Quercetin inhibits a large panel of kinases implicated in cancer cell biology

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Abstract. Flavonoids are polyphenolic secondary metabolites from plants that possess a common phenylbenzopyrone structure (C6-C3-C6). Depending upon variations in their heterocyclic C-ring, flavonoids are categorised into one of the following groups: flavones, flavonols, flavanones, flavanols, anthocyanidins, isoflavones or chalcones. Flavonols include, among others, the molecules quercetin, myricetin and kaempferol. The anticancer activity of flavonols was first attributed to their electron-donating ability, which comes from the presence of phenolic hydroxyl groups. However, an emerging view is that flavonoids, including quercetin, may also exert modulatory actions in cells by acting through the protein kinase and lipid kinase signalling pathways. Data from the current study showed that 2 μM quercetin, a low concentration that represents less than 10% of its IC$_{50}$ growth-inhibitory concentration as calculated from the average of eight distinct cancer cell lines, decreased the activity of 16 kinases by more than 80%, including ABL1, Aurora-A, -B, -C, CLK1, FLT3, JAK3, MET, NEK4, NEK9, PAK3, PIM1, RET, FGF-R2, PDGF-Rα and -Rβ. Many of these kinases are involved in the control of mitotic processes. Quantitative video microscopy analyses revealed that quercetin displayed strong anti-mitotic activity, leading to cell death. In conclusion, quercetin partly exerts its anticancer activity through the inhibition of the activity of a large set of kinases. Quercetin could be an interesting chemical scaffold from which to generate novel derivatives possessing various types of anti-kinase activities.

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Introduction

Phytochemicals have become an important part of the available arsenal of anticancer drugs. Over 75% of non-biological anti-cancer drugs approved between 1981 and 2007 were either natural products or were developed based on such molecules (1,2).

Agelanthus dodoneifolius Polhill & Wiens Danser (Loran-thaceae), previously named *Tapinanthus dodoneifolius* (African mistletoe), is a common plant that both parasitizes plants such as Mimosaceae and Sapotaceae and displays a wide range of medicinal properties (3,4). Phytochemical screenings of the plant have revealed the presence of tannins, anthracenosides, anthraquinones, flavonoids, anthocyanosides, sterols, triterpenes and saponosides (3,4). In this study, we focused primarily on the flavonoid component of *Agelanthus dodoneifolius* and more closely on quercetin.

Flavonoids are polyphenolic plant secondary metabolites that possess a common phenylbenzopyrone structure (C6-C3-C6). According to variations in their heterocyclic C-ring, flavonoids may be categorised as flavones, flavonols, flava-nones, flavanols, anthocyanidins, isoflavones or chalcones (2,5). More than 4,000 types of flavonoids have already been identified (6).

Specific examples of flavonols include quercetin, myricetin and kaempferol (2,5). The anticancer activity of the flavonols was first attributed to their ability to donate electrons from their phenolic hydroxyl groups (2,6). However, although there has been a major focus on the antioxidant properties of flavonoids (7), there is an emerging view that the flavonoids, including quercetin, do not act only as conventional hydrogen-donating antioxidants but may also exert modulatory actions on cells through both protein kinase and lipid kinase signal-ling pathways (8,9). In addition, flavonoids have been reported to act on phosphoinositide (PI) -3-kinase, Akt/protein kinase B (PKB), tyrosine kinases, protein kinase C (PKC) and mitogen-activated protein (MAP) kinase signalling cascades (8,9). As emphasised by Willliams *et al* (8), inhibitory or stimulatory actions at these pathways are likely to profoundly affect cellular functions such as cell proliferation, cell death, and/or

cell migration by altering the phosphorylation state of target molecules and modulating gene expression.

Flavonoids, including quercetin, may also influence the activity of epigenetic-modifying enzymes in cancer cells (10) and/or induce apoptosis via the mitochondrial pathway (7). Quercetin aglycone has been shown to modulate several signal transduction cascades involving the MEK/ERK and NRF2/KEAP1 pathways, which are associated with the processes of inflammation and carcinogenesis (6). Quercetin also induces ubiquitination and down-regulation of HER-2/NEU, a transmembrane tyrosine kinase that acts as a co-receptor together with other EGFR (epithelial growth factor receptor) family members (11). Jagtap *et al* (7) provided a list of about 20 molecular targets of quercetin.

Although several hundred studies have looked at the relationship between quercetin and kinases, to the best of our knowledge, this study is the first to characterize the global antikinase profile of quercetin against a panel of 300 kinases. We have paid particular attention to quercetin because it is one of the components that we identified from *Agelanthus dodoneifolius* as a contributor to the anticancer activity of this plant.

Materials and methods

Plant material. The leaves of *Agelanthus dodoneifolius* (Loranthaceae) were collected during the period from October to November of 2005. The plant was found on a sheanut tree [*Vitellaria paradoxa* CF Gaertn (Sapotaceae)] in the region of Ouagadougou (Burkina Faso). The plant identity was determined by Professor I.J. Boussim from the Plant Ecology and Biology Laboratory at Ouagadougou University. A sample specimen was deposited and bears voucher number 01 and 02.

Extraction of flavonoids. The dried and powdered leaves of *Agelanthus dodoneifolius* (60 g) were treated with boiling methanol (ChemLab, Zedelgem, Belgium) at room temperature. The extract was concentrated to dryness under reduced pressure. The residue was resuspended in boiling water (100 ml) and successively exhausted with petroleum ether (3x100 ml), diethyl ether (3x100 ml; VWR, Leuven, Belgium), ethyl acetate (3x100 ml; ChemLab) and n-butanol (3x100 ml; VWR). Each fraction was dried under reduced pressure and stored in a desiccator prior to the biological assays. The petroleum ether fraction was not used because it contained virtually no flavonoids, as shown by thin layer chromatography (TLC; VWR, Leuven, Belgium).

For the aqueous extracts, the shade dried leaves of *Agelanthus dodoneifolius* were powdered before extraction. A decoction was made by bringing 100 g of powdered leaves to boil in 1 l of distilled water for 15 min. Then, the extract was filtered, frozen and freeze-dried (Christ, Alpha 1-2 LD; Osterode, Germany).

Determining IC_{50} in vitro growth inhibition concentrations. We used one mouse and seven human cancer cell lines that were obtained from the European Collection of Cell Culture (ECACC, Salisbury, UK), the American Type Culture Collection (ATCC, Manassas, VA, USA) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen, (DSMZ, Braunschweig, Germany). The human cancer cell lines included the non-small cell lung cancer (NSCLC) line A549 (DSMZ code ACC107), the melanoma line SKMEL-28 (ATCC code HTB-72), the glioblastoma lines U373 (ECACC code 89081403) and T98G (ATCC code CRL1690), the colon cancer line LoVo (DSMZ code ACC350), the breast cancer line MCF-7 (DSMZ code ACC115) and the prostate cancer line PC-3 (DSMZ code ACC465), and the mouse cancer cell line used was the melanoma line B16F10 (ATCC code CRL-6475). The cancer cell lines under study were cultured in RPMI medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% heat-inactivated foetal calf serum, 4 mM glutamine, 100 µg/ml gentamycin and penicillin-streptomycin (200 U/ml and 200 µg/ml, respectively; Invitrogen).

The overall growth levels of the human cancer cell lines were determined using the colorimetric MTT [3-(4,5-dimethylthiazol-2yl)]-diphenyltetrazolium bromide, Sigma, Belgium) assay (12-17). Briefly, cell lines were incubated for 24 h in 96-microwell plates at concentrations of 10,000-40,000 cells/ml, depending on the cell type, to ensure adequate plating prior to making cell growth determinations. The assessment of cell growth using the MTT colorimetric assay was based on the capability of living cells to reduce the yellow product MTT [3-(4,5)-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide] to a blue product, formazan, by a reduction reaction that occurs in the mitochondria. The number of living cells after 72 h of culture in the presence (or absence for the negative control) of the various compounds was directly proportional to the intensity of the blue colour, which was measured quantitatively by spectrophotometry using a Bio-Rad Model 680XR (Bio-Rad, Nazareth, Belgium) at the 570-nm wavelength (with a reference wavelength of 630 nm). Each experimental condition was run six times (12-17). Quercetin was obtained from Sigma (Bornem, Belgium), and quercetin-3-O-glucuronide (miquelianin) and quercetin-3-O-galactoside (hyperoside) were obtained from Carbosynth (Compton, Berkshire, England).

Computer-assisted phase-contrast microscopy (quantitative video-microscopy). The direct visualisation of the effects of quercetin on human T98G glioblastoma cells was carried out by computer-assisted phase contrast microscopy (quantitative video-microscopy), as detailed elsewhere (13,18-21). T98G glioblastoma cells were monitored for 72 h in the absence (control) or presence of 30 µM quercetin, which is the IC_{50} *in vitro* growth-inhibitory concentration as calculated by the MTT colorimetric assay (Fig. 1Ab). Movies were created from the time-lapse image sequences, which enabled a detailed screen for cell viability to determine whether the compound induced cytostatic or cytotoxic effects (13,18-21). Each experimental condition was performed in triplicate.

Kinase activity determination. We originally treated ProQinase (Freiburg, Germany) with quercetin as a stock solution in 100% DMSO, from which the aliquots were further diluted with water in 96-well microtiter plates directly before use. For each kinase assay, 5 µl from either a 2.10^6 M/10% DMSO or a 3.10^5 M/10% DMSO compound solution was transferred to the assay plates. The final volume in each well of the assay was 50 µl. The final concentrations for quercetin were either 2 or 30 µM.

Table I. Quantitative determination by UPLC-MS/MS analyses on the proportion of various phenolic compounds in various fractions from *Agelanthus dodoneifolius* and the determination of IC$_{50}$ *in vitro* growth-inhibitory concentrations of these fractions in three human cancer cell lines.

Fractions	% of phenolic compounds				IC$_{50}$ *in vitro* growth-inhibitory concentration (μg/ml)		
	Gallic acid	Catechin	Rutin	Quercetin	A549	U373	LoVo
Aqueous decoction	0.4	0.7	0.2	Not detected	>100 (66%)	75	>100 (77%)
Diethyl ether	2.1	10.5	<0.1	0.3	47	98	49
Ethyl acetate	1.0	5.7	0.3	0.2	>100 (56%)	95	50
Butanolic	0.1	0.1	0.8	Not detected	>100 (92%)	>100 (75%)	>100 (56%)

The three human cancer cell lines included the A549 non-small cell lung cancer (NSCLC), the U373 glioblastoma (GBM) and the LoVo colon cancer models. The percentages under parentheses when the IC$_{50}$ values are >100 μg/ml represent the percentages of surviving cancer cells at 100 μg/ml after 3 days of cell culture with the extracts. UPLC-MS/MS is defined as Ultra Performance Liquid Chromatography coupled to tandem mass spectrometry.

A radiometric protein kinase assay (^{33}PanQinase® Activity assay) was used for measuring the kinase activity of the 256 protein kinases under study as detailed previously (15). All kinase assays were performed in 96-well FlashPlates™ (Perkin Elmer, Boston, MA, USA) in a 50 μl reaction volume. The reaction cocktail was assembled in the following order: i) 10 μl of the non-radioactive ATP solution (in H$_2$O), ii) 25 μl of the assay buffer [γ-^{33}P]-ATP mixture, iii) 5 μl of the test sample in 10% DMSO and iv) 10 μl of the enzyme/substrate mixture. The assay mixture for all enzymes contained 60 mM HEPES-NaOH, pH 7.5, 3 mM MgCl$_2$, 3 mM CaCl$_2$, 3 μM Na-orthovanadate, 1.2 mM DTT, 50 μg/ml PEG$_{20000}$, 1 μM ATP/ [γ-^{33}P]-ATP (approximately 6x10^5 cpm per well), protein kinase (variable amounts; 15) and substrate (variable amounts; 15).

All PKC assays except for the PKC-mu and the PKC-nu assays also contained 1 mM CaCl$_2$, 4 mM EDTA, 5 μg/ml phosphatidylserine and 1 μg/ml 1.2-dioleyl-glycerol. The MYLK2, CAMK1d, CAMK2a, CAMK2b, CAMK2d, CAMK4, CAMKk2, DAPK2 and EEF2K assays also contained 1 μg/ml calmodulin and 0.5 mM CaCl$_2$. The PRKG1 and PRKG2 assays also contained 1 μM cGMP (15).

The recombinant human protein kinases were expressed in either Sf9 insect cells or in *E. coli* as either GST-fusion proteins or His-tagged proteins (ProQinase). The kinases were purified by affinity chromatography using either GSH-agarose or Ni-NTH-agarose (ProQinase). The purity of each kinase was determined by SDS-PAGE/silver staining, and the identity of each kinase was verified by mass spectroscopy (ProQinase).

The reaction cocktails were incubated at 30˚C for 60 min. The reaction was stopped with 50 μl of 2% (v/v) H$_3$PO$_4$; plates were then aspirated and washed 2 times with 200 μl of 0.9% (w/v) NaCl. Incorporation of ^{33}P$_i$ was determined with a microplate scintillation counter (Wallac Microbeta, Perkin Elmer, MA). All assays were performed with a Beckman Coulter/Sagian robotic system (Krefeld, Germany).

The median value of the counts in column 1 (n=8) of each assay plate was defined as the 'low control'. This value reflected the non-specific binding of the radioactivity to the plate in the absence of a protein kinase, but in the presence of the substrate. The median value of the counts in column 2 of each assay plate (n=8) was taken as the 'high control', at which concentration there was full activity in the absence of any inhibitor. The difference between high and low control counts was taken as 100% activity.

As part of the data evaluation, the low control value from a particular plate was subtracted from the high control value as well as from all 'compound values' of the corresponding plate. The residual activity (in %) for each well of a particular plate was calculated by using the following formula:

Res. Activity (%) = 100 x [(cpm of compound - low control)/ (high control - low control)]

The Z'-factor for the low and high controls of each assay plate was used by ProQinase as a parameter for assay quality. The ProQinase criterion for repetition of an assay plate was a Z'-factor below 0.4 (15).

Results

Agelanthus dodoneifolius organic extracts contain quercetin and display in vitro anticancer activity. The data in Table I show that both the diethyl ether and ethyl acetate extracts from *Agelanthus dodoneifolius* contained quercetin and displayed *in vitro* anticancer activity, meaning that the IC$_{50}$ growth-inhibitory concentrations were less than 100 μg/ml. In contrast, the two extracts that did not contain detectable amounts of quercetin, both the aqueous decoction and the butanolic extract, displayed no or only weak growth-inhibitory activity (Table I). Therefore, we decided to pursue our investigations by analysing the quercetin antikinase profile as detailed below.

Quercetin displays in vitro anticancer activity. The effect of quercetin on cellular proliferation (Fig. 1Aa) was assayed in seven human and one mouse (B16F10) cancer cell lines, and its IC$_{50}$ *in vitro* growth-inhibitory concentrations were very
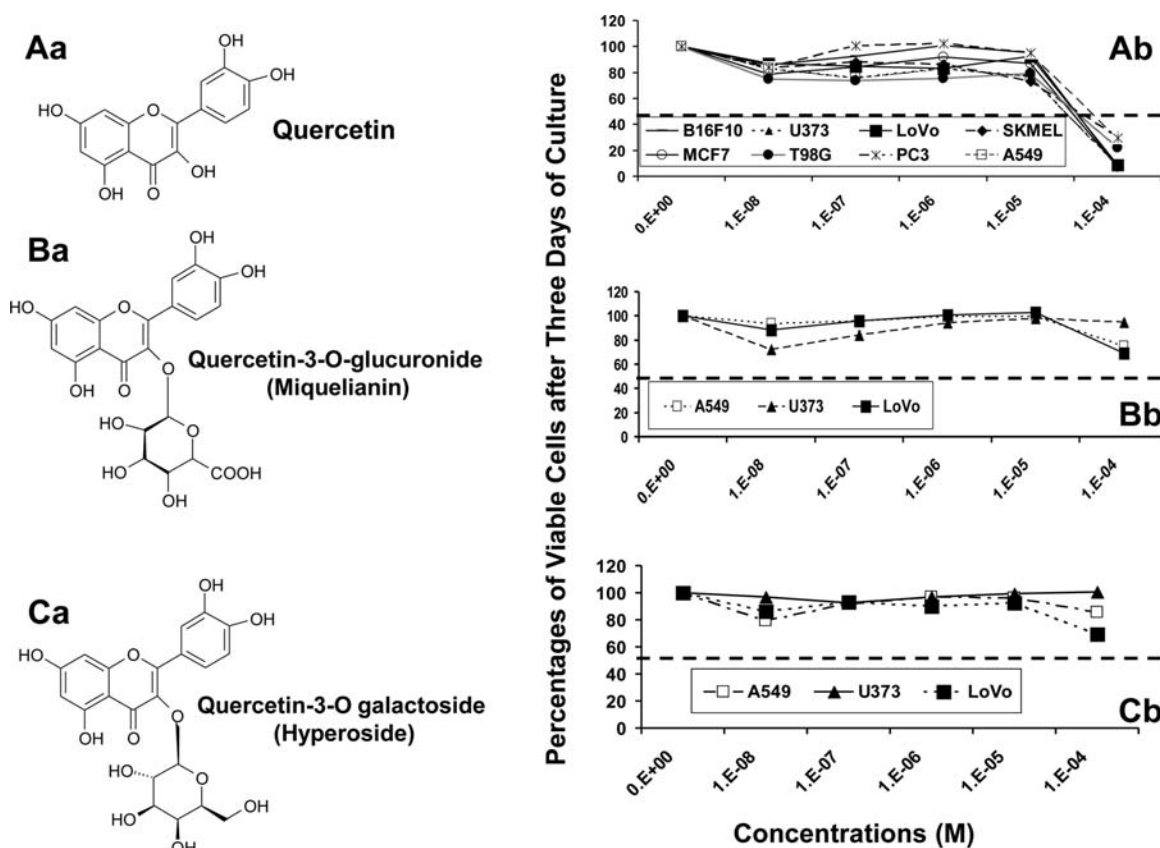
Figure 1. Characterisation of the *in vitro* growth-inhibitory effects induced by quercetin, quercetin-3-O-glucuronide (miquelianin) and quercetin-3-O-galactoside (hyperoside) in seven human (U373, T98G, LoVo, SKMEL-28, MCF-7, PC-3 and A549) and one mouse (B16F10) cancer cell lines.

similar among these eight cancer cell lines: 26±5 μM in B16F10; 31±1 μM in U373; 32±1 μM in MCF-7; 33±2 μM in T98G; 34±2 μM in A549; 33±1 μM in LoVo; 38±2 μM in SKMEL-28; and 44±1 μM in PC-3 cancer cells (Fig. 1Ab).

In contrast, the two quercetin-related glycosylated derivatives quercetin-3-O-glucuronide (miquelianin; Fig. 1Ba) and quercetin-3-O-galactoside (hyperoside; Fig. 1Ca) displayed no *in vitro* anticancer activity (Fig. 1), as evidenced by having IC_{50} growth-inhibitory concentrations of more than 100 μM (Fig. 1Bb and Cb).

Quercetin is a cytostatic rather than a cytotoxic compound. Quantitative video-microscopy analyses found that the growth-inhibitory activity of quercetin as determined by the MTT colorimetric assay (Fig. 1Ab) acted through cytostatic rather than cytotoxic effects (Fig. 2). Indeed, human T98G glioblastoma cells given quercetin at the IC_{50} *in vitro* growth-inhibitory concentration of 30 μM displayed a markedly decreased rate of proliferation. It appears that quercetin blocked T98G cells in the mitotic phase and that this blockade led to cell death (Fig. 2). The cytostatic effects of quercetin treatment on mitosis could be related to the inhibitory effect of quercetin on the NIMA-related kinases (NEK), Aurora kinases and PAK kinases, as discussed below.

Quercetin is a multi-antikinase compound. Quercetin was found to decrease the activity of ~100 kinases by greater than 95% (Fig. 3) at 30 μM, very close to the mean IC_{50} *in vitro*

growth-inhibitory concentration of 34±2 μM that was calculated for the eight cancer cell lines studied (Fig. 1Ab). We then investigated the multi-antikinase effects of a much lower concentration for quercetin, i.e., 2 μM, which represents about 7% of its mean IC_{50} *in vitro* growth-inhibitory concentration (see above). At this low concentration, quercetin still decreased the activity of ~15 kinases by greater than 95% and decreased the activity of a remaining set of ~50 kinases by between 80 and 95% (Fig. 3). In Fig. 4, we report the kinases for which 2 μM quercetin decreased their residual activity by at least 90%. We believe it is reasonable to consider the kinases for which the activity was reduced by at least 90% at a concentration of 2 μM, which is only 7% of the IC_{50} growth-inhibitory concentration found for the various cancer cell lines tested, to probably be specific targets for quercetin. The kinases with barely detectable activity after treatment with 2 μM quercetin were Cdc2-like kinase-1 (CLK1), insulin receptors (INSR-R) and muscle specific kinase (MUSK; Fig. 4).

Quercetin displays specific inhibitory effects in various groups of kinases. We have split the group of ~30 kinases illustrated in Fig. 4 into their respective subgroups of kinases as illustrated in Fig. 5A-L. For example, Fig. 4 shows that 2 μM quercetin reduced the activity of ABL1, which has various mutated forms, by 91%. Fig. 5A illustrates the effect of 2 μM quercetin on ABL1 and its mutated forms. Fig. 4 shows that 2 μM quercetin reduced the activity of Aurora B, which belongs to a sub-group of three kinases, by 92%. Fig. 5B

Human T98G Glioblastoma Cells

Figure 2. Morphological illustrations of the anticancer effects observed after 72 h of culturing human T98G glioblastoma cells with 30 µM quercetin. The cells that are entering mitosis (M) are round, refringent and display a dark spot in their centrum, whereas dying cells (DC) are also refringent but display heterogeneous morphological patterns.

Figure 3. Characterisation of quercetin-induced anti-kinase activity. A schematic illustration of the kinases in the human dendrogram-related kinome that were targeted at two concentrations with analyses of <5% (30 µM) versus <5% and 5-20% (2 µM) of kinase residual activity. The various classes of kinases included tyrosine kinases (TK), tyrosine kinase-like kinases (TKL), serine/threonine protein kinases (STE), casein kinases (CK1), cAMP-dependent protein kinases (AGC), calcium/calmodulin protein kinase II kinases (CAMK) and the CMGC sub-class, which includes cyclin-dependent kinase (CDK), mitogen-activated protein kinase (MAPK), glycogen synthase kinase (GSK), and CDK-like groups of kinases.

shows the result of 2 µM quercetin treatment on the Aurora A, B and C kinases, and so on for the remaining kinases that are illustrated in Fig. 4.

Fig. 5A shows that quercetin displays similar inhibitory activity on the wild-type (wt) and on various mutated forms of the ABL1 kinase. As recently emphasised by Agrawal *et al*
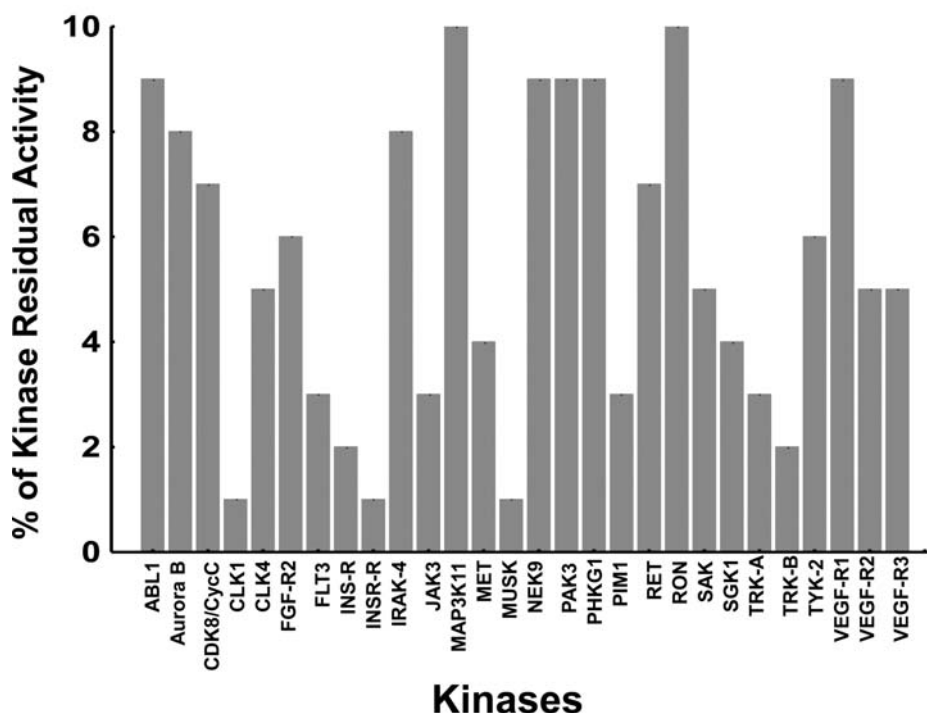
Figure 4. Identification of the kinases whose residual activity dropped below 10% (control = 100%) when treated with 2 μM quercetin.

(22), the drug imatinib mesylate, a Bcr-Abl1 tyrosine kinase inhibitor, has revolutionised treatment for chronic myeloid leukaemia (CML). By directly targeting the Bcr-Abl kinase, imatinib treatment can lead to durable cytogenetic remission and, in turn, improve survival. However, many patients with CML develop resistance, fail to respond, or become intolerant to imatinib due to side effects (22). This response has spurred interest in developing second-generation TKIs to overcome the mechanisms of resistance that lead to treatment failure, specifically Bcr-Abl1 kinase domain mutations (22). Unfortunately, many patients fail subsequent treatment with these agents because they can develop highly resistant mutations such as T315I (22). Fig. 5A clearly indicates that quercetin inhibited both the wild-type and the mutated T315I forms of ABL1.

Although not as potent as some of the specifically-designed Aurora kinase inhibitors (23,24), quercetin was found to significantly inhibit the activity of the three Aurora kinases at 2 μM (Fig. 5B), which is only 7% of the mean IC$_{50}$ *in vitro* growth-inhibitory concentration (see above). Aurora kinases A and B are essential components of the mitotic pathway, ensuring proper chromosome assembly, formation of the mitotic spindle, and cytokinesis (23,24). The role of Aurora C is less clear (23,24). Overexpression of Aurora A and B has been observed in several tumour types and has been linked with a poor prognosis of cancer patients (23,24). The combination of Aurora inhibitors with other chemotherapeutic agents may open new opportunities in cancer chemotherapy (24).

Quercetin markedly inhibited the activity of both the CLK1 and CLK4 kinases at 2 μM but inhibited to a much lower extent the activities of the CLK2 and CLK3 kinases (Fig. 5C). Therefore, quercetin seems to represent a rather specific inhibitor of CLK1 within the CLK subgroup of kinases. The CLK kinases are implicated in the circadian rhythm/biological clock systems (25).

The fms-like receptor tyrosine kinase-3 (FLT3) is important for the normal development of hematopoietic stem cells and cells of the immune system, and it is frequently mutated in patients with acute myeloid leukaemia (AML; 26). Quercetin had similar inhibitory activity on the wild-type form of FLT3 and two of its mutated forms (Fig. 5D).

Flavonoids are already known to inhibit Janus kinase 1 (JAK1) activity (27). Fig. 5E shows that quercetin markedly inhibited JAK3 activity, had intermediate inhibitory activity on JAK2 kinase and had little or no effect on the activity of JAK1 kinase. JAK3 is a non-receptor tyrosine kinase, predominantly expressed in hematopoietic cells, that has been implicated in the signal transduction of the common γ chain subfamily of cytokine receptors (28). JAK3-inactivating mutations result in immunodeficiency syndromes (SCID) in both humans and mice (28). Abnormal activation of JAK3 due to activating mutations is also found in human haematological malignancies, including acute megakaryoblastic leukaemia (AMKL) and cutaneous T cell lymphoma (CTCL) (28).

Fig. 5F shows that 2 μM quercetin greatly decreased the activity of the MET kinase and its mutated forms. MET is a tyrosine kinase receptor that, upon binding to its natural ligand, the hepatocyte growth factor (HGF), is phosphorylated and subsequently activates different signalling pathways involved in proliferation, motility, migration and invasion (29). Canadas *et al* (29) reported that MET is aberrantly activated in human cancer by mutation, amplification or protein overexpression. MET expression and activation have been associated with prognosis and predicted response to MET inhibitors in preclinical models for a number of tumour types (29).
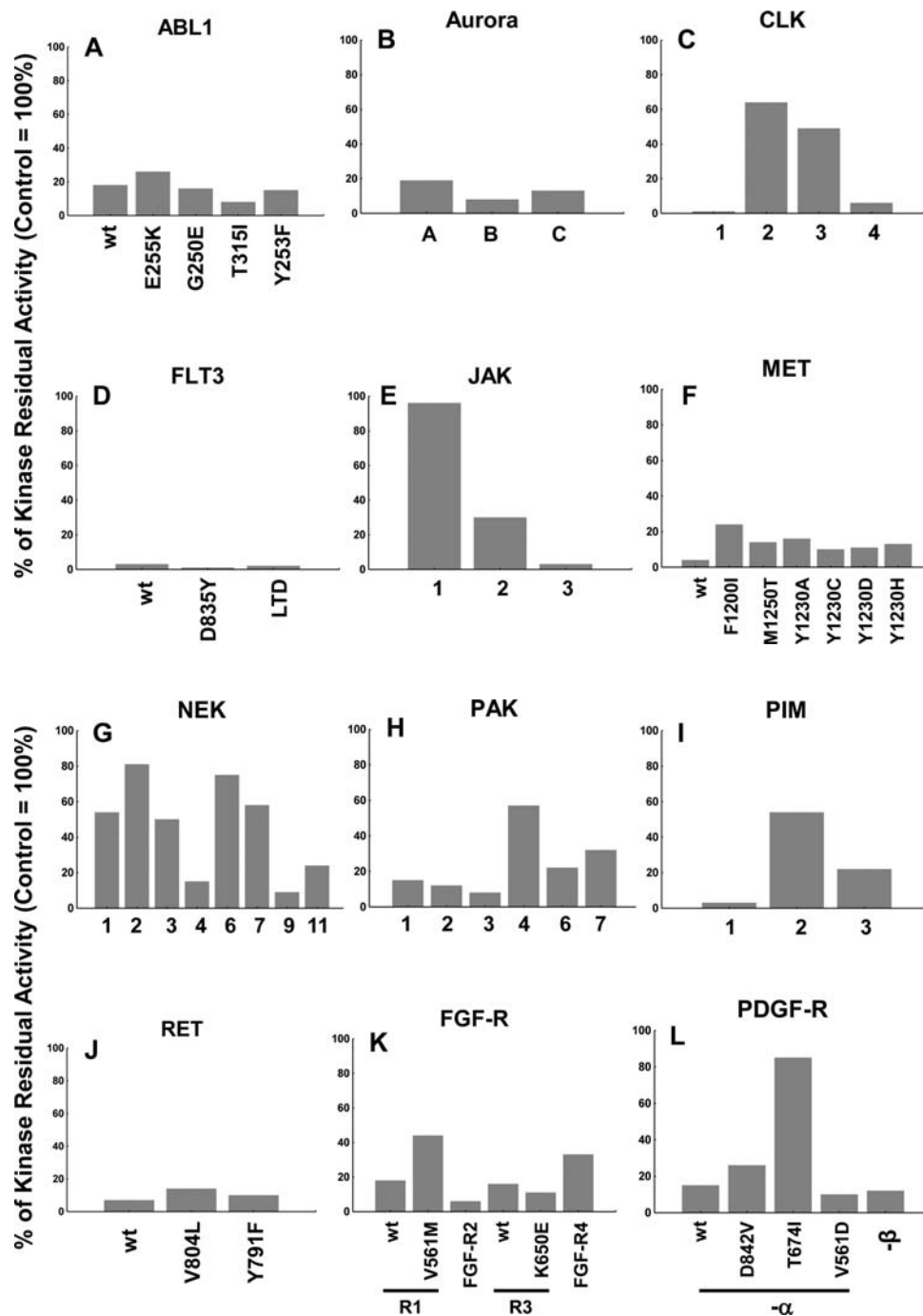
Figure 5. Identification of the kinases that may have been specifically targeted by 2 μM quercetin listed as specific groups of kinases and their mutated forms.

The NIMA-related kinases represent a family of serine/threonine kinases implicated in cell cycle control (30). Mammals contain a large family of eleven NIMA-related kinases, named NEK1 through NEK11, and of these, there is now substantial evidence that NEK2, NEK6, NEK7 and NEK9 also regulate mitotic events (30). As detailed in the discussion, NEK kinases are not yet well studied as potential targets in cancer therapy. Fig. 5G shows that quercetin significantly targeted NEK4 and NEK9 as compared to the remaining 8 NEK kinases under study.

The PAKs (p21-activated kinases) are a family of effector proteins for Rho-family GTPases Rho, Rac and Cdc42 that regulate physiological processes including cell migration, survival, mitosis, transcription and translation, and actin cytoskeleton dynamics (32,33). There are six mammalian PAKs that can be divided into two groups: group I PAKs (PAK1-3) and group II PAKs (PAK4-6; 33). Group I PAKs are activated by extracellular signals through GTPase-dependent and GTPase-independent mechanisms, whereas group II PAKs are constitutively active (32). Fig. 5H shows that 2 μM quercetin lowered the activity by a greater degree in group I than in group II PAKs.

Quercetin at a concentration of 2 μM almost completely obliterated the activity of PIM1 kinase and it also greatly

reduced the activity of the PIM2 and PIM3 kinases (Fig. 5I). The PIM1 kinase is an oncogene implicated in early transformation and tumour progression in haematopoietic malignancies and prostate carcinomas; it is also associated with the more aggressive subgroups of lymphoma (34). PIM kinases are induced by the JAK/STAT-dependent pathway (35). Interestingly, low-dose quercetin also markedly inhibited JAK2 and JAK3 (Fig. 5E) in addition to PIM1 (Fig. 5I).

The data in Fig. 5J show that 2 μM quercetin inhibited the RET kinase and two of its mutated forms to a high degree. The RET kinase has been implicated in the development of multiple endocrine neoplasia (MEN) syndromes and familial medullary thyroid carcinoma (36).

Quercetin (2 μM) greatly inhibited FGF-R activity, having similar effects on the wild-type and mutated forms of FGF-R with maximal quercetin-induced inhibitory activity on the kinase FGF-R2 (Fig. 5K). The FGF-Rs play important roles as signalling molecules governing angiogenesis, an attractive target for drug therapy due to its key role in tumour growth (37). FGFs (fibroblast growth factors) and their receptors also control a wide range of biological functions regulating cellular proliferation, survival, migration and differentiation (38). There is also substantial evidence for the importance of FGF signalling in the pathogenesis of diverse tumour types (38). As for the FGF-Rs, quercetin (2 μM) also greatly inhibited the activity of PDGF-R-α and -β (Fig. 5L). PDGF (platelet-derived growth factor) was one of the first polypeptide growth factors identified that signals through a cell surface tyrosine kinase receptor (PDGF-R) to stimulate various cellular functions including growth, proliferation, and differentiation (39). George (39) has reported that since then, several related genes have been identified constituting a family of ligands and their cognate receptors, as illustrated in Fig. 5L. To date, PDGF expression has been shown in a diverse set of tumour types, from glioblastoma to prostate carcinoma (39).

Discussion

Cancer remains a disease that is devastating because more than 90% of cancer patients die after tumour metastases due to the intrinsic resistance of metastatic cells to apoptosis, knowing that the majority of the currently available anti-cancer drugs are unfortunately apoptosis inducers (40-42). Many cancer types also display intrinsic resistance to proapop-totic stimuli even before metastasising, including NSCLC (43-45), melanoma (46,47), pancreatic cancer (48,49), oeso-phageal cancer (50) and glioma (51,52). Apart from the lack of response to apoptotic drugs, many cancers also develop an acquired chemoresistance during chronic treatments: the multi-drug resistance (MDR) phenotype, which is a phenomenon caused by decreased intracellular drug accumulation in the cancer cells due to enhanced drug efflux (53,54).

The partial inhibition of a small number of kinases appears to be a promising strategy to overcome the intrinsic resistance to apoptosis and/or the acquisition of the MDR phenotype by cancer cells (9,55-57). As single compounds, some poly-phenols such as curcumin, resveratrol and the green tea polyphenol epigallocatechin-3-gallate (EGCG) have the potential to target multiple kinases that have been implicated in cancer cell biology (58-61). This targeting ability is in addition to the multiple anticancer properties of polyphenols that have already been reported, such as anti-oxidative, pro-apoptotic, DNA damaging, anti-angiogenic, and immuno-stimulatory effects (9).

In the current study, the IC$_{50}$ *in vitro* growth-inhibitory concentration of quercetin was determined in seven human and one mouse cancer cell lines by means of the MTT colorimetric assay. Of these eight cancer cell lines, we previously demonstrated high levels of resistance to pro-apoptotic stimuli for the human U373 and T98G glioblastoma cells (50,62), the A549 NSCLC (15,44) and the SKMEL-28 melanoma (47). In the same manner, we demonstrated the sensitivity to pro-apoptotic stimuli of the human MCF-7 breast cancer (63), the PC-3 prostate cancer (63), and the mouse B16F10 melanoma (47) cell lines. We observed no statistically significant (p>0.05) differences in the IC$_{50}$ values for quercetin between cancer cell lines that are sensitive or resistant to pro-apoptotic stimuli (Fig. 1).

Quercetin is commonly present as a glycoside and is converted to glucuronide/sulphate conjugates during intestinal absorption. Therefore, only conjugated metabolites are found in circulating blood (6). Although metabolic conversion attenuates its biological effects, active aglycone may be generated from the glucuronide conjugates by enhanced ß-glucuronidase activity during inflammation (6).

The fact that quercetin (but not the glycosylated forms of quercetin; see Fig. 1) displays significant anticancer activity in cancer cells that are sensitive to proapoptotic stimuli as well as in cancer cells that display various levels of resistance to such triggers may be due at least in part to the fact that this compound exerts marked antikinase activity. As recently covered by Hou and Kumamoto (27), the inhibition of protein kinases has emerged as an important target for cancer chemo-prevention and therapy. Hou and Kumamoto (27) report that accumulated data have shown that flavonoids exert chemo-preventive effects by acting not only as conventional hydrogen-donating antioxidants but also on protein kinase signalling pathways. Recent studies have shown that flavonoids can bind directly to many protein kinases, including Akt/protein kinase B (Akt/PKB), Fyn, Janus kinase 1 (JAK1), mitogen-activated protein kinase kinase 1 (MEK1), phosphoinositide 3-kinase (PI3K), mitogen-activated protein (MAP) kinase kinase 4 (MKK4), Raf1 and ζ chain-associated 70-kDa protein (ZAP-70) kinase. The flavonoids then alter the phosphorylation state of the kinases to regulate multiple cell signalling pathways in the carcinogenesis processes (27). The current study found a significant additional number of kinases that are targets for the flavonoid quercetin. The activities of kinases ABL1, Aurora-A, -B, -C, CLK1, FLT3, JAK3, MET, NEK4, NEK9, PAK3, PIM1, RET, FGF-R2, PDGF-Rα and -Rβ were the most markedly inhibited by quercetin. Indeed, at 2 μM quercetin, a concentration that represents <10% of its IC$_{50}$ growth-inhibitory concentration calculated from eight distinct cancer cell lines (see Fig. 1), decreased the activity of the 16 kinases mentioned above by greater than 80% (see Fig. 5). Many of these kinases are involved in the control of mitotic processes. Indeed, the quanti-tative videomicroscopy analyses reported in Fig. 2 revealed that quercetin displayed anti-mitotic activity, leading to cell death (Fig. 2).

Rodent studies have already demonstrated that dietary administration of quercetin prevents chemically-induced carcinogenesis, especially in the colon, whereas epidemiological studies have indicated that an intake of quercetin may be associated with the prevention of lung cancer (6).

In conclusion, the current study found that quercetin exerts its anticancer activity, at least partly, through the inhibition of the activity of a large set of kinases and that this inhibitory activity occurred *in vitro* at concentrations of about one-tenth of its growth-inhibitory activity. Quercetin may represent an interesting chemical scaffold from which to generate novel derivatives that have a diverse set of antikinase profiles.

Acknowledgements

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