed by centrifugation and washing of the beads to remove non-specifically bound protein. CK2 is a tetramer consisting of two 45 kDa α-subunits and two 25 kDa β-subunits which can be readily detected as two discreet bands on an SDS–PAGE gel. The SDS–PAGE analysis, however, did not detect any photocrosslinked CK2 by silver staining (Fig. 5a). The only bands

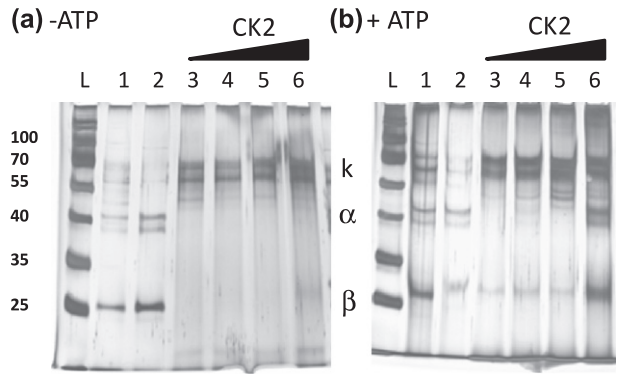

Figure 5. Casein Kinase II pull down by BioQ. Increasing concentrations of CK2 (lanes 3–6: 17.5, 35, 52.5, 70 nM) in 400 μL, 10 mM PBS buffer (pH 7.2), were incubated with 150 μM BioQ and then irradiated at 365 nm for 30 min in the (a) absence or (b) presence of 10 mM $MgCl_2$ and 2 μM ATP. The biotinylated proteins were then pulled down by streptavidin beads and subjected to a denaturing wash prior to electrophoresis. L is a Fermentas prestained protein ladder. Lane 1 in panel (a) and lane 2 in panel (b) were controls that contained 0.05 μg CK2, while lane 2 in panel (a) and lane 1 in panel (b) contained 0.1 μg of CK2. α and β refer to the positions of the α and β subunits of CK2, and k refers to the position of contaminating keratin proteins.

detected corresponded to contaminating keratin proteins in the 45–70 kDa range.

As casein kinase II is an ATP dependent kinase, that is, known to autophosphorylate,[36] it was possible that autophosphorylation was required for CK2 to bind to quercetin. Indeed, we found that ATP was consumed by CK2 in the presence of quercetin and the absence of substrate (Fig. S7). We therefore incubated CK2 and BioQ in the presence of 2 μM ATP in PBS buffer for 30 min, after which the samples were irradiated for 30 min prior to analysis by SDS–PAGE. After much experimentation, we found that a 2% SDS Tris–HCl buffer was sufficient to remove non-specifically bound proteins from the streptavidin agarose beads, without denaturing the streptavidin and causing the release of the biotinylated CK2. Once the concentration of CK2 reached 4 μg/400 μL (70 nM) a significant amount of both α and β subunits could be detected by SDS–PAGE (Fig. 5b, lane 6). The identities of these proteins were confirmed by trypsinization followed by LC–MS/MS and a MASCOT search (Table 2).

3.3. In vivo pull down of proteins from Jurkat cells with BioQ

Having verified that BioQ could be used to pull down a known quercetin binding protein, we carried out pull down experiments with cells that had been incubated with BioQ. In one set of experiments the photocrosslinking step was carried out after cell lysis (Fig. 6) and in a second set of experiments it was carried out before cell lysis (Fig. 7). As can be seen from lanes 1–4 of the 12% SDS–PAGE gel shown in Figure 6, a large number of proteins were pulled down by the streptavidin beads in the absence of the denaturing wash, whether or not the cells were incubated with BioQ and/or heat shocked. With the exception of contaminating keratin proteins, the high protein background was reduced to almost nothing with the use of the denaturing wash as shown in lane 7 for cells that were incubated with BioQ and heat shocked. When cell lysates containing BioQ were UV irradiated for 30 min prior to pull down and followed with the denaturing wash, discrete new bands A–F were observed (lanes 5 & 6). Similar results were obtained when the cells were irradiated prior to lysis (Fig. 7).

To confirm that the proteins appearing in the gel had been photo-modified with BioQ, a western blot with a biotin-specific antibody was carried out (Fig. 6, lanes 8–10, Fig. 7, lanes 9–14). Samples for lanes 8–10 of Figure 6 were identically prepared from heat shocked cells, except that the sample for lane 8 was not UV irradiated and the sample for lane 10 lacked BioQ. Only the samples that contained BioQ and had been irradiated (lane 9) showed bands with the anti-biotin antibody that matched the major bands observed in the corresponding lane of the silver stained gel (lane 6). The same was true for the experiment in which the cells had been irradiated prior to lysis (compare lanes 8 and 12 of Fig. 7).

3.4. Mass spectrometric identification of proteins pulled down from the Jurkat cells

Proteins contained in discrete bands A to F of lane 8 of the gel in Figure 7 that were confirmed to be biotinylated (lane 12) were trypsinized and analyzed by LC–MS/MS. Corresponding sections of lane 6 which had not been subjected to irradiation were similarly analyzed so that contaminating proteins such as keratins and albumins could be excluded from consideration. MASCOT search[37] of the NCBI database led to the identification of the proteins shown in Table 2 that did not appear in the control lane and that had high MASCOT scores (>100) and the expected molecular weight. In the case of

Table 2
LC–MS/MS identification of proteins pulled down with BioQ using MASCOT[a]

Band	App. MW (kD)	Name	Gene ID	GI number	MW (Da)	MASCOT score[b]
Figure 5						
α	45/40	Casein kinase II alpha subunit (CSNK2A1P)	283106	21464270	45,261	369
		Casein kinase II alpha isoform (CSNK2A1)	1457	86156152	45,423	186
β	25	Casein kinase II beta subunit (CSNK2B)	1460	181155	25,242	188
Figure 7						
A	110	Ubiquitin-activating enzyme E1 (UBA1)	7317	23510338	118,858	205
		Spliceosomal protein SAP 130 (SF3B3)	23450	6006515	136,590	252
B	100	Heat shock protein HSP 90-alpha 2 (HSP90AA1)	3320	61656603	98,622	663
C	70	Heat shock protein 70-2 (HSPA2)	3306	23271312	70,237	524
D	50	RuvB-like 2 helicase ATPase (RUVBL2)	10856	5730023	51,026	450
		Mitochondrial ATP synthase beta subunit ATPase (ATP5B)	506	32189394	56,525	447
		Mitochondrial ATP synthase alpha subunit precursor (ATP5A1)	498	4757810	59,828	223
E	40	Eukaryotic translation initiation factor 3, subunit 5 epsilon (EIF3F)	8665	4503519	37,654	179
		Mutant β actin (ACTB)[c]	60	28336	42,128	657

[a] The target proteins were identified by comparison of the proteins detected in an unirradiated control sample band with an irradiated sample band so that contaminating keratins and other proteins were excluded from consideration. Proteins were also excluded if they did not match the observed molecular weight range, or had MASCOT scores less than 100.

[b] The MASCOT score corresponds to $-10 * \log(P)$ where P is the probability that the match to the database is wrong.

[c] Beta actin was also observed in the control lane, but because it is a known quercetin binding protein, it is included. Though MASCOT matched the protein to a mutant or beta' actin sequence, all the peptides identified also match the wild type sequence (GI: 46397333), except for one peptide that does not completely match either the wild type or mutant sequence.

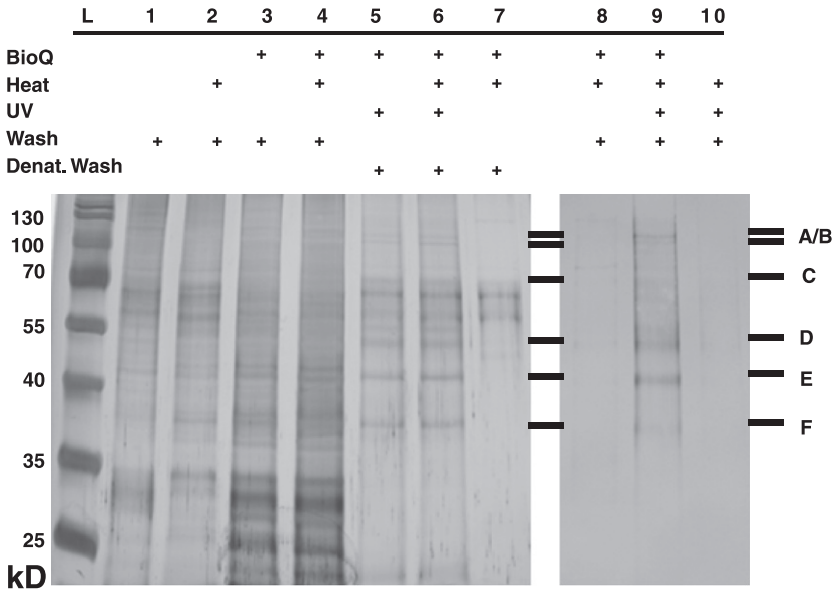

Figure 6. SDS–PAGE of proteins pulled down from Jurkat cells incubated with BioQ that were UV irradiated following lysis. Left: Silver stained 12% SDS–polyacrylamide electrophoresis gel of proteins. Lanes 1 and 2: proteins pulled down from lysates of normal or heat shocked Jurkat cells by the streptavidin beads in the absence of BioQ and UV irradiation. Lanes 3 and 4: proteins pulled down from lysates of normal or heat shocked cells that had been incubated with 150 μM BioQ in the absence of UV irradiation. Lanes 5 and 6: proteins pulled down from lysates of normal or heat shocked cells that had been incubated with BioQ and UV irradiated for 30 min following lysis, and then subjected to a denaturing wash. Lane 7: proteins pulled down under the same conditions as for lane 6, except that the lysate was not UV irradiated. Right: Western blot of corresponding lanes with anti-biotin antibody. Legend: heat, heat shock applied after incubation with BioQ but before lysis; wash, washing beads with cell lysis buffer; denaturing wash, washing beads with 2% SDS buffer.

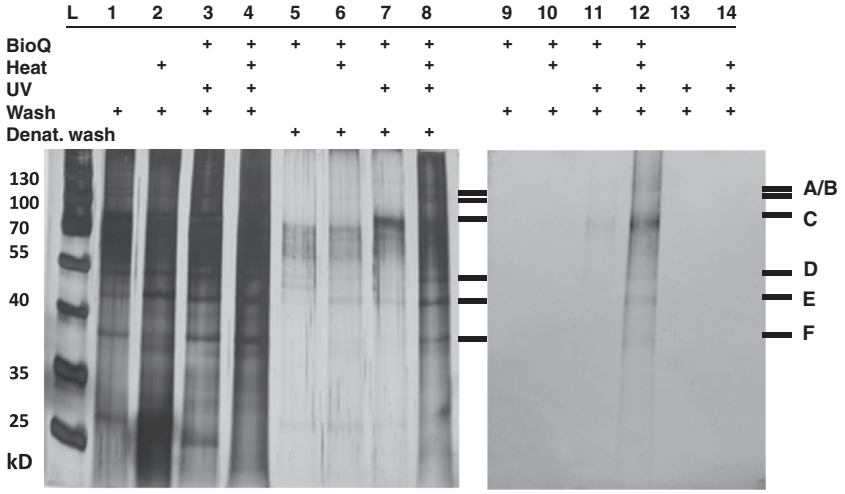

Figure 7. SDS–PAGE of proteins pulled down from Jurkat cells incubated with BioQ that were irradiated prior to lysis. Left: Proteins pulled down with streptavidin beads from Jurkat cells under similar sets of conditions as in the gel in Figure 6. Right: Western blot with anti-biotin antibody. Legend the same as in Figure 6.

band A, two proteins, ubiquitin-activating enzyme E1 and spliceosomal protein SAP 130, were identified as potential quercetin target proteins. For band B, heat shock protein 90 was identified and for band C, heat shock protein 70 was identified. Three proteins were detected in band D, RuvB-like 2 and both alpha and beta subunits of mitochondrial ATP synthase. Band E contained a protein that matched eukaryotic translation initiation factor 3. Band E also contained beta actin, which may also have been pulled down by BioQ despite the fact that it was present in the control lane, because it is also a known target for quercetin.[18,38]

4. Discussion

The strategy for coupling photoaffinity and affinity purification agents to a biologically active ligand to facilitate isolation of a target protein is a powerful approach to identifying potential therapeutic targets.[19,20,25] The general idea appears to have been first described over 20 years ago for the isolation of ACTH receptors,[39] and successfully implemented a few years later for isolation of a melanocyte-stimulating hormone receptor.[40] In both cases, biotin had been selected as the affinity purification agent because of its high affinity for streptavidin, which in principal would enable facile separation of a protein bearing a biotinylated ligand from non-target proteins. In practice, however, it is difficult to find conditions that reduce the non-specific binding of the proteins to the streptavidin without dissociating the biotinylated ligand protein complex, as we found this to be the case for BioQ. This led to the idea of using a photoaffinity crosslinker to covalently link the biotinylated ligand to the target protein, and permit the use of more stringent wash conditions.

In our case, quercetin is known to have intrinsic photoreactivity, and is able to crosslink to malate dehydrogenase,[28] thereby only

necessitating the attachment of biotin to create a pull down probe. We had already determined that the O7 position of quercetin did not interfere with the ability of quercetin to inhibit HSP70 induction in vivo, or to inhibit CK2 and CAMK2, two of the presumed target proteins, and so this site was chosen for biotinylation.[26] As we had found for a simpler derivative, quercetin biotinylated at the O7 position retained its ability to penetrate cells and inhibit heat shock induction of HSP70 (Fig. 4) as well as to inhibit CK2 and CAMK2 (Table 1). Initial attempts to demonstrate photocrosslinking of BioQ to malate dehydrogenase, or to BSA which quercetin is also known to bind,[41] failed. It is possible that derivatization of the O7 position interfered with binding to these proteins, or that there were no crosslinkable protein side chains at the binding site. We did confirm, however, that the BioQ was photoreactive, and could be photobleached by irradiation with a UVA light source (Fig. S4).

In a very limited study of two commercially available peptides used as standards for mass spectrometry, we found that irradiation of one of them (angiotensin I) with BioQ resulted in the formation of an adduct between a fragment of BioQ and the peptide (Figs. S5 and S6). The site or nature of the crosslink could not, however, be rigorously established at this time. One can tentatively rule out Arg, Pro, Lys, Gln, Phe, Gly, Leu, and Met as crosslinkable side chains as no reaction was observed with substance P, suggesting that either Asp or Tyr or His was involved in the crosslinking reaction of angiotensin I.

We therefore turned to CK2 kinase as a model target protein, which we had shown was inhibited by BioQ. Initial photocrosslinking experiments with CK2 and BioQ in the absence of ATP and substrate, however, did not result in any detectable photocrosslinking. When ATP was added to the reaction, photocrosslinking took place, suggesting that CK2, which is known to autophosphorylate,[36] must be in its phosphorylated state to bind quercetin. Further experiments established that ATP was consumed by CK2 in the presence of quercetin and in the absence of substrate (Fig. S7). In contrast to many other enzymes that are inhibited by quercetin binding to an ATP binding pocket, CK2 may be inhibited by another mechanism. One possibility is that quercetin is binding to the allosteric cleft between α/β surface of CK2,[42] where the interaction of α, β subunits is believed crucial to the function of CK2 kinase. In accord with this idea, BioQ pulled down both subunits, though more efficiently for the β subunit (Fig. 5).

BioQ was also examined for its ability to specifically pull down target proteins from normal and heat shocked Jurkat cells as shown in Figures 6 and 7. We found that discrete, biotin-containing protein bands could be obtained by using a photocrosslinking step in combination with a denaturing wash step. Protein identification was only carried out on the heat shocked cells that were irradiated prior to lysis (lane 8, Fig. 7), in order to avoid artifacts that may have resulted as a consequence of lysing the cell. LC–MS/MS analysis of trypsinized protein bands from lane 8 in comparison to the control lane 6 led to the identification of a number of potential and known target proteins of quercetin shown in Table 2. Five of these proteins have known or putative ATP binding domains (UBA1, HSP90AA1, HSPA2, RUVBL2 and ATP5B/ATP5A1), which is in accord with quercetin's known ability to inhibit ATPases and kinases.[10] The remaining proteins, SF3B3 and EIF3F are not associated with nucleotide binding domains, and may bind to quercetin through some other site.

Of the proteins that were pulled down by BioQ, only mitochondrial ATP synthase has been previously shown to be a target of quercetin, which binds between the α, β and γ subunits and thereby inhibits ATPase activity.[29] ATP synthase has been found to be upregulated in breast cancer, and the ATP synthase inhibitor aurovertin B is being investigated as a therapeutic agent.[43] RuvB-like 2, is an ATPase as well as a putative helicase that has been found to be overexpressed in hepatocellular carcinoma and required for cell viability.[44] Beta actin, is also a known target of quercetin,[18,38] and though it was identified in Band E, we cannot unambiguously conclude that it was pulled down by BioQ because it was also present in the control lane.

Both splicesomal protein (SAP130) and eukaryotic translation initiation factor 3 were also identified as potential targets and are known to be important in translation or post-translation protein synthesis steps.[45,46] As such, these proteins may play a role in the expression of inducible heat shock proteins 70 and 90,[47] which quercetin is known to inhibit.[48] The spliceosome is a known target of some anticancer compounds,[49] while the eukaryotic initiation factor has been found to be upregulated in breast and prostate cancer, and thus represents a new therapeutic target.[45,50] The identification of HSP70-2 and HSP90 as possible quercetin targets could also explain the decreasing level of these proteins in quercetin treated cells,[51,52] which might result from the deactivation of these proteins and their subsequent degradation or aggregation in the cell. Both HSP70 and HSP90 proteins are of current interest as anticancer targets.[53–56]

Another potential target of quercetin that we identified was the ubiquitin-activating enzyme E1 which initiates protein degradation through the E1–E2–E3 mediated proteosome pathway.[57] Binding to this enzyme could explain the observation that quercetin inhibits the ubiquitin-proteosome pathway for degradation of unfolded proteins.[58] Currently, ubiquitin-activating enzyme E1 is being investigated as a therapeutic target for treatment of leukemia and multiple myeloma.[59] Inhibition of the proteosome pathway, together with the heat shock protein pathway for repairing unfolded proteins could induce cell apoptosis, which may be one of the mechanisms behind the antitumor activity of quercetin.

5. Conclusion

We have shown that quercetin derivatized with biotin at the 7-hydroxyl position, BioQ, can be used as a photoaffinity agent to pull down possible quercetin target proteins from cells which can then be identified by bottom up mass spectrometry. Many of the proteins identified as possible targets of quercetin have also been found to be potential therapeutic targets for cancer, and all might play a role in the apoptotic and antitumor activities of quercetin. It is conceivable that the anticancer activity of quercetin is due to simultaneous but low level inhibition of multiple therapeutic targets, which might explain how a molecule with low specificity and toxicity can achieve a positive therapeutic effect.

The O7 hydroxyl site for attaching biotin to quercetin was originally chosen because it did not substantially interfere with quercetin's ability to inhibit heat induced HSP70 induction or the inhibition of CK2 and CAMK2 kinase activities, which are thought to be involved in heat shock transcription factor activation. Surprisingly, neither of these kinases was detected in the assay, possibly because they were only present at much lower levels than the other proteins. In this case, screening against a phage display library of proteins[24] with a more uniform protein distribution might enable one to identify quercetin binding proteins that are at too low a level to be detected by BioQ in vivo. A phage display library might also be able to provide the amount of protein needed for more detailed analysis of the protein binding sites by mass spectrometric analysis.

Though we have identified a number of possible quercetin targets, further work will be needed to confirm them as actual targets, with the exception of mitochondrial ATP synthase,[29] and beta actin[18,38] which have already been shown to be targets of quercetin. It is also likely that a number of targets were not pulled down by the BioQ probe, either because the position of the biotin interfered

with binding, or because of an inefficient photocrosslinking reaction. The mechanism and amino acid specificity of the photocrosslinking reaction will also need to be further investigated. To screen for all the possible targets of quercetin, it might be necessary to attach biotin along with an extrinsic photocrosslinking agent to other positions of quercetin. In any case, our approach appears to be a fruitful one for beginning to unravel the biomolecular basis for the medicinal effects of a ubiquitous natural product in our diet.

6. Experimental section

6.1. General

Anhydrous dimethyl formamide and acetone were from EMD Chemicals Inc. Anhydrous dichloromethane was freshly distilled from calcium hydride. All other reagents were directly purchased from Omni Solvent or Sigma Aldrich unless otherwise specified. Analytical thin layer chromatography was performed on Aldrich Silica gel 60 F_{254} plates (0.25 mm) and visualized by UVG-54 mineral light UV lamp at 254 nm. Flash column chromatography was conducted using E. Merck silica gel 60 (40–63 μm). ^{1}H NMR and ^{13}C NMR spectra were carried out on Varian Mercury-300 MHz, 500 MHz or 600 MHz spectrometers. UV–vis spectra were obtained on a Varian Cary 100 Bio spectrophotometer. High capacity streptavidin agarose resin, silver SNAP stain kit, and avidin-conjugated horseradish peroxidase were from Pierce Thermo Fisher Scientific Inc., and a Western blot kit was from Bio-Rad Laboratories. ECL staining reagents as well as mouse anti-actin antibody and horseradish peroxidase conjugated anti-mouse secondary antibody were from GE Healthcare Systems. Silver staining was carried out by a standard protocol[60] or with the Pierce Silver Stain Kit (Pierce). Protease inhibitor cocktail tablets were from Roche Pharmaceuticals.

6.2. 7-*O*-Carbo-*tert*-butoxymethyl-quercetin tetra-acetate 2

Anhydrous potassium carbonate (2.0 g, 15 mmol) and potassium iodide (0.166 g, 1 mmol) were added to a solution of quercetin penta-acetate **1** (1.0 g, 1.95 mmol)[61] and *tert*-butyl chloroacetate (3.0 g, 19.5 mmol) in 50 mL of anhydrous acetone . The suspension was refluxed for 2 h at which point TLC indicated the reaction was near completion. The reaction mixture was then filtered to give a yellowish clear solution, concentrated in a rotatory evaporator, and the residue purified by flash column chromatography (ethyl acetate/hexane 4:3). The resulting solid was recrystallized with acetone/hexane to give **2** as a white solid in 65 % yield (0.66 g, 1.27 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.70 (dd, J = 8.0, 2.0 Hz, 1H), 7.66 (d, J = 2.0 Hz, 1H), 7.34 (d, J = 8.0 Hz, 1H), 6.80 (d, J = 2.0 Hz, 1H), 6.67 (d, J = 2.0 Hz, 1H), 4.60 (s, 1H), 2.40 (s, 3H), 2.32 (s, 9H), 1.49 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 169.7, 168.3, 168.2, 168.1, 166.8 (6 C=O), 162.3, 158.2, 153.6, 151.2, 144.5, 142.4, 134.2, 128.3, 126.7, 124.2, 124.0, 112.0, 109.2, 100.1, 83.6 ($C(CH_3)_3$), 66.2 (OCH_2–COO), 28.3 (3 CH3), 21.4(CH_3CO), 21.0 (2CH_3CO), 20.8 (CH_3CO). MS ($C_{29}H_{28}O_{13}$), LRESI: [M+H$^+$] 585.2, [M+Na$^+$] 607.1, [M+K$^+$] 623.1, [M+H$^+$−CH_2CO] 543.1. HRESI: [M+H$^+$] 585.1606, calcd 585.1603.

6.3. 7-*O*-Carboxymethyl quercetin tetra-acetate 3

To 200 mg of **2** (0.38 mmol) in 4 mL anhydrous dichloromethane under nitrogen was slowly added 1 mL anhydrous TFA. After stirring for 3 h at room temperature the solution was concentrated by rotatory evaporation under vacuum. The residue was dissolved in chloroform, washed with water and then brine until the aqueous layer became neutral. The organic phase was concentrated in a rotary evaporator and purified by flash column chromatography (ethyl acetate/hexane/acetic acid 4.5:4.5:1) to afford **3** as a pale white solid in 72% yield (142 mg, 0.27 mmol). ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ 7.90 (dd, J = 8.0, 2.0 Hz, 1H), 7.86 (d, J = 2.0 Hz, 1H), 7.49 (d, J = 8.0 Hz, 1H), 7.20 (d, J = 2.0 Hz, 1H), 6.82 (d, J = 2.0 Hz, 1H), 4.95 (s, 2H), 2.34–2.30 (m, 12H). ^{13}C NMR (151 MHz, $(CD_3)_2CO$) δ 169.9, 169.0, 168.4, 168.1, 168.0, 168.0 (6 C=O), 163.1, 158.5, 153.6, 151.4, 145.3, 143.2, 134.3, 128.5, 126.9, 124.8, 124.3, 111.7, 109.6, 100.5, 65.7 (OCH_2–COO), 20.7, 20.2, 20.1, 20.0 (4 CH_3CO). MS ($C_{25}H_{20}O_{13}$), LRESI: [M+Na$^+$] 551.1, HRESI: [M+Na$^+$] 551.0820, calcd 551.0802.

6.4. Quercetin-3,5,3′,4′-tetra-acetate 4

Imidazole (93.2 mg, 1.37 mmol) and 181 mg thiophenol (1.2 equiv) were added to a solution of quercetin penta-acetate (700 mg, 1.37 mmol) in anhydrous dichloromethane at 0 °C. After 1 h, TLC indicated that the reaction was near completion, and the reaction was diluted with dichloromethane, and concentrated to dryness under vacuum in a rotatory evaporator. The resulting residue was dissolved in ethyl acetate washed with 1 N HCl and then brine and concentrated to dryness. Flash column chromatography of the residue (ethyl acetate/hexane: 4:3) afforded a yellow oil from which **4** was obtained as a pale white solid in 65% yield (418 mg, 0.89 mmol) following treatment with methanol. Compound **4** gave spectroscopic data in accord with previously published data.[33]

6.5. BioQ tetra-acetate 5

6.5.1. Method I

Compound **3** (20 mg, 0.038 mmol) was first incubated with EDC (9.6 mg, 0.05 mmol) and *N*-hydroxysuccinimide (5.7 mg, 0.05 mmol) in 50% MES buffer/acetonitrile (0.1 M, pH 5.5) for 20 min. Compound **6** (20 mg, 0.053 mmol)[31] was then added and stirred under nitrogen for 12 h at room temperature after which it was concentrated to dryness under vacuum. The resulting residue was added to 50 mL of ethyl acetate and washed with saturated ammonium chloride and brine three times and then concentrated under vacuum. Purification by step gradient flash chromatography (5–8% methanol in dichloromethane) afforded **5** in 15% yield (5 mg, 0.006 mmol).

6.5.2. Method II

Compound **4** (20 mg, 0.042 mmol) and compound **7** (30 mg, 0.055 mmol)[31] were dissolved in 5 mL of anhydrous dimethyl formamide under a nitrogen atmosphere in the presence of 4 Å molecular sieves. Cesium carbonate (17 mg, 0.05 mmol) was then added at 0 °C and the solution was stirred at room temperature for 1.5 h during which time the flask was wrapped with aluminum foil to protect the reactants from light. Hydrochloric acid (1 M) was then added to a pH of about 6 to stop the reaction. The solution was concentrated under vacuum and the resulting residue was purified by flash column chromatography (5% methanol in dichloromethane) to afford compound **5** in 87% yield (33 mg, 0.037 mmol). ^{1}H NMR ($CDCl_3$ 600 MHz) δ 7.73 (dd, J = 8.0, 2.0 Hz, 1H), 7.70 (d, J = 2.0 Hz, 1H), 7.36 (d, J = 8.0 Hz, 1H), 7.07 (s, H–N in ethylene glycol, 1H), 6.90 (d, J = 2.0 Hz, 1H), 6.72 (d, J = 2.0 Hz, 1H), 6.45 (NH in biotin, 1H), 6.31 (NH in biotin, 1H), 4.64 (s, CH_2, 2H), 4.51 (m, 1H), 4.31 (m, 1H), 3.62–3.39 (m, 12H), 3.15 (m, 1H), 2.88 (dd, 12.0 Hz, 4.2 Hz, 1H), 2.68 (d, 13.8 Hz, 1H), 2.47–2.33 (m, 12H, 4 CH_3CO), 2.21 (m, 2H), 2.09 (acetic acid), 1.67–1.61 (m, 4H), 1.43–1.41 (m, 2H). ^{13}C NMR (151 MHz DMSO-d_6) δ 174.7, 173.6, 169.9, 169.7, 168.1, 167.9, 167.8, 167.2 (8 C=O), 164.5, 161.2, 157.9, 153.5, 151.0, 144.4, 142.3, 133.9, 127.8, 126.4, 124.0, 123.8, 112.0, 109.2, 100.0, 70.2, 70.1, 69.6, 69.5, 67.7, 62.0, 60.6, 55.0, 40.3, 39.2, 39.0, 35.6, 27.7, 25.2, 21.1, 20.7, 20.7, 20.6 (4 CH_3). MS

($C_{41}H_{48}O_{16}N_4S$) LRESI: [M+H$^+$] 885.3, [M+Na$^+$] 907.3, [M−CH_2CO+H$^+$] 843.3; HRESI: [M+H$^+$] 885.2827, calcd 885.2786, [M+Na$^+$] 907.2618, calcd 907.2686.

6.6. BioQ 9

A solution of compound **5** (30 mg, 0.034 mmol) in 10 mL tetrahydrofuran/phosphate buffer/methanol (4.5:4.5:1, pH 7.4) was treated with *N*-methyl-2-dimethylamino-acetohydroxamic acid (4.5 equiv, 20 mg, 0.15 mmol) under a nitrogen atmosphere for 12 h. The solution was then acidified with hydrochloric acid (1 M) to pH 5, concentrated under vacuum, extracted with 50 mL ethyl acetate, and washed with saturated ammonium chloride. The residue from the organic phase was purified by high performance liquid chromatography on a Waters Xterra Prep MS C18 column (10 μm, 7.8 × 300 mm) with a 30 min 0–50% and 10 min 50–100% linear gradient of solvent B (1% acetic acid in acetonitrile) in solvent A (1% acetic acid in water) to afford compound **9** eluting at 32 min in 74% yield (18 mg, 0.025 mmol). The purity was determined by analytical HPLC to be 98.3% when detected at 280 nm, 97.6% at 300 nm, and 100% at 254 nm. ^{1}H NMR (DMSO-d_6 300 MHz) δ 8.20 (s, N–H), 7.82 (s, N–H), 7.72 (d, J = 2.0 Hz, 1H), 7.56 (dd, J = 8.0 Hz, 2.0 Hz, 1H), 6.89 (d, J = 8.0 Hz, 1H), 6.70 (d, J = 2.0 Hz, 1H), 6.41 (d, J = 2.0 Hz, 1H), 6.40 (NH in biotin, 1H), 6.36 (NH in biotin, 1H), 4.63 (s, CH_2, 2H), 4.29 (m, 1H), 4.11 (m, 1H), 3.49–3.07 (m, 13H), 2.80 (dd, 12.0 Hz, 4.2 Hz, 1H), 2.58 (d, 13.8 Hz, 1H), 2.05 (m, 2H), 1.91 (s, acetic acid), 1.58–1.04 (m, 6H). ^{13}C NMR (151 MHz DMSO-d_6) δ 176.4, 172.5, 167.4, 163.5 (4 C═O), 163.1, 160.7, 156.2, 148.3, 147.8, 145.5, 136.5, 122.2, 120.4, 116.0, 115.6, 104.8, 98.4, 93.1, 69.9 (2C overlap), 69.6, 69.2, 67.6 (2C overlap), 61.4, 59.6, 55.8, 38.8, 38.7, 35.5, 28.6, 28.4, 25.6. MS ($C_{33}H_{40}O_{12}N_4S$) LRESI: [M+H$^+$] 717.2, [M+Na$^+$] 739.3, [M+K$^+$] 755.2. HRESI: [M+H$^+$] 717.2442, calcd 717.2363; [M+Na$^+$] 739.2256, calcd 739.2263; [M+K$^+$] 755.1984, calcd 755.3343.

6.7. 2D NMR Spectroscopy of BioQ 9

^{1}H and ^{13}C NMR spectra were recorded on Mercury-300, Inova-500 and Inova-600 (Varian Assoc., CA) NMR instruments while 2D NMR spectra were recorded on an Inova-600 spectrometer. All the data were processed with VNMR series software (Varian Assoc., CA) with standard functions and the chemical shifts were referenced to TMS. Proton spectra were collected with an 8000 Hz spectral width and 32 K data points while carbon spectra were collected with a 30000 Hz spectral width and 64 K data points. The COSY experiments were obtained with 512 real points in the F1 dimension and 2000 complex points in the F2 dimension. HMBC (heteronuclear multiple bond correlation) spectra were collected with 10.2 μs 90° ^{1}H pulse width and a 14.0 μs 90° ^{13}C pulse width. HMQC (heteronuclear multiple quantum correlation) spectra were recorded using a 0.3 s ^{1}H–^{13}C nulling period.

6.8. Kinase inhibition and autophosphorylation assays

The kinase inhibition and autophosphorylation assays were carried out with the PKLight assay kit for ATP (Lonza Rockland, Rockland ME), following a previously reported procedure.[26] To test for kinase inhibition of CK2, CK2 was incubated with 300 μM peptide substrate (RRRADDSDDDDD), 100 μM ATP in 20 mM Tris–HCl, 50 mM KCl, and 10 mM $MgCl_2$ at 30 °C for 30 min, with increasing concentrations of BioQ. To test for kinase inhibition of CAMK2, preactivated CAMK2 was mixed with increasing concentrations of BioQ, 300 μM autocamtide-2 (KKALRRQETVDAL), 100 μM ATP in pH 7.5 50 mM Tris–HCl, 10 mM $MgCl_2$, 2 mM DTT, and 0.1 mM Na_2EDTA at 30 °C for 30 min. Autophosphorylation of CK2 (0.035, 0.07 and 0.35 μM) was assayed in 1 μM ATP, 10 μM quercetin, 20 mM Tris–HCl, 50 mM KCl, 10 mM $MgCl_2$ at 30 °C for 10 and 40 min, with or without 300 μM peptide substrate (RRRADDSDDDDD).

6.9. Assay for HSP70 heat induction inhibition in Jurkat cells

Assays were carried out as previously described for other quercetin derivatives.[26]

6.10. Model peptide photocrosslinking study

Angiotensin I peptide (NRVYIHPFHL, 100 μM) and Substance P (RPKPQFFGLM, 100 μM) were mixed with 4 mM BioQ in 200 μL of 10 mM PBS buffer, pH 7.2, and irradiated. The protein solution was then irradiated for 30 min in a glass tube that was immersed in ice 10 cm away from a 450 W UV medium pressure mercury arc lamp in a water cooled immersion apparatus that was shielded with Wood's glass which transmits ultraviolet between 320 and 400 nm with a peak transmittance at 365 nm. After irradiation the sample was diluted 50–100 fold and subjected to LC–MS/MS analysis.

6.11. Casein kinase II pull down assay

The indicated concentrations of casein kinase II and150 μM BioQ in 400 μL of 0.01 M PBS buffer/protease cocktail (pH 7.2) or 0.01 M PBS buffer (pH 7.2)/protease cocktail containing 2 μM ATP/Mg^{2+} were photocrosslinked for 30 min as described in procedure 6.11 and then immediately incubated with 20 μL of streptavidin agarose beads for 1 h during which time the color of the beads changed from white to yellow due to binding of BioQ or BioQ photoreaction products. The beads were then centrifuged at 14000 rpm for 5 min and the supernatant removed. The beads were then washed with lysis buffer (10 mM phosphate buffer, pH 7.2, 100 mM NaCl, 0.2% Triton X-100, and protease cocktail) or denaturing buffer (20 mM Tris·HCl, 2% SDS, pH 7.4) and shaken well for 3 min before each spin-down. The washing procedure was repeated at least three times. The beads were then resuspended in 20 μL of 2× SDS–PAGE gel loading buffer (50 mM Tris·HCl, 100 mM dithiothreitol, 8 M urea, 2% SDS, 10% glycerol) and boiled for 10 min to denature the streptavidin and release the biotin conjugates. Samples were loaded onto an SDS polyacrylamide gel and electrophoresed at 125 V for about one hour at which point the 10 kD protein marker would reach the bottom of the gel. The SDS polyacrylamide gel was then incubated overnight in a 100 mL solution of ethanol/glacial acetic acid/water (30:10:60) with gentle shaking to fix the proteins. After washing with deionized water, the gel was silver stained either by a published procedure,[60] or using the Pierce Silver Stain Kit (Pierce).

6.12. Pull down assay from Jurkat cells

Jurkat cells were exponentially grown in RPMI media with 10% fetal bovine serum, until about 15 × 10^6 cells were obtained, after which the cells were harvested and treated with 3% aqueous DMSO or 150 μM BioQ in 3% aqueous DMSO for 2 h. Then the cells were subjected a 43 °C heat shock for 30 min and allowed to recover at 37 °C for 1 h when the level of activated heat shock factor in cells typically reaches a maximum. The Jurkat cells were then cooled to 0 °C while being transported to the location of the UV lamp (10 min), where they were irradiated at 4 °C as described in procedure 6.11 immediately before or after cell lysis. The Jurkat cells were lysed by sonication in a cold room in 2 mL of cell lysis buffer (10 mM phosphate buffer, pH 7.2, 100 mM NaCl, 0.2% Triton X-100, and protease inhibitors) and centrifuged at 5000 rpm for 10 min.

The supernatant was then incubated with the streptavidin beads and processed as described in procedure 6.12. For the detection of biotin, proteins were transferred from SDS–polyacrylamide gels to nitro-cellulose membranes which were then immersed in 3% BSA overnight at 4 °C to block non-specific binding. After washing with PBS Tween buffer, the blots were incubated with horseradish peroxidase (HRP) conjugated anti-biotin antibody for 1 h. The blots were visualized with a 50 mL solution containing 30 mg of 4-chloro-1-napthol and 0.01% hydrogen peroxide which produces a purple color at sites of horseradish peroxidase (HRP) activity.

6.13. In-gel trypsin digestion

All equipment was first thoroughly washed with 70% ethanol followed by deionized water using non-latex gloves and a face mask. Silver stained bands were immediately excised with a gel cutter or scalpel blade, taking only the silver stained gel area. The excised bands from (Lane 8, Fig. 7), as well as corresponding sections from the control lane without irradiation (Lane 6, Fig. 7) were further cut into small pieces and placed into microfuge tubes, into which destaining buffers supplied with the silver SNAP stain kit were added. After destaining, gel pieces were washed at least three times with 100 mM sodium bicarbonate until the gel color turned a pale white. Acetonitrile (200 μL) was added to each tube for 5 min to dehydrate the slices, and then removed, after which the samples were dried by centrifugal evaporation in a Speedvac. Oxidized cysteines were reduced by adding 50 μL of 10 mM DTT/50 mM ammonium bicarbonate to the dried slices for 30 min at room temperature. After decanting the DTT solution, the gel slices were incubated with 50 mM iodoacetamide solution in 50 mM ammonium bicarbonate for 30 min at room temperature in the dark to cap the freshly reduced cysteines. The gel slices were then washed three times with 100 mM ammonium bicarbonate and two times with 50 mM ammonium bicarbonate in 50% acetonitrile. The gel slices were dehydrated with 200 μL of acetonitrile for 5 min and then 500 μL acetonitrile for 20 min. After removal of the acetonitrile, the samples were completely dried in a centrifugal evaporator and subjected to trypsin digestion. Promega sequencing grade trypsin (10 ng) was dissolved in 1 mL ice-cold 50 mM ammonium bicarbonate and 20–50 μL was added to each sample on ice and incubated in a cold room for 1 h at which point the gel pieces had completely rehydrated and swelled. Excess trypsin solution was then removed and enough 50 mM ammonium bicarbonate was added to cover the gel pieces to keep them hydrated while being digested overnight. After 12 h, the bicarbonate solution was transferred to microfuge tubes on ice and the gel pieces were extracted with 1% trifluoroacetic acid in 50% aqueous acetonitrile for 10 min with sonication, and then combined with the first extract. The extraction was repeated again with 10% trifluoroacetic acid in 50% aqueous acetonitrile. The combined extracts were lyophilized in a SpeedVac and resuspended in 0.1% TFA, followed by μC-18 zip-tip (Millipore) desalting to provide clean peptide fragments for LC–MS/MS analysis.

6.14. LC–MS/MS of trypsinized proteins

A 75 μm i.d. fused-silica capillary column was packed with C18 reverse-phase material (Magic, 5 μm, 300 A, Michrom, CA) and pre-equilibrated with 100% solvent A (0.1% aqueous formic acid). Then 5 μL of digested sample was loaded onto the column and subjected to a linear 50 min gradient of 2–50% solvent B (3% water, 97% acetonitrile, 0.1% formic acid) in solvent A, followed by a linear increase to 85% solvent B in 5 min at a flow rate of 260 nl/min, followed by a 5 min re-equilibration in solvent A. The effluent was directly sprayed into an LTQ-Orbitrap mass spectrometer (Thermo Fisher, Waltham, MA), using a PicoView PV-500 nanospray source (New Objective, Woburn, MA). A full mass spectrum of eluting peptides was obtained with the FT mass spectrometer component at high mass resolving power (100,000 for ions of m/z 400). Ions submitted for MS/MS were placed in a dynamic exclusion list for 8 seconds. The MS/MS collections for the six most abundant eluting ions were performed with wide-band activation, in the LTQ mass spectrometer at a normalized collision energy of 35%, with a 2 Da isolation width. Raw data was uploaded to MASCOT licensed version (Matrix Science, MA) and submitted to identity search through NCBInr 20090707 database, with 9,252,587 sequences (226,521 from Homo sapiens). Carbamidomethyl cysteine was treated as a fixed modification, and methionine single oxidation as variable modification, and up to one missed trypsin cleavage was allowed. Monoisotopic mass was used, with a peptide mass tolerance of ±20 ppm, and fragment mass tolerance of ±0.6 Da, using an ESI-FTICR instrument type. MS/MS results on the model peptide were manually analyzed with Xcalibur software (Thermo scientific, USA).

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Supplementary data

Supplementary data (two dimensional NMR spectra of BioQ, irradiation studies of quercetin and BioQ, and autophosphorylation studies of CK2) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.07.005. These data include MOL files and InChiKeys of the most important compounds described in this article.

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