JAK2 and AMP-kinase inhibition *in vitro* by food extracts, fractions and purified phytochemicals

Harry Martin [ad], Elaine J. Burgess [bd], Wendy A. Smith [cd], Tony K. McGhie [a], Janine M. Cooney [cd], Rona C. M. Lunken [ad], Erika de Guzman [e], Tania Trower [cd] and Nigel B. Perry [bd] [1]

Abstract

We have identified a range of food phytochemicals that inhibit Janus Kinase 2 (JAK2) and Adenosine Monophosphate Kinase (AMPK). A mutated and dysregulated form of JAK2, a tyrosine kinase, is associated with several diseases including Crohn's disease. Using an *in vitro*, time-resolved fluorescence (TR-FRET) assay, we tested 49 different types of food extracts, plus 10 concentrated fractions of increasing hydrophobicity from each extract, to find foods containing JAK2 inhibitors. The food extracts tested included grains, meat, fish, shellfish, dairy products, herbs, mushrooms, hops, fruits and vegetables. Several fruits were potent inhibitors of JAK2: blackberry, boysenberry, feijoa, pomegranate, rosehip and strawberry, which all contain ellagitannins, known inhibitors of kinases. These fruits are in the Rosales and Myrtales plant orders. No other foods gave >1% of the maximal JAK2 inhibitory activities of these fruits. AMPK, a sensor and regulator of energy metabolism in cells, is a serine-threonine kinase which is reported to be activated by various flavonoid phytochemicals. Using a TR-FRET assay, we tested various fruit extracts for AMPK activation and inhibition. Ellagitannin containing foods scored highly as AMPK inhibitors. Despite several reports of AMPK activation in whole cells by phytochemicals, no extracts or pure compounds activated AMPK in our assay.

Abbreviations

AMP   Adenosine monophosphate
AMPK   Adenosine monophosphate kinase
ATP   Adenosine triphosphate
DMSO   Dimethyl sulfoxide
EDTA   Ethylenediaminetetraacetic acid
EGTA   Ethylene-glycol-tetraacetic acid
IC_{50} half maximal inhibitory concentration
JAK2   Janus kinase 2
PKA   Protein kinase A
TR-FRET Time resolved, Förster resonance energy transfer

Introduction

Natural foods may contain anti-inflammatory molecules. For example the concentration of salicylates in cumin occur at 1.5 % by weight[1] while oleocanthal, found in olive oil, has ibuprofen-like activity.[2] Systemic inflammation is associated with cancer, obesity, aging and diabetes. [3, 4] Several dietary phytochemicals are reported to alleviate inflammatory diseases. For example, resveratrol and capsaicin reduce obesity associated inflammation in mice[5, 6], and orally administered d-limonene was found to reduce inflammation in rat colitis and to lower plasma markers of inflammation in humans.[7]

The inflammatory bowel condition known as Crohn's disease has several genetic susceptibility markers including Nucleotide-binding oligomerization domain-containing protein 2, Autophagy-related protein 16, tumour necrosis factor α and JAK2.[8] JAK2 is a protein tyrosine kinase and mediates signal transduction following cell stimulation by inflammatory cytokines including interleukins 2 through 6 and Interferon-γ.[9, 10] An acquired mutation of JAK2 (V617F) is associated with the bone-marrow diseases polycythemia vera, essential thrombocythemia, idiopathic myelofibrosis[11] and also with unregulated lymphocyte activation in Crohn's disease.[12] V617F JAK2 lacks the normal autoregulation conferred by the JH2 pseudokinase domain of JAK2.[13]

Adenosine Monophosphate-activated Protein Kinase (AMPK) is a cellular energy sensor, present in almost all eukaryotes. An increasing AMP:ATP ratio leads to allosteric activation of AMPK, which can also be activated by phosphorylation by upstream kinases.[14] AMPK plays a fundamental role in controlling metabolic rate, glucose consumption and appetite [15] and has therefore attracted a great deal of attention from medicinal chemists and drug designers.

Several phytochemicals that are JAK2 inhibitors[16] are also activators of AMPK, e.g. genistein and resveratrol[17-19]. Other phytochemicals reported to activate AMPK in a variety of cell-based and animal models include apigenin, [20] anthocyanins [21] and ellagic acid.[22] Ellagic acid is a known kinase inhibitor due to its affinity for the kinase ATP binding site.[23] In a comparison of 12 kinases, this affinity ranged from 40 nM to greater than 40 µM.[24] In our recent study of boysenberry ellagitannin inhibition of JAK2 we found that ellagic acid had an IC_{50} of 92 nM.[16]

To find further foods with JAK2 inhibitory activity, we have conducted a large scale screen of 49 common and New Zealand sourced food products. These foods included fruits, vegetables, herbs, fish shellfish, meats and grains which we tested for JAK2 inhibitory activity as well as 10 fractions of increasing hydrophobicity prepared from each food sample using solid phase extraction. The JAK2

and AMPK studies were performed on different samples, as projects running in parallel. Although JAK2 inhibition and AMPK activation are fundamentally different physiological targets, we found similar activities in selected foods and phytochemicals.

Although several previous reports have identified ellagitannins as inhibitors of kinases, we show that the overriding indicator of whether a food has strong JAK2 inhibitory capacity is the presence of ellagitannins in the food. With 49 different types of food included in this study we believe this is the most comprehensive analysis of its kind. We also show that several phytochemicals that are reported to be AMPK activators in whole cell systems are, in fact, inhibitors of AMPK in a direct biochemical assay of AMPK.

Materials and methods

Materials and reagents

Activated JAK2 (SH1, SH2) was supplied by Invitrogen Inc. AMPK (α1,β1γ1) was supplied by Promega Inc. ULight^{TM} substrates, Europium-coupled anti-phosphotyrosine antibody, 384-well proxiplates and Lance detection buffer were supplied by Perkin Elmer Inc. The ULight^{TM} substrate sequences for the JAK2 and AMPK assays were CAGAGAIETDKEY$\underline{Y}$TVKD and CHMRSAM$\underline{S}$GLHLVKRR respectively. The phosphorylation target amino acids are underlined. Europium-labelled antibodies Eu-W1024 anti-phosphotyrosine (PT66) and Eu-anti P acetyl CoA Carboxylase ser79 used in the JAK2 and AMPK assays respectively, were supplied by PerkinElmer Inc. The AMPK activating compound A769662 was supplied by Reagents Direct. All other reagents were supplied by Sigma Inc. Foods were purchased from retail outlets or obtained direct from producers.

Food extraction and fractionation procedure (JAK2 assay)

Freeze-dried ground food material (10 g) was extracted overnight by shaking with 96% EtOH, 4% H_2O (100 ml), then filtered to give an extract, which was stored at -20 °C. An aliquot of extract (50 ml) was coated onto 2 g C18 (Aldrich octadecyl-functionalized silica gel) by rotary evaporating at 30 °C and applied to a 5 g C18 Isolute SPE cartridge preconditioned with EtOH, 1:1 EtOH:H_2O, then H_2O (10 ml each). Fractions were eluted with 2 x 10 ml of H_2O, 1:4 EtOH:H_2O, 1:1 EtOH:H_2O, 4:1 EtOH:H_2O, EtOH, and EtOAc to give 12 10 ml fractions. Aliquots (1 ml) of extract and fractions were dried at 20 °C in Eppendorf Safelock^{TM} tubes and stored at -20 °C until assayed. The samples were dissolved in 250 µl of DMSO then diluted

into the JAK2 assay to achieve a final concentration of 0.83% DMSO. To simplify the set up for robotic assays, the first two fractions were pooled after suspension in DMSO, as were the last two fractions, thus reducing the sample number per food to 12 (whole extract, 10 fractions and one control sample e.g. staurosporine). The foods extracted, fractionated and assayed are summarised in Table 1.

Production of fruit extracts for AMPK assay

Polyphenol-rich extracts were prepared for apple, blackcurrant, blueberry, boysenberry, feijoa and kiwifruit as previously described.[23] Briefly, fruit were extracted with 4:1 EtOH: H_2O, the polyphenolic compounds were bound to XAD-7 resin, eluted with EtOH and dried to a powder using a rotary evaporator. The dried extracts were dissolved in DMSO (10 mg/mL).
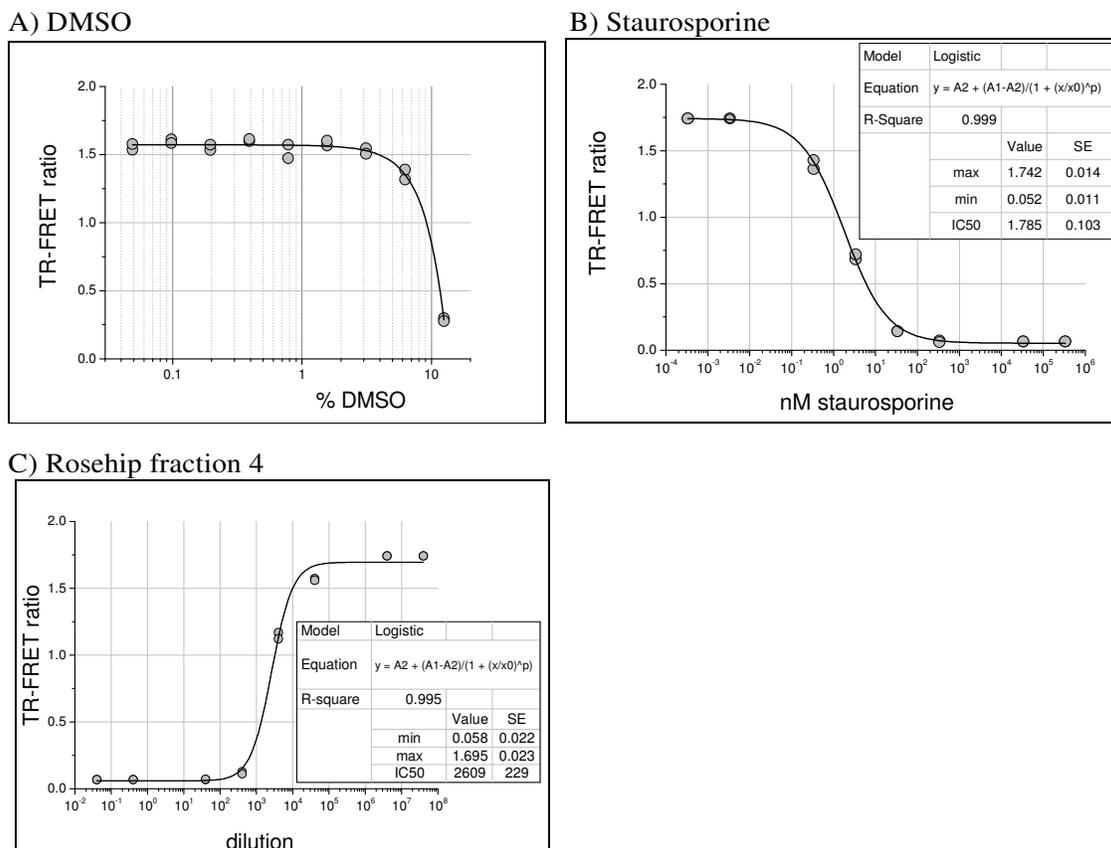
JAK2 and AMPK assays

The JAK2 and AMPK assays used Perkin-Elmer Lance® Ultra fluorescence resonance energy transfer (TR-FRET).[11] Briefly, the phosphorylated amino acid on the fluorescently tagged (Ulight™) JAK2 or AMPK peptide substrate is detected by the proximity of europium chelated to an anti-phospho-amino acid antibody. Standard reaction buffer contained 1 nM kinase; 200 nM ULight substrate; 100 µM Mg ATP; 50 mM HEPES; 9 mM $MgCl_2$; 2 mM dithiothreitol; 1 mM EGTA and 0.01 % Tween® 20. The kinase reaction was stopped by the addition of europium antibody to a final concentration of 2 nM in Lance detection buffer and EDTA to a final concentration of 10 mM. Kinase and antibody reaction phases were incubated for 60 min at 20–25 °C. Europium fluorescence was read at excitation and emission wavelengths of 345 nm and 625 nm and the phosphorylated peptide product was read at 665 nm. All statistical analyses (Sigmoidal curve fitting and IC_{50} estimates) were performed with Origin® software v7.5 (OriginLab, Northampton, MA, USA). IC_{50} estimates were derived using the logistic equation (y = A2 + (A1-A2)/(1 + (x/x0)^p)). Each IC_{50} is produced with a standard error of the IC_{50} estimate. Although the "time resolved" component of TR-FRET is effective at reducing interference from sample fluorescence, some concentrated samples nonetheless contained components that substantially altered the emission of either the europium or the ULight fluors. To detect these artefacts samples were read for 'normal' (i.e. non-time resolved) ULight fluorescence (excitation 625 nm, emission 665 nm) as soon as substrate was added. In addition, interference with Europium emission was detected as soon as the antibody was added by reading at 345 nm excitation and 625 nm emission. Any microplate wells showing more than 15% decrease in fluorescence, compared to DMSO controls, were excluded from TR-FRET analyses.

For JAK2 analysis, samples dissolved in DMSO were diluted into assay buffer on a Biomek 2000 liquid handling system and assayed at four ten-fold dilutions ranging from 1/125 to 1/125000. AMPK analysis was on a much smaller scale than JAK2, and therefore carried out manually. Adenosine monophosphate and the thienopyridone compound A769662 are allosteric AMPK activators that bind at different sites on AMPK.[25] These compounds served as positive controls in our search for AMPK activating phytochemicals.

Results and discussion

In the JAK2 assay, samples dissolved in DMSO were diluted to a final concentration of 0.83% DMSO in assay buffer. This concentration had no effect on JAK2 activity since DMSO was tolerated up to 2% (Figure 1A). For JAK2, an IC_{50} of 1.8 nM for staurosporine was measured using the robotic assay, close to the 0.9 nM determined by the JAK2 suppliers (Figure 1B). Rosehip samples, which contain ellagic acid, [26] a known phytochemical kinase inhibitor, [16] were tested to confirm that the automated JAK2 assay was functioning. Figure 1C shows the dose response curve for a fraction 4 which gave 50% inhibition at a dilution factor of 2609.

Fig. 1 Assay validation results (JAK2 activity)

A) DMSO

B) Staurosporine

Model	Logistic		
Equation	$y = A2 + (A1-A2)/(1 + (x/x0)^p)$		
R-Square	0.999		
		Value	SE
	max	1.742	0.014
	min	0.052	0.011
	IC50	1.785	0.103

C) Rosehip fraction 4

Model	Logistic		
Equation	$y = A2 + (A1-A2)/(1 + (x/x0)^p)$		
R-square	0.995		
		Value	SE
	min	0.058	0.022
	max	1.695	0.023
	IC50	2609	229

Once the assay protocol was validated we analysed 49 different types of food extract (Table 1). In some cases several varieties of one type of food were tested. For example, four types of apple were tested and some of these were separated into skin and flesh before extraction and fractionation because of differences in phytochemical contents[27]. The crude extracts were assayed alongside 10 reversed-phase fractions designed to concentrate and separate food components ranging from water soluble compounds to lipids[27]. This fractionation can potentially separate active inhibitors from masking compounds, and the activity profile assists with dereplication of known inhibitors. The screening results are summarised in Table 1.

Table 1 JAK2 screening results. 'Fraction with max. activity' indicates which of the 10 fractions, or extract "E", gave most JAK2 inhibition. The activity is shown as logarithm (base 10) of the dilution factor required for 50% inhibition, or "-" for no inhibition at any dilution. Repeated extracts are shown with suffices '1' and '2'. All activities higher than 3 (a dilution factor of 1000) consistently had standard error (SE) values less than 10% of the IC_{50} value. Less active samples would occasionally give higher SE values. Samples scoring SE/IC_{50} values between 10 - 25% are marked with an asterisk in Table 1. Weak inhibitors giving SE/IC_{50} values higher than 25% were scored as inactive.

| Food type	Sample	Fraction with max. activity	Activity	Food type	Sample	Fraction with max. activity	Activity
fruit	Apple, Cox's Orange	4	1.5*	grain	Bread, brown, gluten free	8	1.9*
fruit	Apple, Crab apple, flesh	3	2.5	grain	Bread, white, gluten free	8	1.3*
fruit	Apple, Crab apple, skin	4	2.7	grain	Oats, bran	8	2.1*
fruit	Apple, Merton Russet, flesh	2	1.5	grain	Oats, rolled, plus Omega 3	-	-
fruit	Apple, Merton Russet, skin	4	2.6	grain	Oats, rolled	-	-
fruit	Apple, Niagara, flesh	3	1.6				
fruit	Apple, Niagara, skin	8	2.5	herbs and sundry	Hops 1	3	2.7
fruit	Apple, Royal Gala, flesh	2	1.8*	herbs and sundry	Hops 2	10	2.0
fruit	Apple, Royal Gala, skin	4	1.5*	herbs and sundry	Mushroom, saffron milk cap	-	-
fruit	Avocado, organic	6	1.5*	herbs and sundry	Olive, leaf		-
fruit	Avocado	5	1.3*	herbs and sundry	Parsley	1	1.9*
fruit	Blackberry	3	5.3	herbs and sundry	Rosemary 1	7	2.6
fruit	Blackcurrant	1	2.8	herbs and sundry	Rosemary 2	7	2.8*
fruit	Blueberry	E	2.3	herbs and sundry	Sage	4	2.4*
fruit	Boysenberry	4	5.0	herbs and sundry	Tea, green 1	-	-
fruit	Cherry	7	2.8*	herbs and sundry	Tea, green 2	6	1.8
fruit	Feijoa 1	3	5.1				
fruit	Feijoa 2	4	5.1	meat	Beef	7	1.5
fruit	Gooseberry	-	-	meat	Deer velvet	1	2.4
fruit	Grapefruit	-	-	meat	Kidney, pig	8	2.2*
fruit	Kiwifruit, gold, crush	8	1.1*	meat	Lamb	1	1.7*
fruit	Kiwifruit, green, crush	5	1.3*				
fruit	Kiwifruit+boysenberry, crush	1	4.9	seafood	Arrow Squid, flesh	8	1.7
fruit	Kiwifruit, Actinidia callosa var	6	1.6	seafood	Arrow Squid, intestine	8	3.0
fruit	Kiwifruit, A. chinensis Hort 16A	2	1.6	seafood	Arrow Squid, tentacles 1	8	3.0
fruit	Kiwifruit, A. chinensis var rufopulpa	3	1.8	seafood	Arrow Squid, tentacles 2	8	2.7
fruit	Kiwifruit, A. chrysantha	2	2.5	seafood	Crayfish, flesh	8	1.8
fruit	Kiwifruit, A. deliciosa (DA36-01)	8	1.6	seafood	Crayfish, exoskeleton	8	2.1
fruit	Kiwifruit, A. deliciosa (diploid DA)	1	1.7	seafood	Crayfish, intestine	7	2.4
fruit	Kiwifruit, A. deliciosa (Hayward)	7	1.6	seafood	Fish, Alfonsino, flesh	8	1.8*
fruit	Kiwifruit, A. glaucophylla	7	1.4	seafood	Fish, Alfonsino skin, skeleton, head	8	2.2*
fruit	Kiwifruit, A. indochinensis	7	1.7	seafood	Fish, Eel, flesh	8	1.3*
fruit	Kiwifruit, A. latifolia	4	1.6	seafood	Fish, Salmon, flesh 1	-	-
fruit	Kiwifruit, A. purpurea	8	1.7	seafood	Fish, Salmon, flesh 2	-	-
fruit	Kiwifruit, A. setosa	E	2.5	seafood	Fish, Salmon, flesh, smoked 1	8	2.7
fruit	Lemon	8	2.3	seafood	Fish, Salmon, flesh, smoked 2	8	2.6*
fruit	Orange	8	2.3	seafood	Fish, Salmon, flesh, smoked 3	7	1.9
fruit	Pomegranate	2	6.0	seafood	Fish, Salmon, intestine	8	2.4
fruit	Rosehip 1	4	3.4	seafood	Fish, Salmon, skeleton+head	7	2.5
fruit	Rosehip 2	2	3.8	seafood	Fish, Snapper - intestine	7	-
fruit	Strawberry	4	6.0	seafood	Oyster, Bluff	8	2.4
fruit	Tomato	8	1.8*				
fruit	Tomato, paste	6	1.3	vegetable	Broccoli	-	-
fruit	Wolfberry	-	-	vegetable	Carrots	-	-
				vegetable	Kumara	-	-
dairy	Colostrum, Cow	-	-	vegetable	Onions	-	-
dairy	Milk, goat	1	1.5	vegetable	Pumpkin	8	2.3
dairy	Milk, cow, Protein powder, casein	11	1.2*				
dairy	Milk, sheep	-	-				

Only the most active sample in the set of 11 for each food extract is reported in Table 1.
Occasionally the unfractionated whole extract scored as the most active material. During the

fractionation procedure the samples are concentrated. Therefore it is possible that during the assay's aqueous dilution steps, the higher concentration of inhibitor in the fractions may cause it to precipitate thereby causing an underestimate of its quantity. The whole extracts contain a complex mixture of hydrophobic and hydrophilic molecules from plant and animal tissues. There is the potential for the formation of an emulsion in the whole extract which artificially increases the 'apparent solubility' of the inhibitors during aqueous dilution. In addition, the pure fractions have been subjected to more exposure to light and possibly oxidation by the air or may be chemically labile outside of their original environment. We suppose that insolubility and instability of phytochemicals might sometimes reduce the inhibitory activity of the fractions. In addition, although this study does not look into synergistic mechanisms of inhibition, it is formally possible that more the presence of different kinase inhibitors could act in combination with each other in the whole extract and that this synergy is lost in the fractions.

The most potent JAK2 inhibitors were readily detected because they caused inhibition at dilutions $\geq 1\mathrm{x}10^5$, presumably well beyond any possible interference from other components. The distribution of highest JAK2 inhibitory activity across extract and fractions summarised in Figure 2 with IC_{50} values on a log scale to facilitate comparison. Samples with activities higher than $1\mathrm{x}10^6$ could not be scored accurately in the high throughput screening process and have been shown in Figure 2 as having an activity of only $1\mathrm{x}10^6$. This occurred in some fractions of pomegranate and strawberry samples.

Fig. 2 Distribution of highest JAK2 inhibitory activity across extract and fractions (● foods known or suspected to contain ellagitannins)

Jak2 inhibition by fractionated foods
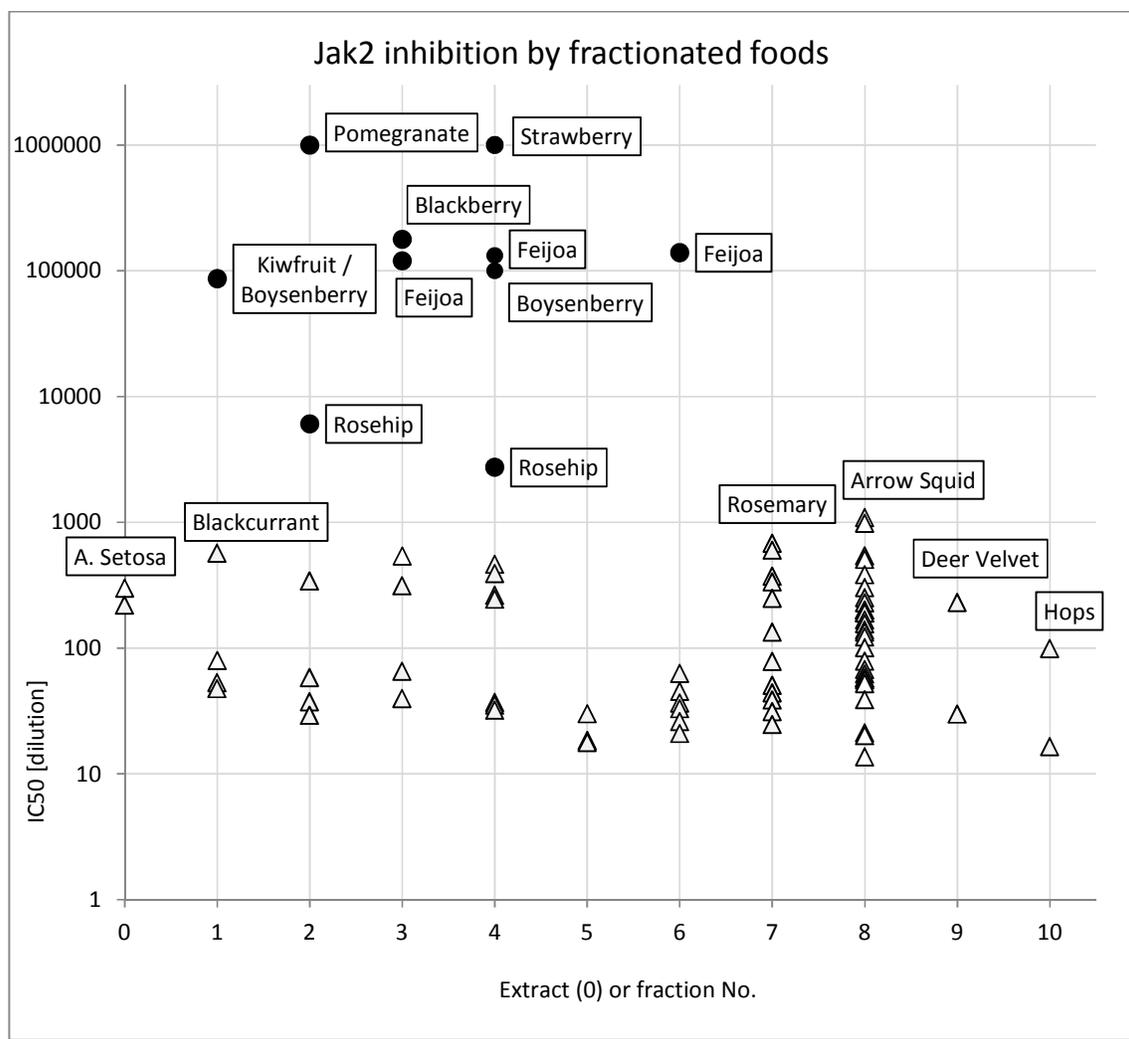
It is apparent that the data fall into two groups, with several fruit samples two to three orders of magnitude more active than the other foods (Fig. 2). Labelling foods reported in the literature as containing ellagic acid or ellagitannins[28, 29, 30] with solid black circles showed that all the JAK2 inhibitory activity found in this screening was attributable to these phytochemicals. Note that kiwifruit extracts were not active, but a combined kiwifruit and boysenberry "crush" was active due to the boysenberry ellagitannins (Table 1). [16]

The distribution of the JAK2 inhibitory activity across the fractions was characteristic, peaking around fractions 2-4, but did vary slightly between ellagitannin containing fruits (Fig. 3). The peak of inhibitory activity for pomegranate, rich in punicalagin,[29] occurred in fractions 2 and 3 whereas the berry fruits, rich in Sanguiin H-6, [29] showed peak activity in slightly less polar fractions (Fig. 3).
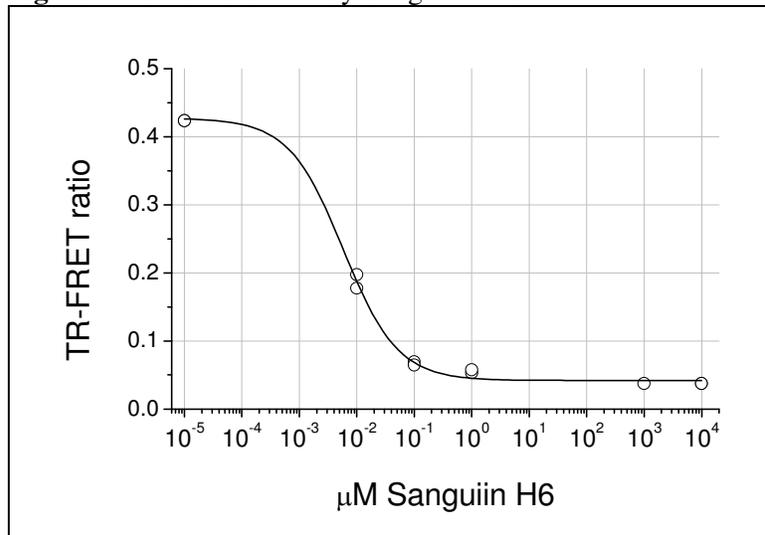
Fig. 3 JAK2 inhibitory activity in selected active ellagitannin containing samples

* Dilution factors above 1×10^6 were not scored accurately – shown by the dashed line to the right hand Y-axis
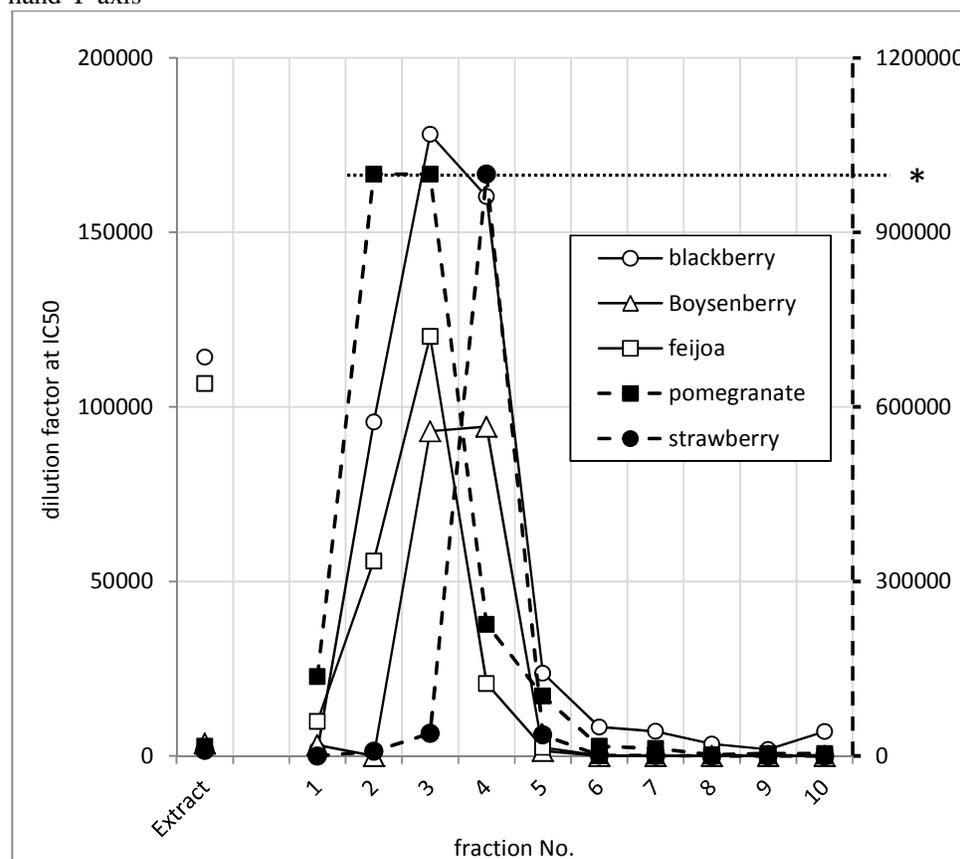
Sanguiin H-6 purified from boysenberry extracts[31] was found to have an IC_{50} of 58 ± 6.1 nM in this JAK2 assay (Fig. 4).

**Fig. 4:** Inhibition of JAK2 by Sanguiin H-6

Ellagitannins are known to be converted by hydrolysis to ellagic acid and subsequently to urolithins in the gut, [32] and ellagic acid is known as a strong inhibitor of kinases. [23] Healthful properties have been ascribed to ellagic acid including anti-cancer[33, 34] and anti-inflammatory activity in animal models of gut inflammation.[35, 36] The concentration of ellagitannins in raspberries and blackberries is reported to be as high as 2 mg/kg [37], most of which is hydrolysed to ellagic acid by acid hydrolysis *in vivo*. [38] However, ellagic acid bioavailability is reported to be very low. In rats the plasma concentration reached only 0.2 µg/ml after an oral dose of 0.8 g/kg.[39] In a human study when volunteers consumed around 350 mg of an ellagic acid/ellagitannin mixture in the form of pomegranate drink, the maximum plasma concentration achieved was around 30 ng/ml. [40] A contributory factor to this poor bioavailability may be that ellagic acid binds non-specifically to enterocyte protein and DNA. In a study using the human enterocyte cells Caco2, 93 % of cellular ellagic acid was irreversibly bound to macromolecules.[41] However, although ellagic acid has poor bioavailability, the increased permeability of the gut in Crohn's disease [42] coupled with the high affinity of ellagic acid for JAK2, may contribute to the beneficial effects of ellagic acid in animal models of digestive tract cancer and inflammation.

AMPK

AMPK assay conditions were validated by confirming inhibition by staurosporine and activation by AMP and the synthetic small molecule activator A76966243 (Fig. 5).

Fig. 5: Inhibition of AMPK by staurosporine, apigenin and ellagic acid. Activation by AMP and A769662
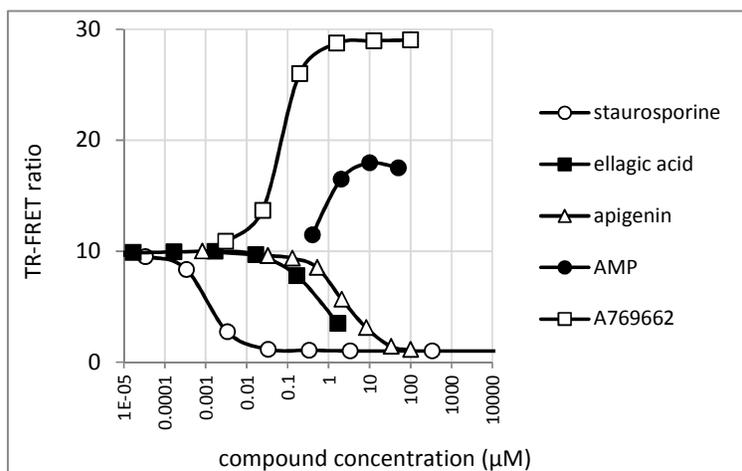
Compared to JAK2, the activity of AMPK was slightly more sensitive to DMSO, but this was well tolerated below a concentration of 1 % (data not shown). 50 % of maximum activation was achieved at 69 ±3 nM by A769662 and at 0.9 ±0.03 µM by AMP. These values are close to the values reported for A79662 [43] and AMP. [44] A769662 was capable of increasing substrate phosphorylation by approximately 3-fold whereas the maximum increase in activity observed with AMP was nearer to 1.5 fold. The greater amplification of AMPK by A769662 compared with AMP and the slight inhibition of AMPK by AMP in the hundred-micromolar range is in agreement with published results.[43] Figure 5 also shows the inhibitory effect of ellagic acid (IC_{50}= 0.67 ±0.028 µM) and apigenin (IC_{50} = 2.53 ±0.22 µM) on AMPK. Due to limited ellagic acid solubility, it was not tested above a concentration of 1.67µM.

A panel of 14 purified phytochemicals were assayed for AMP activation or inhibition. The panel included resveratrol and genistein which are reported to activate AMPK. [17-19] These compounds gave clear inhibition of the TR-FRET signal at concentrations of 100 µM and 10 µM. The results are shown in Table 2 along with the ellagic acid and apigenin IC_{50} values. Ellagic acid is clearly the most active inhibitor having a sub-micromolar IC_{50}. However, both apigenin and cyanidin-3 glucosyl-rutinoside are potent inhibitors with IC_{50} values of 2.5 µM and 3.2 µM respectively.

Table 2: Phytochemical inhibition of AMPK (IC_{50}s)

phytochemical	µM	SE
apigenin	2.53	0.22
curcumin	-	-
cyanidin 3-glucosyl-rutinoside	3.2	0.61
cyanidin 3-rutinoside	198	74.1
cyanidin chloride	31.5	11.2
delphinidin 3-rutinoside	546	125
ellagic acid	0.67	0.028
genistein	1474	154

idaein	47.7	5.07
kaempferol 3-glucoside	215	26.4
quercetin	12.3	2.82
quercetin-3 beta-glucoside	114.4	13.8
quercitrin	44.9	4.45
resveratrol	95.1	24.2

To determine whether crude extracts could activate or inhibit AMPK, a selection of fruit extracts were assayed. Figure 6 shows that apple and kiwifruit slightly reduced the AMPK rate, but this inhibition was too slight to be quantifiable. Blackcurrant and blueberry extracts gave intermediate AMPK inhibition: IC_{50}s were achieved at extract dilution factors of 700 and 2600 respectively. Boysenberry and feijoa extracts were much more potent inhibitors of AMPK. Boysenberry extract inhibited AMPK by 50% at a dilution of 40,000 and feijoa at a dilution of 130,000.

Fig. 6: Fruit extract effects on AMPK
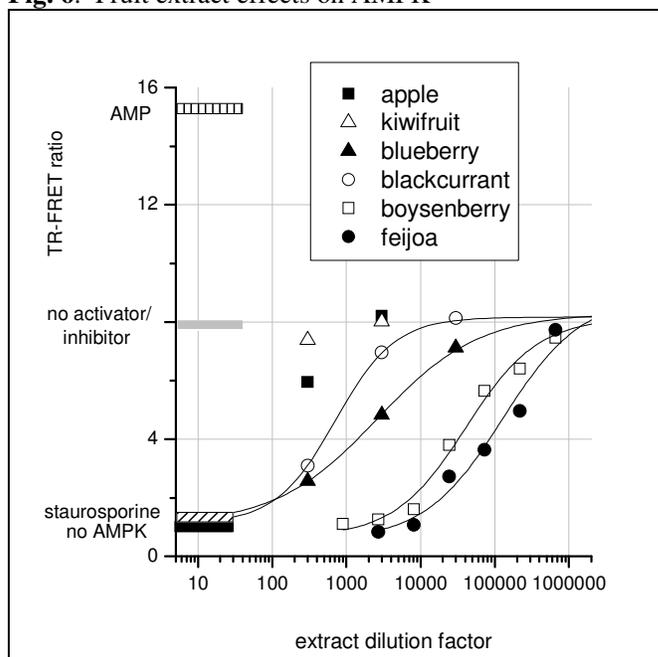
It should be noted that these extracts were produced differently from the extracts tested in the JAK2 assay. However, as with JAK2, strong AMPK kinase inhibition by a fruit extract correlates with presence of ellagitannins. The inhibition of AMPK by ellagic acid and by ellagitannin containing extracts agrees with published data showing 'broad-spectrum' kinase inhibition by ellagic acid. [16, 24] JAK2, a tyrosine kinase, and casein Kinase II, a serine threonine kinase, are both inhibited in the nanomolar range by ellagic acid. However, the observed inhibition of AMPK by apigenin and resveratrol are somewhat at odds with published data. The data reported here are from a direct assay on purified AMPK, whereas apigenin and resveratrol treatment of keratinocytes [20] and adipocytes [18] leads to AMPK activation within the cells. It therefore seems likely that, although these compounds are AMPK activators in whole cells, they do not exert their effects directly on AMPK. Conceivably

these AMPK activating phytochemicals may exert their influence on AMPK regulators within the cells. For example AMPK activity is suppressed by dephosphorylation of threonine 172 by phosphatase 2A. [45] Thus inhibition of an AMPK control protein may lead indirectly to AMPK activation. An alternative explanation may be that apigenin and resveratrol are able to directly inhibit protein kinase A (PKA). [46] PKA phosphorylates AMPK at serine-173 and thereby prevents AMPK activation via phospho-threonine-172. Thus inhibition of PKA might conceivably result in AMPK activation depending on the relative affinities of the phytochemicals for AMPK compared with its control proteins phosphatase 2A or PKA.

Ellagitannins have a widespread occurrence in human foods[28] including several not tested in this study (Table 3).

Table 3 Botanical classification of some known ellagitannin/ellagic acid containing foods

Food / plant material	This study	Species / Order	Ellagitannin reference
Blackberry	yes	*Rubus fruticosus /* Rosales	37
Boysenberry	yes	*Rubus ursinus* × *idaeus/* Rosales	31
Pomegranate	yes	*Punica granatum /* Myrtales	40
Rosehip	yes	Rosa rugosa / Rosales	26
Strawberry	yes	*Fragaria* × *ananassa /* Rosales	47
Muscadine Grape	no	*Vitis rotundifolia /* Vitales	48
Oak (for wine casks)	no	*Quercus petraea /* Fagales	49
Walnut	no	*Juglans regia /* Fagales	50
Almond	no	*Prunus amygdalus /* Rosales	51

We predict that these other foods could be JAK2 inhibitors *in vitro*, although ellagitannin structures vary depending on their source. Sanguiin H-6 is the major ellagitannin in the order Rosales, castalagin is the major ellagitannin component of chestnut and oak (the order Fagales) while punicalagin is a major component of pomegranate ellagitannins (order Myrtales). Many toxic plant species also produce ellagitannins. For example *Euphorbia* species contain ellagitannins but also highly toxic phorbol esters and the structurally related ingenols.[52]

Ellagitannins are also known inhibitors of various tyrosine and serine/threonine kinases including JAK2[16], human epidermal growth factor receptor [33] Protein Kinase C [53], Protein Kinase A and Casein Kinase II. [54] Here we show that ellagitannins are also potent inhibitors of AMPK and we report that the ellagitannin Sanguiin H-6 inhibits JAK2 with an IC_{50} of 58nM.

Conclusions

We have shown that potent JAK2 and AMPK inhibitory compounds are common in the human diet. JAK2 and AMPK inhibitory activity is strongly associated with the presence of ellagitannins in these foods. Future studies will be required to determine if dietary ellagitannins have sufficient bioavailability to ameliorate inflammatory bowel disease or influence metabolism through effects on AMPK.

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