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Defining Key Structural Determinants for the Pro-Osteogenic Activity of Flavonoids

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ABSTRACT

Epidemiological studies suggest that fruits and vegetables may play a role in promoting bone growth and preventing age-related bone loss, attributable, at least in part, to phytochemicals such as flavonoids stimulating osteoblastogenesis. Through systematically screening the effect of flavonoids on the osteogenic differentiation of human mesenchymal stem cells *in vitro*, and correlating activity with chemical structure using comparative molecular field analysis (CoMFA), we have successfully identified important structural features which relate to their activity, as well as reliably predicting the activity of compounds with unknown activity. Contour maps emphasised the importance of electronegativity, steric bulk, and a 2-C−3-C double bond at the flavonoid C-ring, as well as overall electropositivity and reduced steric bulk at the flavonoid B-ring. These results support a role for certain flavonoids in promoting osteogenic differentiation, thus their potential for preventing skeletal deterioration, as well as providing a foundation for the lead optimisation of novel bone anabolics.

Bone remodelling is a highly dynamic process with approximately 5% of cortical and 20% of trabecular bone turned over annually.[1] Skeletal integrity is dependent on the balance between osteoclastic-resorption and osteoblastic-formation of bone during cycles of remodelling, however this balance is lost with age. Trabecular bone loss is evident from the age of thirty,[2] and by the age of fifty, up to 42% of total lifetime trabecular bone is lost.[3] Contributing to this imbalance is a reduction in the level of bone formation during remodelling cycles, thought to occur as early as twenty years of age,[4] and is partly associated with reduced osteoblast differentiation and deposition of matrix components.[5] Preventative intervention strategies are therefore paramount for maintaining skeletal homeostasis, and thus life-long skeletal health.

Epidemiological evidence suggests a positive association between fruit and vegetable intake and the accrual and maintenance of bone mass.[6] Although the exact role(s) of fruit and vegetables remain unclear, much evidence from *in vitro* and *in vivo* studies suggest that flavonoids and related dietary phytochemicals could play an important role in supporting skeletal homeostasis via direct actions on osteoclasts and osteoblasts. The flavonols quercetin and kaempferol have gained considerable attention, with *in vitro* reports of their anti-osteoclastogenic activity,[7-9] and ability of both quercetin[10, 11] and kaempferol[10-14] to promote osteoblast differentiation and function in several *in vitro* osteogenesis models. These observations are reinforced *in vivo* where complete recovery of ovariectomy-induced bone loss was observed following rutin (quercetin-3-rutinoside) supplementation in ovariectomised (OVX)-rats,[15] and quercetin in OVX-mice,[16] the latter of these studies associating the outcome predominantly with quercetin promoting bone formation. Osteogenic transplant studies also highlight the bone-anabolic properties of quercetin; promoting engraftment and bone formation in calvarial defect models.[10, 17] Similarly, kaempferol has been demonstrated to protect against OVX-induced bone loss in rats, both by reducing the rate of skeletal turnover, and by increasing bone forming capacity; with a greater number of osteoprogenitor cells in bone marrow examined *ex-vivo*.[13] Further to this,

kaempferol injection into the calvarial periosteum of newborn rats has been shown to promote osteogenesis.[18] These studies are further supported by the bone anabolic effects of kaempferol- and quercetin-rich *Ginkgo biloba* in rat models of OVX- and glucocorticoid-induced osteoporosis.[19-21] Collectively, a convincing argument exists for kaempferol and quercetin's potential to support skeletal homeostasis via anti-osteoclastogenic and pro-osteogenic effects. Less attention however has focussed on other flavonols, of which there are over 600.[22]

Alongside flavonols, flavanones have attracted attention due to the positive effects of a hesperetin glycoside-enriched diet on bone health in OVX-mice,[23] OVX-rats,[24, 25] and healthy young rats,[25] with reports of supressed osteoclast number and bone turnover, and promotion of osteoblastic activity. Certain flavones[26, 27] and flavanols[28] have also been seen to have the potential to influence bone health *in vivo*, adding to the weight of literature exclaiming flavonoids as possible therapeutic agents for the prevention of bone thinning. Although a compelling argument exists for certain flavonoids exerting bone-anabolic effects, the wide-ranging *in vitro* models of osteogenesis examined and concentrations tested (Table S1, Supporting Information), make identifying which flavonoids have the greatest effects, and the structural basis for such activity difficult. Moreover, results using animal cells and transformed cell lines may be influenced by interspecies variation[29] and variable phenotypic profiles [30] respectively. Thus we investigated the influence of flavonoids and related dietary phytochemicals on the osteogenic differentiation of primary human bone marrow-derived mesenchymal stem cells (hMSCs), the common precursor of the osteogenic lineage.

We report the differential effects of a panel of 21 flavonoids and related phytochemicals, spanning the major flavonoid subclasses, on osteogenic differentiation within hMSC cultures. Using alkaline phosphatase (ALP) activity, an established osteogenic marker expressed between the late osteoprogenitor and preosteoblast stages of differentiation[31, 32] we have employed three-dimensional quantitative structure activity relationship (3D QSAR) modelling with comparative molecular field
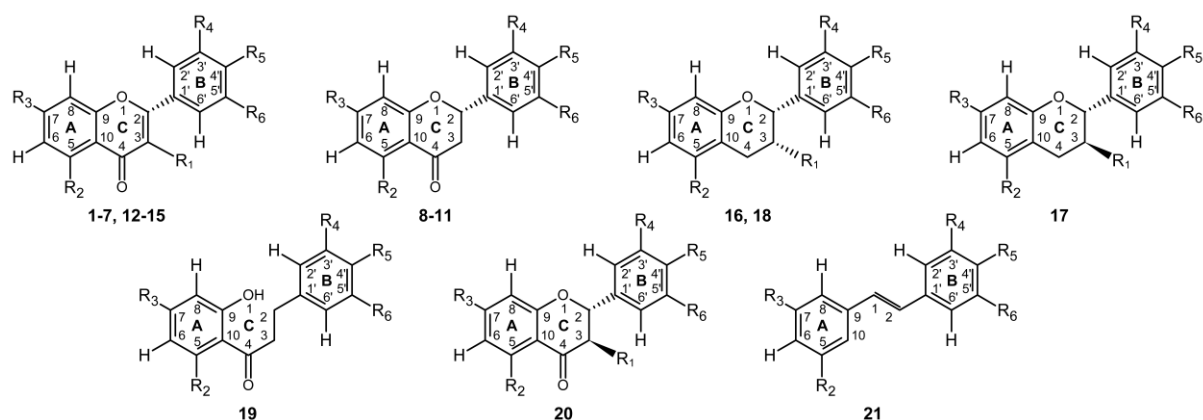
analysis (CoMFA) to relate the observed pharmacological activity of these compounds to their chemical structures.

Here we describe, for the first time, the effects of multiple flavonoids on the osteogenic differentiation of hMSCs, and discuss the structural basis for their activity. Briefly, seven flavonols (tamarixetin, kaempferide, kaempferol, galangin, quercetin, fisetin, and isorhamnetin) and two flavanones (hesperetin and naringenin) stimulated osteogenic differentiation of hMSCs. CoMFA contour maps emphasised the importance of electronegativity, steric bulk, and a 2-C–3-C double bond at the flavonoid C-ring, as well as overall electropositivity and reduced steric bulk at the flavonoid B-ring.

RESULTS AND DISCUSSION

The Effect of Flavonoids on the Osteogenic Differentiation of hMSCs. Twenty-one flavonoids, and related dietary compounds (Figure 1), were screened for effects on osteogenic differentiation, using ALP activity as a marker of osteogenic differentiation (Figure 2).

No flavonol increased ALP activity compared to the vehicle control (VC) following 1 µM treatment; significant increases were observed following 5 and 10 µM treatments with all flavonols except myricetin (**7**). 5 µM treatment with active flavonols increased ALP activity to approximate levels of between 2.37- and 1.52-fold compared to VC in the following order of magnitude: kaempferol (**2**) > tamarixetin (**1**) > quercetin (**4**) > fisetin (**5**) > galangin (**3**) > isorhamnetin (**6**). The order of magnitude was slightly different following 10 µM treatment (tamarixetin (**1**) > kaempferol (**2**) > galangin (**3**) > quercetin (**4**) > fisetin (**5**) > isorhamnetin (**6**)) with increases of between 3.01- and 1.32-fold over the VC; only tamarixetin (**1**), kaempferol (**2**) and galangin (**3**) induced a dose-dependent response between 5 and 10 µM. In contrast to other flavonols tested, at 5 and 10 µM, myricetin (**7**) significantly reduced ALP activity by approximately 0.74- and 0.62-fold of the VC.

Figure 1. Structure of compounds screened in the model of osteoblast differentiation. Compounds are numbered 1 to 21 and their respective substituents (R-groups) are highlighted in the table. Numbers within skeletal structures represent atom nomenclature.

#	Compound	R_1	R_2	R_3	R_4	R_5	R_6
1	tamarixetin	OH	OH	OH	OH	OCH_3	H
2	kaempferol	OH	OH	OH	H	OH	H
3	galangin	OH	OH	OH	H	H	H
4	quercetin	OH	OH	OH	OH	OH	H
5	fisetin	OH	H	OH	OH	OH	H
6	isorhamnetin	OH	OH	OH	OCH_3	OH	H
7	myricetin	OH	OH	OH	OH	OH	OH
8	hesperetin	-	OH	OH	OH	OCH_3	H
9	naringenin	-	OH	OH	H	OH	H
10	eriodictyol	-	OH	OH	OH	OH	H
11	homoeriodictyol	-	OH	OH	OCH_3	OH	H
12	diosmetin	H	OH	OH	OH	OCH_3	H
13	apigenin	H	OH	OH	H	OH	H
14	flavone	H	H	H	H	H	H
15	luteolin	H	OH	OH	OH	OH	H
16	epicatechin (2R,3R)	OH	OH	OH	OH	OH	H
17	catechin (2R,3S)	OH	OH	OH	OH	OH	H
18	EGCG (2R,3R)	Gal	OH	OH	OH	OH	OH
19	phloretin	-	OH	OH	H	OH	H
20	taxifolin (2R,3R)	OH	OH	OH	OH	OH	H
21	resveratrol	-	OH	OH	H	OH	H

No flavanone significantly increased ALP activity above the VC following 1 or 5 µM treatment, with only hesperetin (**8**) and naringenin (**9**) inducing a significant increase following 10 µM treatment (1.72- and 1.53-fold respectively). Neither eriodictyol (**10**) nor homoeriodictyol (**11**) had a significant effect on ALP activity.
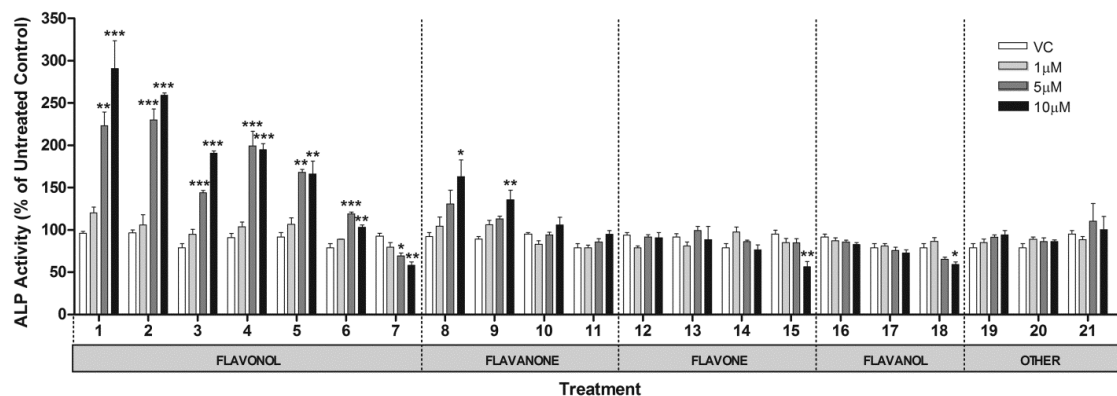
Figure 2. The effects of dietary flavonoids and related phytochemicals on the osteogenic differentiation of hMSCs. Passage three MSCs were sub-cultured in osteogenic differentiation (Os.D) medium and treated with 0-10 μM phytochemicals at days 0, 3, and 6. Cultures were assayed for ALP activity and cell number at day 9. Values are expressed as means of percentage of untreated control from 3 independent triplicate experiments with error bars representing SEM and asterisks representing significance compared to the vehicle control value (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$). Phytochemical names corresponding to treatment numbers are displayed in Figure 1.

Of the flavones tested in this study, diosmetin (**12**), apigenin (**13**) and flavone (**14**) had no effect on ALP activity at any concentration. Luteolin (**15**) induced a significant decrease in ALP activity following 10 μM treatment to approximately 0.59-fold of the VC.

No flavanol tested significantly increased ALP activity at any concentration. Whilst (2*R*,3*R*)-epicatechin (**16**) and (2*R*,3*S*)-catechin (**17**) had a negligible effect, (2*R*,3*R*)-epigallocatechin-3-*O*-gallate (**18**) induced a significant decrease in ALP following 10 μM treatment to approximately 0.75-fold of the VC.

Of the related compounds tested, neither phloretin (**19**), (2*R*,3*R*)-taxifolin (**20**), nor resveratrol (**21**) had an effect on ALP activity at any concentration tested.

The Effect of Flavonoids on the Proliferation and Viability of hMSCs. Effects of treatments on cell number (Figure S1, Supporting Information) and viability (Table S2, Supporting Information) were also examined. Of the compounds tested, only kaempferol (**2**) and hesperetin (**8**) significantly increased cell number above the level of the VC. Four flavonols, four flavones, one flavanol, and the stilbene resveratrol decreased cell number. With the exception of the flavanols (2*R*,3*R*)-epicatechin (**16**) and (2*R*,3*R*)-epigallocatechin-3-*O*-gallate (**18**), no treatment significantly reduced cell viability suggesting anti-proliferative activity of a number of flavonoids, most notably within the flavone subclass with decreases in cell number as low as 0.43±0.05-fold of the VC.

QSAR Model Generation for the Relationship Between Flavonoid Structure and ALP Activity. Using 3D QSAR modelling with CoMFA, models were developed that predict the effect of phytochemical treatment at 5 and 10 μM concentrations on ALP activity in hMSC cultures. The training set consisted of ALP activity data (expressed as log10 ALP fold increase over the vehicle control) for 18 compounds (refer to Figure 1 for training set structures). Kaempferol (**2**), eriodictyol (**10**) and EGCG (**18**) data were removed to establish a test set. Both low-energy (built) conformers and PDB-extracted conformers (Table S2, Supporting Information) for each compound were aligned to flavone by overlapping atoms 1, 5-10 and 1′- 6′. This allowed a satisfactory superimposition of all major areas of the compound; importantly, B- and C-rings of flavonoids, where the majority of structural variation in the training set occurred (Figure 3).
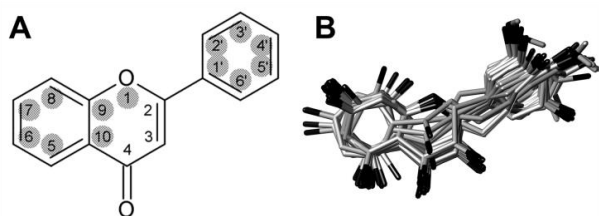

Figure 3. Alignment rule. Conformers were aligned by common core alignment to flavone, overlapping atoms 1, 5-10 and 1′- 6′ (shaded: panel A). This delivered an alignment with appropriate

superimposition of flavonoid A-, B-, and C-rings (see lateral view: panel B), as well as directional alignment of functional groups (oxygen atoms of hydroxyl and methoxy groups highlighted in black).

Statistical models were generated using partial least squares (PLS) regression analysis. Linear regression plots between observed activity within the training set and predicted activity based on molecular descriptors had a good correlation at both 5 μM ($r^2 = 0.768$; Figure 4A, Table 1) and 10 μM ($r^2 = 0.767$; Figure 4B, Table 1), signifying that variances in molecular fields account for approximately 77% of the variances in training set activity. Table S3, Supporting Information presents all CoMFA-generated values including residual values (mean observed value minus mean predicted value) and standard deviations of prediction between different conformers processed per compound. Both models had an optimum component number of 5 (Table 1).
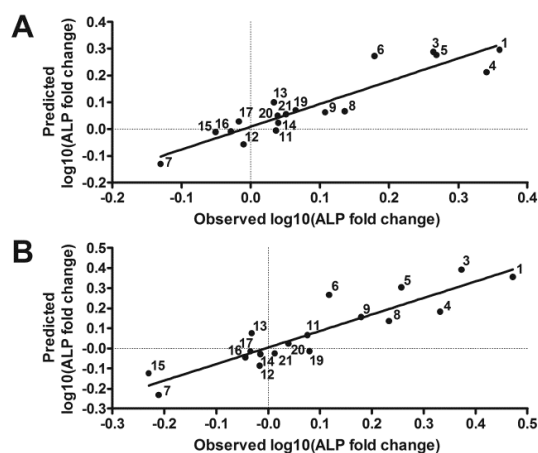

Figure 4. Linear correlation between observed and predicted values generated from CoMFA for ALP activity at 5 μM (A) and 10 μM (B). Observed values (x-axis) are correlated with CoMFA-generated predicted values based on steric and electrostatic field variations. Predicted values are expressed as the mean of all individual conformer predictions and numbers indicate compounds (as listed in Figure 1). Corresponding CoMFA-generated values are presented in Table S3, Supporting Information.

Table 1. Partial Least Squares Analysis for CoMFA Models

	$r^{2\,a}$	$q^{2\,b}$	SE ^{c}	comp. ^{d}
5 μM model	0.768	0.466	0.077	5
10 μM model	0.767	0.420	0.098	5

^{a}correlation coefficient; ^{b}cross-validated correlation coefficient; ^{c}standard error for the prediction of log10(ALP fold change); ^{d}number of components used in CoMFA model.

The predictive potential of the model was quantified using leave-one-out cross-validation. q^2 values for 5 and 10 μM models were 0.466 and 0.420 with standard errors of prediction of 0.077 and 0.098 respectively (Table 1). Although these appear low, the q^2 value is a conservative estimate as its calculation involves removing data so true predictability can be underestimated.[33] Therefore, models were further validated using a test set of molecules.

Test Set of Molecules for Model Validation. The test set consisted of three compounds with known activity ("known compounds": kaempferol (**2**), eriodictyol (**10**) and EGCG (**18**)), previously removed from the training set, and three with unknown activity, ("blind compounds": kaempferide (**T1**), pinocembrin (**T2**) and tricetin (**T3**)), which were predicted using the QSAR models and assayed in the model of osteoblast differentiation at a later date (see Figure 5 for structures).
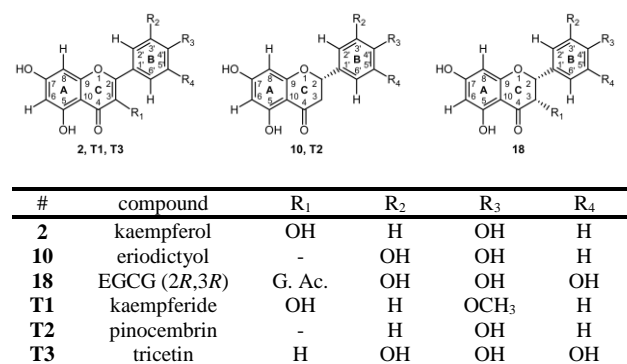

#	compound	R_1	R_2	R_3	R_4
2	kaempferol	OH	H	OH	H
10	eriodictyol	-	OH	OH	H
18	EGCG (2R,3R)	G. Ac.	OH	OH	OH
T1	kaempferide	OH	H	OCH$_3$	H
T2	pinocembrin	-	H	OH	H
T3	tricetin	H	OH	OH	OH

Figure 5. Test Set Compound Structures. The structures and respective substituents (R-groups) of test compounds with known activity (**2**, **10** and **18**) or unknown activity (**T1**, **T2** and **T3**) are indicated. Numbers within skeletal structures represent atom nomenclature.

QSAR model predictions for compounds with known activity correctly identified kaempferol (**2**) as being active, eriodictyol (**10**) as having a negligible effect and EGCG (**18**) as having a detrimental effect on ALP activity in hMSCs after 9 days in culture. Both 5 and 10 μM models are relatively conservative in their prediction of the level of ALP activity in response to kaempferol (**2**) with predicted values approximately 78% and 76% of the mean observed activity in cultures treated with 5 and 10 μM kaempferol (**2**) respectively. Models also overestimated the detrimental effect of EGCG (**18**) with predicted activity approximately 1.9- and 2.6-fold lower than actual activity in 5 and 10 μM models respectively (Table 2).

Table 2. Known Test Set Predicted Values and Observed ALP Activity Values.

#	5 μM model			10 μM model		
	obsd. [d] (%)	pred. [e] (%)	res. [f] (%)	obsd. [d] (%)	pred. [e] (%)	res. [f] (%)
2 [a]	237*	185	52	268*	205	63
10 [b]	98	110	-16	111	107	4
18 [c]	84	44	40	75*	29	46

[a]kaempferol (**2**), [b]eriodictyol (**10**), [c]EGCG (**18**). [d]observed; [e]predicted; [f]residual. *$p<0.05$ compared to control when tested experimentally.

Of the blind compounds within the test set, kaempferide (**T1**) was predicted to increase ALP activity at 5 μM (180% of VC) and 10 μM (248% of VC), pinocembrin (**T2**) to have a slight detrimental effect on ALP activity at 5 μM (81% of VC) but negligible effects at 10 μM (96% of VC), and tricetin (**T3**) to decrease ALP activity at 5 and 10 μM (90% and 64% of VC respectively).

When tested experimentally, kaempferide (**T1**) increased ALP activity in hMSC cultures dose-dependently with significant elevation following treatment with 1, 5 and 10 μM to 143±6%, 237±23% and 281±30% of the VC respectively (Figure 6). As with kaempferol (**2**), QSAR models underestimated activity, with predicted values 71% and 88% of the observed value in 5 and 10 μM models respectively (Table 3), but successfully identified an active compound. Pinocembrin (**T2**) had no significant effect on ALP activity at any concentration tested (Figure 6). Although the exact values

for pinocembrin (**T2**) predicted by QSAR models are lower than actual values for ALP activity (Table 3), the successful prediction of an inactive compound is highlighted. Finally, tricetin (**T3**), whilst having no significant effect on ALP activity at 1 or 5 µM, significantly decreased ALP activity following 10 µM treatment to a level of 79±7% of the VC (Figure 6). These predictions accurately described tricetin (**T3**) as a compound that has a detrimental effect on osteogenic differentiation at the higher concentration tested (Table 3).
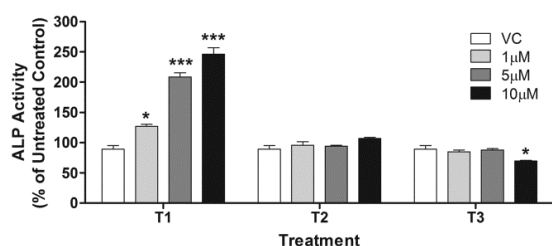

Figure 6. The effects of blind test compounds on the osteogenic differentiation of hMSCs. Passage 3 MSCs were sub-cultured in Os.D medium and treated with 0-10 µM kaempferide (**T1**), pinocembrin (**T2**) and tricetin (**T3**) at days 0, 3, and 6. Cultures were assayed for ALP activity at day 9. Values are expressed as means of percentage of control from 3 independent triplicate experiments with error bars representing SEM and asterisks representing significance compared to the vehicle control value (***$p < 0.001$; *$p < 0.05$).

Table 3. Blind Test Set Predicted Values for ALP activity.

#	5 µM model			10 µM model		
	obsd.[d] (%)	pred.[e] (%)	res.[f] (%)	obsd.[d] (%)	pred.[e] (%)	res.[f] (%)
T1[a]	237*	170	67	281*	248	33
T2[b]	107	81	26	121	96	25
T3[c]	99	90	9	79*	64	15

[a]kaempferide (**T1**), [b]pinocembrin (**T2**) [c]tricetin (**T3**). [d]observed; [e]predicted; [f]residual. *$p<0.05$ compared to control when tested experimentally.

In summary, QSAR models successfully predicted kaempferol (**2**) and kaempferide (**T1**) as being active, eriodictyol (**10**) and pinocembrin (**T2**) as having little effect, and EGCG (**18**) and tricetin (**T3**) as having little or a detrimental effect. The observed results follow these predictions, although estimates for active compounds in the test set are relatively conservative at both 5 and 10 μM (summarised in Figure 7A and C respectively). The linear correlation between observed and predicted activity for known and blind predictions is relatively good for 5 μM (r^2=0.895) and 10 μM (r^2=0.995) models (Figure 7B&D), demonstrating the predictive capacity of QSAR models.
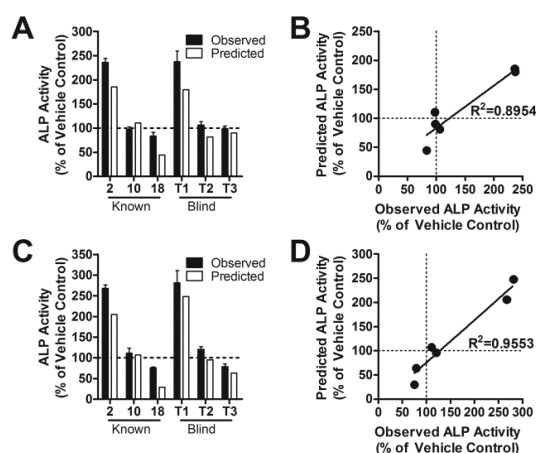

Figure 7. Correlation between observed and predicted activities in the test set. Observed and predicted values for the ALP activity of known and blind compounds (kaempferol (2), eriodictyol (**10**), EGCG (**18**), kaempferide (**T1**), pinocembrin (**T2**), tricetin (**T3**) are presented as percentage of vehicle control in response to 5 μM (A) and 10 μM treatment (C). The respective linear correlations between observed and predicted values are represented for the 5 μM (B) and 10 μM (D) QSAR models.

Examination of Structure-Activity Relationships. CoMFA contour maps for 5 and 10 μM models were examined for regions where variations in steric and electrostatic molecular fields contribute to changes in training set compound activity. Maps for both models were essentially

identical (inset Figure 8Ai), also reflected by the similarity in r^2 values (0.768; 5 µM and 0.767; 10 µM). Therefore, the following description of contours is considered applicable to both models.
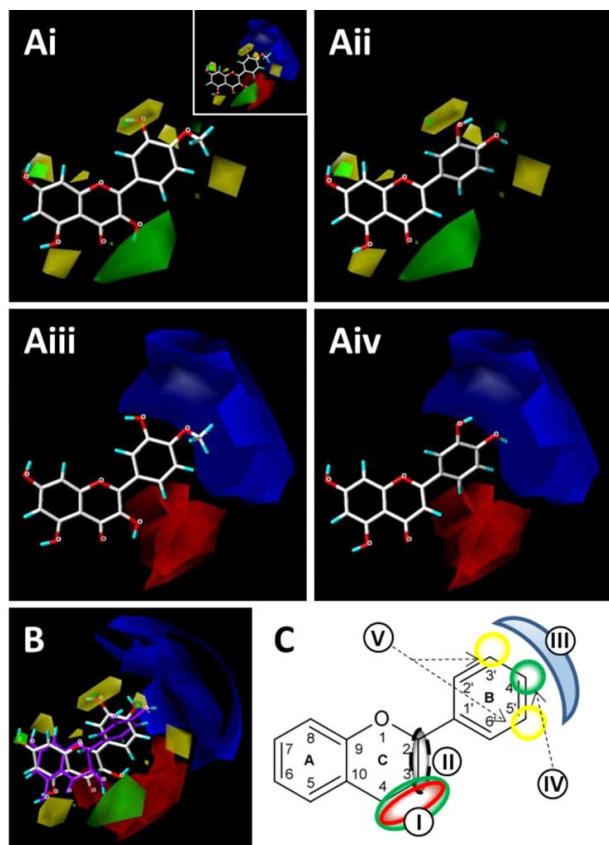

Figure 8. QSAR contour maps derived from CoMFA for ALP activity in response to flavonoid treatment. A: Contour maps representative of the most active compound: tamarixetin (**1**) (Ai & Aiii) and least active compound: luteolin (**15**) (Aii & Aiv) in the 10 µM training set. Inset figure represents contours for the 5 µM training set. B: Contour maps displaying the influence of a 2-C−3-C double bond on conformation. Green and yellow contours highlight steric fields (green: steric bulk is favourable, yellow: steric bulk is unfavourable). Blue and red contours highlight electrostatic fields (blue: positive charge is favourable, red: negative charge is favourable). Contours are calculated as the model Stdev*Coeff. C: A summary of molecular regions, derived from CoMFA, important for the osteogenic activity of flavonoids; (I) steric bulk (shaded green) and electronegativity (shaded red) at the C-3 and

C-4 positions of the C-ring; (II) unsaturation of the 2-C–3-C of the C-ring (shaded grey) ensuring planarity; (III) an overall positive charge at the B-ring (shaded blue); (IV) steric bulk at the C-4′ position of the B-ring (shaded green); and (V) reduced steric bulk at the C-3′ and C-4′ positions of the B-ring (shaded yellow).

Figure 8 presents the contour maps for the most active training set conformer: tamarixetin (**1**), and the least active conformer: luteolin (**15**). Steric fields (Figure 8Ai-ii) suggest that the largest region where steric bulk is favourable (green) is at the C-3 and C-4 positions of the C-ring. Contributing to steric bulk here is the hydroxyl group at the third carbon (OH-3), present in the flavonol tamarixetin (Figure 8Ai) but not in the flavone luteolin (Figure 8Aii). This region is also associated with a red contour suggesting negative charge is favourable, supporting the importance of the OH-3 group (Figure 8Aiii-iv). The 3.1-, 2.8-, and 3.6-fold greater activity of the flavonols tamarixetin (**1**), kaempferol (**2**) and quercetin (**4**) compared to their respective flavone analogues diosmetin (**12**), apigenin (**13**) and luteolin (**15**) at 10 μM highlight the benefit of a OH-3. Also associated with these contours is the ketone group at the fourth position of the C-ring (oxo-4), although the only compounds without an oxo-4 group (catechin (**17**) and epicatechin (**18**)) are non-planar due to 2-C–3-C bond hydrogenation, so a direct comparison cannot be made.

Indeed, a 2-C–3-C double bond at the C-ring appears crucial for flavonol activity, as exemplified by the comparison of quercetin (**4**) with taxifolin (**20**). Whilst quercetin has a 2-C–3-C double bond with associated planarity, taxifolin does not, resulting in flexibility and subsequent inactivity. An overlay of quercetin and taxifolin (purple) is displayed in Figure 8B showing the unfavourable conformation of taxifolin, with OH-3 and oxo-4 groups positioned away from regions of steric- and electronegative-favourability. In the absence of a OH-3 group however, it appears favourable to have a hydrogenated 2-

C–3-C bond as highlighted by the comparison of the flavanones hesperetin (**8**) and naringenin (**9**), both active at 10 μM, with the flavones diosmetin (**12**) and apigenin (**13**), both inactive at 10 μM.

At the B-ring, there is a small steric-favourable region at the C-4′ position that corresponds well with the methoxy group present in tamarixetin (**1**) (Figure 8Ai) but not in luteolin (**15**) (Figure 8Aii). The benefit of C-4′-methylation is supported by the 1.4-fold greater activity of tamarixetin (**1**) compared to quercetin (**4**) at 10 μM, and the high activity of the test compound kaempferide (**T1**). There is also a small steric-favourable region at the C-3′ position, likely associated with the plane that conformers are laying within contours, with the luteolin conformer's OH-3′ being brought closer to the yellow unfavourable region (Figure 8Ai-ii). The steric-unfavourable contour at the 3′ position can be further associated with the detrimental effect of bulky substitution here as illustrated by the 1.4-fold lower activity of isorhamnetin (**6**) (OCH$_3$-3′) compared to quercetin (**4**) (OH-3′) at 10 μM. Also unfavourable is steric bulk at the 5′ position of the B-ring (Figure 8Ai-ii), where substitution here (e.g. myricetin (**7**)) was associated with a low level of activity.

Also at the B-ring, there is a large blue contour covering the 3′, 4′ and 5′ positions: a region where positive charge is favourable for activity whereas negative charge is unfavourable. This is highlighted by comparing the most active (Figure 8Aiii) and least active (Figure 8Aiv) compounds in the training set, where an OH-3′, OCH$_3$-4′ configuration conveys better activity than OH-3′, OH-4′ substitution. A similar relationship occurs within flavonol and flavanone subclasses (i.e. tamarixetin (**1**) > quercetin (**4**); hesperetin (**4**) > eriodictyol (**10**)). Multiple hydroxylation at the B-ring was associated with the attenuation of activity as exemplified by myricetin (7), EGCG (**18**), and tricetin (**T3**) where hydroxyl groups at 3′, 4′ and 5′ positions, conveying strong electronegativity, were unfavourable for activity.

In addition to contours at the C and B rings, there are also steric-favourable and unfavourable regions at the fifth and seventh positions of the A-ring (Figure 8Ai-ii). The majority of compounds within the training set were hydroxylated at these positions so this is likely due to compound flexibility.

This is illustrated by the comparison of quercetin (**4**) with its non-planar analogue taxifolin (**20**) (Figure 8B); where the OH-7 of quercetin is closer to the steric favourable region, the OH-7 and OH-5 of taxifolin are closer to steric unfavourable regions.

CoMFA reveals two major molecular regions that contribute to osteogenic activity within the training set (Figure 8C). Perhaps the most important is at the C-ring, with OH-3 and oxo-4 groups conveying favourable steric bulk and electronegativity, as well as an unsaturated 2-C–3-C bond resulting in planarity. These characteristics are common to flavonols which exhibited the greatest activity within the training set. The second major region is at the B-ring, where steric bulk is unfavourable at all positions except from the 4′ position where bulky substitution may be favourable. Accompanied with this is an overall positive charge of the B-ring suggesting that multiple hydroxylation is unfavourable for activity. Although the exact target(s) for flavonoids in our model of osteoblast differentiation are unknown, these variances in steric and electrostatic interactions are likely to govern binding affinity to target protein(s) and subsequently effect their biological activity.[34]

Conclusions. Seven flavonols (tamarixetin (**1**), kaempferol (**2**), kaempferide (**T1**), galangin (**3**), quercetin (**4**), fisetin (**5**), and isorhamnetin (**6**)) and two flavanones (hesperetin (**8**) and naringenin (**9**)) were found to significantly augment ALP activity in hMSC cultures here for the first time. All other compounds belonging to flavones, flavanols, dihydroflavonols, dihydrochalcones or stilbenes did not significantly increase ALP activity above the vehicle control.

Although many of the compounds tested in our model have previously been investigated for their effect on osteoblastogenesis, outcomes have been highly variable (summarised in Table S1, Supporting Information), demonstrating the requirement for comprehensive screening within a single model. Through executing this, valuable insights have been realised with respect to the molecular properties of flavonoids that convey osteogenic activity in hMSC cultures. The consistent effects of flavonols in our model corroborate the promising observations of kaempferol (**2**) and quercetin (**4**) increasing

osteogenic differentiation *in vitro*[10-14]. This, accompanied by the osteogenic effects of the two flavonols in *in vivo* models,[10, 13, 15-21] highlights an excellent potential for dietary flavonoids belonging to this subclass to promote skeletal health, either by maximising bone mass accrual during growth, or modulating skeletal homeostasis throughout life, thus reducing the lifetime risk of fragility fractures.[2]

We have also demonstrated the capacity for QSAR models to predict the osteogenic activity of compounds; detailing their potential for the prediction of other flavonoids and related compounds with unknown activity, and also for the lead development of novel bone-anabolic treatments. Natural products have been the source of lead compounds for the development of drugs targeted at a number of conditions.[35] Gaining an understanding of the key molecular regions of flavonoids that convey pro-osteogenic activity forms a basis to make analogues with improved pharmacological and/or pharmacokinetic properties. The predominant treatments for osteoporosis are the anti-resorptive bisphosphonates, and although in recent times new bone-anabolic treatments have been developed, these are associated with numerous side-effects[36] highlighting the continued need for better anabolic treatments for bone-metabolic disorders.

EXPERIMENTAL SECTION (full methods available in Supporting Information Methods)

Human Mesenchymal Stem Cell (hMSC) Culture. To assess the impact of test compounds on osteoblast differentiation, proliferation and cell viability, hMSCs, derived from bone marrow aspirates of healthy donors, were cultured at high density in media containing osteogenic supplements (Os.D medium) prior to treatment with test compounds or controls.

Phytochemicals. Of the 24 purchased phytochemicals examined in this study, 22 were determined by HPLC to be ≥95% pure and two were ≥90% pure. All solubilised phytochemicals were serially diluted in Os.D medium to achieve test compound concentrations of 10, 5 and 1 µM. Treated wells were adjusted to have a final concentration of 0.1% (v/v) DMSO and all assays also included a vehicle

control (0.1% (v/v) DMSO only), a positive control (50 nM calcitriol) and an untreated control. All tests within individual experiments were performed in triplicate. Treated cultures were maintained for nine days with media changes and re-application of compounds every three days.

Determination of Osteogenic Differentiation (ALP activity), Cell Number and Viability. Cultures were assessed for ALP activity and cell number on day 9, a time point in our model found to be optimal for minimal variability, and for cell viability on days 1, 4, and 7 (24 hours after every treatment). ALP activity was determined using the *para*-nitrophenyl phosphate-based colorimetric method as described in [37], and data was normalised to time and cell number (μM pNP/min/20,000 cells). Cell number was determined using the methylene blue-based proliferation assay as described in [38] and cell viability was determined using the Alamar blue cytotoxicity assay as described in [39]. All data were expressed as the percentage of the untreated control value.

Quantitative Structure-Activity Relationship (QSAR) Study. Three-dimensional (3D) QSAR models were developed using SYBYL-X (v1.3) molecular modelling software (Tripos). CoMFA descriptors were selected to correlate steric and electrostatic field energy variations with ALP activity in response to 5 and 10 μM treatments. The training set of compounds consisted of 18 phytochemicals with their associated biological activity and the predictive capacity of models was validated using a test set of six compounds.

Statistical Analyses. Statistical analysis was carried out using GraphPad Prism (v4.00). Data are expressed as means of percentage of the untreated control from three independent triplicate experiments ± standard error of the mean (SEM). Statistical comparisons were made using one-way Analysis of Variance (ANOVA; ALP activity and cell number) or two-way ANOVA (cell viability) followed by Bonferroni post hoc tests. *P*-values < 0.05 between treatments and the vehicle control were considered significant (*$p < 0.05$; **$p < 0.01$; ***$p < 001$). For 3D QSAR, statistical analyses were

generated using SYBYL-X (v1.3) (Tripos), and regression models produced using PLS analysis with leave-one-out cross-validation.

ASSOCIATED CONTENT

Supporting Information. (i) A summary of the osteogenic effect of flavonoids from previous *in vitro* studies; (ii) The effects of dietary The effects of dietary flavonoids and related phytochemicals on the proliferation of hMSCs; (iii) The effects of dietary The effects of dietary flavonoids and related phytochemicals on cell viability in hMSC cultures, (iv) CoMFA-generated values for ALP in QSAR models; (v) Known test set predicted and observed ALP activity values; (vi) Blind test set predicted values for ALP activity; (vii) Training set conformer predictions; (viii) Supporting Information Methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare there are no conflicts of interest.

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TABLE OF CONTENTS GRAPHIC

Mesenchymal Stem Cell

Osteoblast

21 Flavonoids and Related Compounds