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Cytotoxicity of Cholesterol Oxides and the Consequences of Relative Molecular Similarity to cGMP Nucleotide

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Abstract

Cholesterol and cholesterol oxides impact on the functional properties of cells, in respect of the intracellular and extracellular distribution of compounds across cell membranes, carcinogenesis and drug resistance. Abnormal levels of cholesterol oxides and steroids in cancerous tissues promote interest in steroid receptor cross-talk during cell-signalling and the steroid metabolome of cancer patients. The research literature links the cytotoxic properties of oxysterols to interference with the NO/cGMP pathway. cGMP participates in cell-signalling and has a molecular structure that relates to cancer-inducing and cancer-preventing agents. This study uses a molecular modelling approach to compare the structures of cholesterol oxides to cGMP. Cholesterol and cholesterol oxide structures fit to a cGMP structural template in several ways, some of which are replicated by corticosteroids and gonadal steroid hormones. The results of this study support the concept that cholesterol oxides modulate cell apoptosis and autophagy via the NO/cGMP pathway and in conjunction with steroid hormones participate in modulating regulation of cell function by cGMP.

Keywords

Cholesterol Oxides, cGMP, Oxysterols, Molecular Similarity, Apoptosis, Autophagy, Cancer

1. Introduction

Several major diseases are attributed to the malfunction of cell mitochondria, endoplasmic reticula and the cell membrane transport proteins that keep the intracellular environment in a state of functional homeostasis. Cholesterol, the precursor of steroid hormones, bile acids and oxysterols, dictates fluidity and

permeability within the cell membrane [1]. Modulation of the cholesterol component of the cell membrane influences the conformation and ATPase activity of P-glycoprotein (MDR1), the ATP-dependent membrane efflux pump [2]. Cholesterol accumulation and oxysterol synthesis within mitochondria decrease the opening-sensitivity of the permeability transition pore [3]. Desensitisation of transporter mechanisms within cells and mitochondria generates pro-apoptotic oxidative stress and mitochondrial dysfunction of relevance to the development of diabetes and cancer, and cancer pharmacology [3] [4].

Investigations of cholesterol as a risk factor in malignancy are complicated by its precursor role and widespread distribution in many chemical forms. Studies on cholesterol oxysterols have demonstrated the promotion of various cancers, pro-apoptotic and cytotoxic effects on tumour cells, and interaction with chemotherapy and steroid hormone receptors [5] [6] [7]. Cell membrane (GPCR) and nuclear receptors (LXR) exist for oxysterols and may participate in the promotion of apoptosis [8]. In endothelial and blood monocyte cultures, the apoptosis-inducing activities of 7β-hydroxycholesterol (7β-HC) and 7-ketocholesterol (7-KC) are rated as greater than those of 25-HC and 5,6-epoxycholesterol (5,6-EC) [9] [10]. Cytotoxic concentrations of these oxysterols induce oxiapoptophagy: ROS (reactive oxygen species) associated cell death with the characteristics of apoptosis and autophagy [11] [12]. Lethal autophagy is another description of cell death attributed to cholesterol metabolites [13]. A wide range of small molecular weight compounds of natural and synthetic origin protect against the cytotoxicity of oxysterols in cell culture. Such compounds include vitamin E (α-tocopherol), docosahexaenoic acid (DHA), ascorbic acid, biotin and methylfumarate [14] [15] [16] [17] [18].

Nucleotide dependent enzyme pathways are integral to cell-signalling and there is considerable interest in the manipulation of tumour cGMP levels to improve outcomes *in vitro* and in clinical settings. cGMP modulates cell ROS and SOCE (store-operated calcium entry), nucleotide binding domains (NBDs), tumour cell apoptosis, autophagy and mitochondrial dysfunction [19]. The presence of cGMP binding sites on multi-drug resistant proteins (MRPs) links the nucleotide to the development of chemotherapy resistance [20]. Some cancer patients receive cGMP-targeted phosphodiesterase medication, although there is considerable debate on the therapeutic value of the nucleotide [21]. A similarly confusing picture is characteristic of *in vitro* studies on the role of cGMP. Individual cell apoptosis-modulating agents including carcinogens, chemotherapy drugs, endogenous hormones and phytochemicals both induce and protect against apoptosis. Such contradictory findings may relate to the molecular similarity of these compounds relative to the nucleotide. The alternative *in-silico* fits of their molecular structures to a cGMP structural template may demonstrate their propensity for engaging with alternative biochemical pathways at NBDs [22].

The major role of cGMP in maintaining mechanisms of cell homeostasis ge-

nerates interest in the capacity of steroid hormones and endogenous sterols to directly influence functional properties of the nucleotide and promote cytotoxicity, as a consequence of altered nucleotide: steroid ratios within cells. The potential for molecular interaction may be evident within compound structures as relative molecular similarity in comparison to the nucleotide; data that may contribute to a better understanding of the impact of biochemical defects within cells and identify compounds suitable for therapy. The aim of this study is to investigate and report on nucleotide relative molecular similarity within the structures of cholesterol, oxysterols, and the compounds modulating their apoptotic and autophagic properties.

2. Materials and Methods

2.1. Compound Structures

The compounds selected for investigation are primarily oxysterol compounds identified in cancer patient tissues [6] [7] and the above small molecular weight inhibitors of oxysterol cytotoxicity. Compounds structures are taken from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/).

2.2. Molecular Modelling

The Nemesis software program (Oxford Molecular version 2.1) is used to build compound structures from the program fragment file and minimise energy values by conformational analysis. The molecular structures used for fitting are minimum energy conformers in an uncharged form. The conformation of the cGMP structure is described by the torsion angle (bond angle formed by 4 adjacent atoms) C8N9C1'O9-33˚ (**Figure 1**). The Nemesis program fits paired molecular structures on a three-point basis. Fitting-points, comprised of atoms of similar type and partial charge within compound and nucleotide structures, are identified in the text and table with respect to the nucleotide labels. Colour-coded atoms in the figures identify ligand fitting-points: carbon-green, nitrogen-blue, oxygen-red, sulphur-yellow. Bond order within the molecular structures is not shown to improve on presentation. The Nemesis program computes goodness-of-fit values, in respect of inter-atomic distance at each fitting-point and root mean square (RMS) value. The sequence of fitting points (given in **Table 1**, left to right) provides the fit with the lowest RMS value.

3. Results

Figure 1 gives six fits of the cholesterol structure (templates 2 - 7) on the cGMP structure (template 1) which all have fitting-points on the purine and ribose-phosphate moieties of the nucleotide. These fits are similar in respect of goodness-of-fit values (**Table 1**). The common sterol core structure of 4 cyclic rings (template 2) enables oxysterol structures, such as 25-HC and 27-HC, to replicate the fits of cholesterol; template 2 and 3 fits are also given by testosterone and template 4 by estradiol (not shown). The keto group of 7-KC (8 and 9) contri-

butes to more exclusive fits, with template 8 using the same fitting points as the chemotherapeutic drug doxorubicin (12). Apoptosis inhibitory compounds, ascorbic acid (10) and dimethylfumarate (11) fit to the ribose-phosphate moiety of cGMP occluding the nucleotide cyclised ring. The O5 lactone ring fit of withaferin A (13), a herbal medicine, contributes to the limited superimposition of this structure on the nucleotide template.

Cholesterol oxides with side-chain C20, C22 and C24 hydroxyl groups target O6, O7 and O8 fitting-points on the cGMP template (**Figure 2**) which are not available to the cholesterol structure; 20α-HC fits at O6 and O8, 22(R)-HC and 24(S)-HC at O7 and O8 respectively. Also given are the fits of cholesterol 5,6-epoxide (5) and metabolites of the epoxide: cholestane-3β,5α,6β-triol (6) (cholestane-triol) and tumour promoter 6-oxo-cholestan-3β,5α-diol (OCDO) (7). The cholestane-triol fit is also given by 11-ketotestosterone (not shown). Dendrogenin (8) is an epoxide metabolite derived by enzymatic conjugation with histamine. Two fits of the LXR agonist and androgen receptor antagonist T0901317 are given (9, 10). Of the T0901317 fits, template 10 is more similar to the fit of 22(R)-HC; both LXR agonists induce apoptosis in a similar manner.

Table 1. Values for fitting compound structures to the cGMP template.

Compounds	Fitting points	Interatomic distances (Å)	RMS (Å)
3-methyladenine	O6C5C8	0.02, 0.03, 0.02	0.0067
3-methyladenosine	C6C1'N7	0.01, 0.02, 0.03	0.0048
3-methyladenosine	N7C8O7	0.09, 0.05, 0.11	0.0040
5,6α-epoxide	C4O8C3'	0.05, 0.06, 0.09	0.0225
7-ketocholesterol	C1'C2C6	0.02, 0.06, 0.07	0.0086
7-ketocholesterol	C4C4'C1'	0.11, 0.10, 0.06	0.0084
17β-estradiol	O3C1'C4'	0.10, 0.03, 0.09	0.0024
20αHC	C4'C3'O6	0.14, 0.14, 0.03	0.0105
20αHC	O6C6O8	0.08, 0.11, 0.04	0.0024
22(R)HC	C4C1'O7	0.04, 0.06, 0.02	0.0093
24(S)HC	C8C2'O7	0.02, 0.12, 0.13	0.0102
27HC	C4C1'C3'	0.03, 0.06, 0.04	0.0117
α-tocopherol	C3'O9O8	0,12, 0.08, 0.07	0.0201
ascorbic acid	C4'C2'O3	0.07, 0.07, 0.07	0.0027
betulinic acid	C2'C1N9	0.09, 0.05, 0.11	0.0205
betulinic acid	O7C4'C6	0.08, 0.10, 0.17	0.0073
betulinic acid	C4'C2'N1	0.09, 0.06, 0.02	0.0072
biotin	O9C4'O8	0.06, 0.11, 0.05	0.0000
bufalin	C2O7C2'	0.07, 0.08, 0.10	0.0188

Continued

bufalin	C6C2O7	0.14, 0.04, 0.12	0.0060
cholestane-triol	O8C3'C4	0.06, 0.08, 0.09	0.0184
cholesterol	C4'C2'C2	0.09, 0.04, 0.05	0.0054
cholesterol	C4'C1'C2	0.11, 0.09, 0.06	0.0188
cholesterol	C3'C2'C4	0.05, 0.09, 0.09	0.0141
cholesterol	C4'C2'C2	0.10, 0.07, 0.06	0.0056
cholesterol	C3'C1'C4	0.05, 0.06, 0.03	0.0176
cholesterol	C4'C1'C8	0.07, 0.03, 0.05	0.0038
cortisol	C6C5C2'	0.08, 0.04, 0.04	0.0065
cortisol	C4C1'C3'	0.05, 0.07, 0.07	0.0180
cycloheximide	C3'C2'C6	0.04, 0.09, 0.09	0.0076
cytosine arabinoside	C3'C4'C8	0.01, 0.01, 0.02	0.0003
dendrogenin	C6C5C2'	0.14, 0.08, 0.08	0.0150
DHEA	C4C1'C3'	0.04, 0.06, 0.08	0.0101
diethylstilbestrol	O3C1'C4	0.06, 0.05, 0.02	0.0049
dimethylfumarate	O2C3'O5	0.03, 0.09, 0.10	0.0028
diosgenin	O8C3'C6	0.06, 0.07, 0.12	0.0056
docosahexaenoic acid	O7O5C2	0.13, 0.11, 0.07	0.0090
doxorubicin	C1'C2C6	0.06, 0.06, 0.12	0.0013
fenoldopam	N1C4O3	0.10, 0.13, 0.04	0.0105
fulvestrant	C4C3C1'	0.07, 0.09, 0.07	0.0151
lumisterol	C6C5C2'	0.07, 0.06, 0.01	0.0002
mifepristone	C5C4C2'	0.10, 0.12, 0.03	0.0143
OCDO	C3'C2'C6	0.14, 0.13, 0.10	0.0182
OCDO	C6C5C2'	0.14, 0.08, 0.08	0.0153
QW-1624F2-2	C5C4O7	0.08, 0.07, 0.01	0.0006
riociguat	O6C6C7	0.06, 0.07, 0.01	0.0047
staurosporine	C1'C8C2	0.03, 0.05, 0.05	0.0048
T0901317	O9C8C2'	0.11, 0.08, 0.04	0.0034
T0901317	C3'C1'C5	0.05, 0.03, 0.08	0.0003
tadalafil	N7C8C4'	0.10, 0.10, 0.06	0.0143
tesmilifene	O3C3'C6	0.05, 0.02, 0.07	0.0001
vitamin D	C5C4C7	0.07, 0.07, 0.01	0.0001
vitamin D	C8N9O7	0.10, 0.11, 0.05	0.0064
withaferin A	C6C2O5	0.14, 0.04, 0.13	0.0073

The template fits of cortisol (1) and OCDO (2) in Figure 3 are the same as that given by dendrogenin in Figure 2. OCDO binds to the glucocorticoid receptor (GR) and shares this property, and similar nucleotide template fit, with the anti-progesterone mifepristone (3). The C4C1'C3' fits of structures 4, 5 and 8 are replicated by cholesterol and dehydroepiandrosterone (DHEA) and differ from those given by estradiol (6) and diethylstilbestrol (7). A more exclusive fit of DHEA (9) is not available to cholesterol-based compounds lacking an oxygen substituent. The molecular structures of DHEA and estradiol are very similar; in comparison to the estradiol fit (6) the structure of DHEA (9) is inverted. Diosgenin (10), a commercial source of DHEA, superimposes along the C6-O8 axis of the cGMP template.

Molecular structures of the pentacyclic triterpenoid betulinic acid and buffalin (Figure 4) also superimpose along the length of the cGMP template. Bufalin (4)

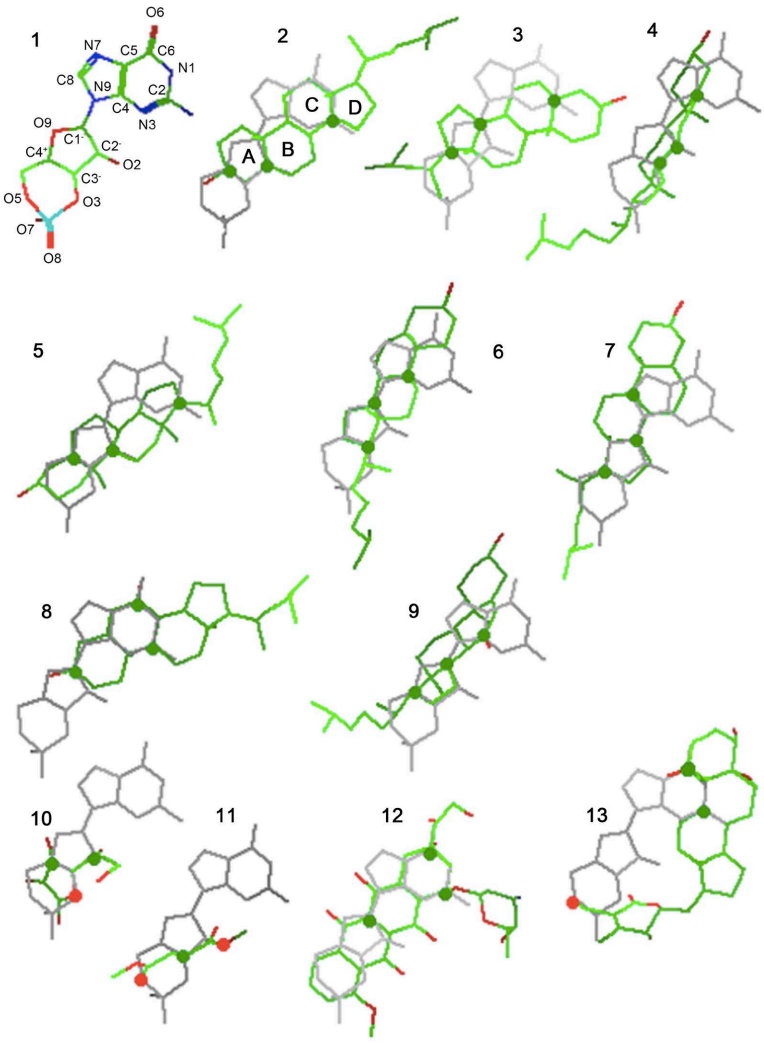

Figure 1. Fits of cholesterol, cholesterol oxides and modulators of apoptosis to cGMP template (grey). 1: cGMP, 2 - 7: cholesterol, 8: 7-ketocholesterol, 9: 7-ketocholesterol, 10: ascorbate, 11: dimethylfumarate, 12: doxorubicin, 13: withaferin A.

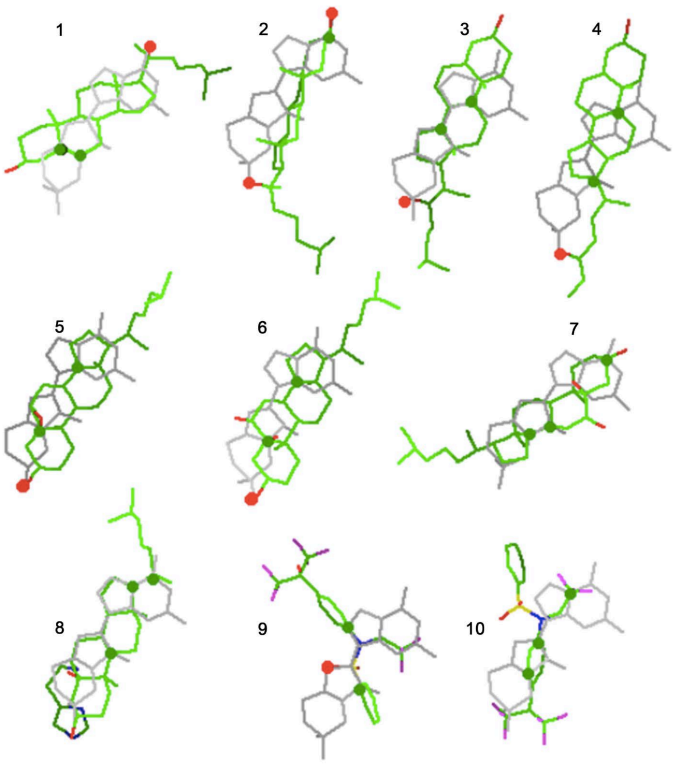

Figure 2. Fits of cholesterol oxides, dendrogenin and compound T091317 to cGMP template (grey). 1: 20*α*-HC, 2: 20*α*-HC, 3: 22(R)-HC, 4: 24(S)-HC, 5: 5,6,*α*-epoxide, 6: cholestane-triol, 7: OCDO, 8: dendrogenin, 9: T091317, 10: T091317.

has a similar template fit to withaferin A (Figure 1). An additional fit of betulinic acid with an O7 fitting-point (C6C4'O7) is not shown as the C6 fitting distance (0.17 Å) is rather high (Table 1). Biotin (5) is a compound that shares antioxidant properties with alpha-tocopherol (6) and DHA (7). Neither biotin or alpha-tocopherol have fitting-points on the nucleotide purine ring. Tesmilifene (8) a novel potentiator of chemotherapy, displays a unique nucleotide template fit. Structural analogues of vitamin D (9, 10) include compound QW-1624F2-2 (11) and the ring-closed structure of lumisterol (12) which provides the cortisol fit given in Figure 3.

Figure 5 includes the structures of compounds in clinical use that increase cGMP levels (1, 2, 3) or find use as pro-apoptotic anti-tumour agents (4, 5, 6). Fenoldopam, tadalafil and riociguat respectively have properties of a peripheral dopamine agonist, PDE5 inhibitor and anti-hypertensive activator of guanylyl cyclase. Minimum energy conformers of the three drugs demonstrate relative molecular similarity to the structure of cGMP, their pharmacological effector molecule. The fits of the three pro-apoptotic structures leave the nucleotide cyclised ring unobstructed. In contrast to some of the above structures that superimpose over the length of the nucleotide template, derivatives of adenine (7, 8, 9) with properties of autophagy inhibition share a preference for the purine ring.

Nucleotide template fitting-values given in Figures 1-5 (Table 1) range from 0.01 - 0.14 Å (interatomic distance) and 0.001 - 0.0225 Å (RMS value).

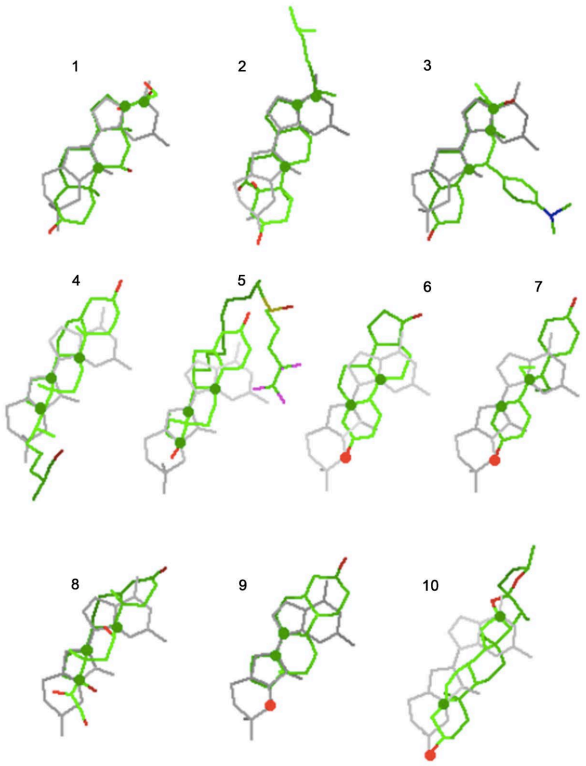

Figure 3. Fits of cholesterol derivatives and drug structures to cGMP template (grey). 1: cortisol, 2: OCDO, 3: mifepristone, 4: 27-HC, 5: fulvestrant, 6: estradiol, 7: diethylstilbestrol, 8: cortisol, 9: DHEA, 10: diosgenin.

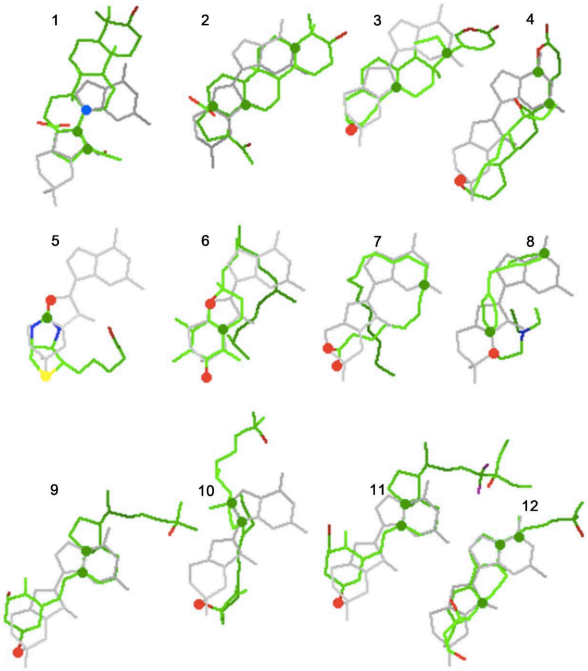

Figure 4. Fits of apoptosis and autophagy modulating compounds to cGMP template (grey). 1: betulinic acid, 2: betulinic acid, 3: bufalin, 4: bufalin, 5: biotin, 6: *α*-tocopherol, 7: docosahexaenoic acid, 8: tesmilifene, 9: vitamin D, 10: vitamin D, 11: QW-1624F2-2, 12: lumisterol.

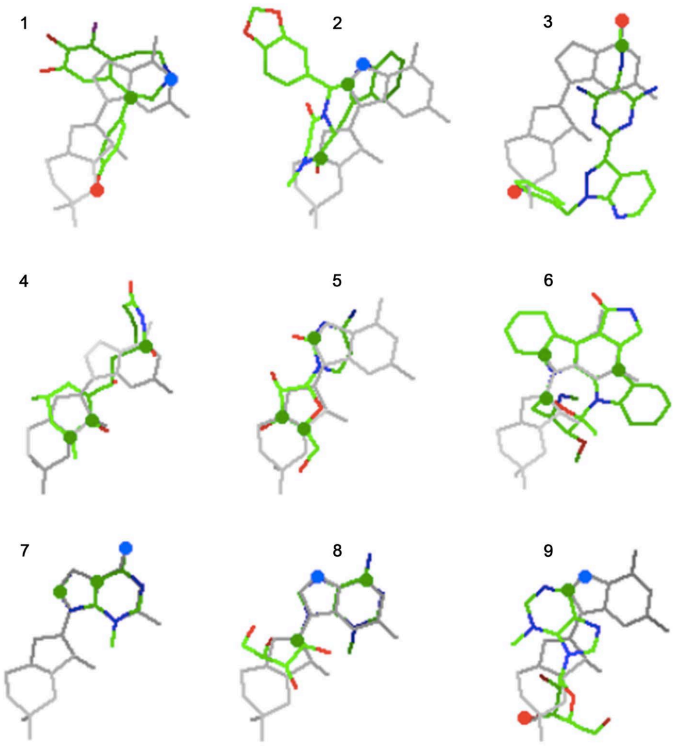

Figure 5. Fits of cGMP modulating compounds and adenine derivatives to cGMP template (grey). 1: fenoldopam, 2: tadalafil, 3: riociguat, 4: cycloheximide, 5: cytosine arabinoside, 6: staurosporine, 7: 3-methyladenine, 8: 3-methyladenosine, 9: 3-methyladenosine.

4. Discussion

Cholesterol provides the core ring structure of steroid hormones and identifies with the structure of cGMP, as illustrated by the cholesterol superimposed nucleotide templates, in respect of molecular size and specific intra-atomic distances. The cholesterol fits are replicated by DHEA, a compound with the property of alleviating oxidative-stress and apoptosis [23] [24] and a potent inhibitor of prostate and mammary gland cancer in the rat [25]. Some template fits of the cholesterol structure are also replicated by cortisol, testosterone and estradiol. Although cholesterol is not a compound with solely benign effects on cell function, cholesterol oxides have a greater capacity for rendering cells dysfunctional via modulation of oxidative stress mechanisms, mitochondrial function and MRPs. The effects of 27-HC on cells include autophagy induction in promonocytic cultures [26], ROS-linked apoptosis in haematopoietic cells [27], decreased colon cancer cell proliferation [28] and block of docetaxel-induced prostate epithelial cell apoptosis [29]. 27-HC is a selective modulator of estrogen receptors [27]. The anti-proliferative effects of 22(R)-HC and 24(S)-HC on breast and prostate cancer cell-lines are also mediated by the AR antagonist T0901317 [30]. All three afore-mentioned compounds are LXR (liver X receptor) agonists, which induce expression of ATP-binding cassette (ABC) transporters involved in cholesterol efflux, promoting reduced intracellular cholesterol levels and cell

proliferation, and stimulation of apoptosis [31]. Although 22(R)-HC and T0901317 structures do not have identical nucleotide template fitting-points their relative molecular similarity to cGMP may be sufficient to modulate cGMP function in the same way. Oxygen and hydroxyl substituents on the core steroid ring structure enable additional fits to the nucleotide template, unavailable to cholesterol and oxysterols with side-chain substituents, and may provide greater affinity to protein receptors of cGMP. 7-KC and cholestane-triol both promote LXR agonist-induced apoptosis of breast and prostate cancer cells [8] whereas cholangocytes cultured in low concentrations of cholestane-triol become resistant to hydrogen peroxide-induced apoptosis [32].

The impact of oxysterols on NO/cGMP biochemistry is evident in studies which have evaluated the cytotoxicity of 7-KC, 25-HC and cholestane-triol on red blood cells, fibroblasts and endothelial cells [33] [34] [35]. The small molecular weight compounds (biotin, vitamin C, α-tocopherol, dimethylfumarate and DHA) that protect against the cytotoxic effects of oxysterols are, in terms of structure, very different in comparison to the oxysterols and each other but not in respect of relative molecular similarity to cGMP. The effects of α-tocopherol and biotin on oxidative stress have been attributed to protection of the redox state of guanylate cyclase and influence on the protein kinase G pathway [36] [37]. DHA induces production of endothelial cell nitric oxide (NO) and the expression of genes associated with down-regulation of PDE5 [38] [39]. α-tocopherol, DHA and dimethylfumarate prevent the cytotoxicity induced in cell cultures by 7-KC [14].

Studies on murine oligodendrocyte death induced by 7β-HC demonstrate attenuation of impaired mitochondrial function and dysfunctional lipid metabolism by biotin, DHA and α-tocopherol [17]. Several earlier studies established a relationship between vitamin D and cGMP through the investigation of guanylyl cyclase activation. Barsony and Marx [40] describe the rapid accumulation of intracellular cGMP in dermal fibroblasts near activated vitamin D receptors. The accumulation of vitamin D and cGMP, in response to adrenocorticosteroids, correlated with GR binding-affinity. Vitamin D induces autophagy with inhibition of oxidative stress and apoptosis in pancreatic beta-cells and breast cancer cells [41] [42]. Supplementation with the vitamin, in the clinical setting, may improve the effectiveness of chemotherapeutic drugs [43].

Several larger compound structures (diosgenin, betulinic acid, bufalin, withaferin A) with template fits that span the length of the nucleotide structure are recognised for their lethal autophagy properties. The generation of ROS and JNK activation by bufalin, a β-hydroxy steroid, induces apoptosis and autophagy in a number of tumour cell lines [44]. Betulinic acid, another compound with apoptotic and autophagic means of inducing cell death has an affinity for estrogen receptors (ER) and stimulates NO generation [12] [45]. Diosgenin inhibits prostate cancer cell proliferation by inducing autophagy and apoptosis [46]. There are, however, some concerns that the cell apoptosis observed when auto-

phagy is inhibited by chemotherapy, radiation, or compounds such as 3-methyladenine and 3-methyladenosine, may not be autophagy related [47] [48]. Tesmilifene-induced apoptosis of MDR cells, associated with increased superoxide and reduced ATP levels, enhances the cytotoxicity of several chemotherapeutic drugs *in vitro* and *in vivo* [49].

The anti-myeloma activity of cholesterol 5,6-epoxide is attributed to oxiapoptophagy; metabolism to OCDO stimulates breast cancer cell growth via GR [12] [50]. Tumour cell proliferation by OCDO, present in higher concentrations in breast cancer tissue, is inhibited by mifepristone [51]. Dendrogenin, a non canonical LXR ligand, degrades hydrogen peroxide and cholesterol 5,6-epoxides via catalase stimulation and lethal autophagy [52]. The nucleotide templates demonstrate the same fit for cortisol, OCDO, mifepristone and dendrogenin. Recent clinical studies question the balance of corticosteroid treatment effects in breast cancer. Cortisol treatment of ER+ tumours is associated with reduced cell proliferation, whereas cortisol inhibition of chemotherapy-induced apoptosis may promote the development and metastasis of ER− tumours [53]. Hydroxycholesterol template fits are replicated by cortisol, progesterone and testosterone, and the dendrogenin fit is replicated by cortisol and progesterone. Additional pro- and anti-apoptotic nucleotide template fits of cortisol, progesterone and testosterone are given in a previous study [54].

Following a systematic review of steroid metabolism in cancers, Anh *et al.* [55] identified estradiol, DHEA, cortisol and estrogen metabolites as oncosteroids. The significance of steroid receptors in cancer subtypes is receiving more attention [55] [56] following recognition that ratios of cancer promoting and inhibitory steroids and their metabolites may determine the status and progression of tumours. There is extensive steroid crosstalk between receptors (GR, ER, PR, AR) during cell-signalling. GR recognises glucocorticoid and progesterone structures with similar affinity, whereas AR binds to the ER element with an antagonistic effect on estrogen [53]. GR has a tumour suppressive role in attenuating AR dependent transcription in prostate cancer. Following an investigation of a substantial metabolome of 36 urinary metabolites in patients with a familial risk of breast cancer, Houghton *et al.* [57] identified six glucocorticoids associated with an increased risk (49% - 161%), including tetrahydrocortisone (THE) and tetrahydrocortisol (THF). Androsterone (AN) and 11-hydroxy-androsterone (11OHAN) were associated respectively with increased risks of 70% and 90%, whereas E1and E2 reduced breast cancer risk. The results are indicative of raised androsterone and cortisol levels in breast cancer patients, as glucocorticoids are all cortisol derived. In regard to the present study, THE and THF structures provide the same **Figure 3** cortisol fit, as the steroid A ring is not involved in template fitting. THE and THF structures are not able to replicate cortisol template fits that use the oxygen fitting-points demonstrated in a previous study [54]. Cortisol, DHEA, AN and 11OHAN replicate the template fits of cholesterol (**Figure 1**, templates 3 and 7); template fit 7 is also given by 11-ketotestosterone.

Research literature documenting the role of cGMP in the transformation of healthy cells into cancerous tissue is limited and confusing. PDE5 inhibitors increase intracellular cGMP by blocking enzymatic hydrolysis of the nucleotide and ABCC5-mediated efflux, producing favourable conditions for the promotion of tumour cell apoptosis and reduced growth [58]. On the other hand, *in vitro* studies report that the NO/cGMP pathway attenuates apoptosis in beta-cells, neuronal cells and prostate cancer stem cells [59] [60] [61]. cGMP age-related disruption of mitochondrial homeostasis is also evident in rats [62]. Breast cancer studies demonstrate the benefits of dopamine and D1R agonists in controlling aggressive tumour cells, *in vitro* and in mouse models, via the cGMP/PKG pathway [63]. Stehle *et al.* [21] implicate the heterogeneity within tumour cell cGMP signalling, attributable to tumour cell type and environment, for contributing to the contradictory results of cancer therapy. A similarly confusing picture is encountered *in vitro*, as the same compounds are reported to possess both positive and negative effects on cell apoptosis [22]. Some variability may be explained by the chemical milieux within which experimental studies are undertaken. Lipids, steroids and other cell products may interfere with agents under investigation, an even more complex problem for studies *in vivo*. Another factor is the assumed nature of an effector agent; for example, a PDE antagonist or dopamine agonist may not necessarily work on the basis of their known properties but on the basis of their molecular structures. A limitation of the present study is that goodness-of-fit values do not provide evidence of pharmacological affinity and in this respect it is difficult to identify the importance of comparative structures for a cGMP binding site, for example cholesterol versus oxysterols structures with ring or side-chain oxygens. A central role for cGMP in the regulation of apoptosis and autophagy is evident, however, from the observation that so many endogenous and exogenous modulators demonstrate relative molecular similarity to the nucleotide structure. Many of these compounds are unlikely to satisfy the enzyme, MDR, ion-channel and conformational change-mediated functions of the nucleotide.

The compound structures linked to the modulation of apoptosis and autophagy processes demonstrate characterisic nucleotide template fitting patterns. The fitting pattern is simplest for the above small molecular weight inhibitors of apoptosis that block the nucleotide cyclised ring. In contrast stimulators of apoptosis fit without blocking the cyclised ring, as is evident for the template fits of hydroxycholesterol compounds in **Figure 2** (templates 1 - 4) and the pro-apoptotic structures in **Figure 5**. Autophagy inhibitors, 3-methyladenine and 3-methyladenosine have a fitting preference for the nucleotide guanine ring. The stimulators of autophagy and lethal autophagy (diosgenin, betulinic acid, bufalin, withaferin A, dendrogenin) relate to the complete nucleotide structure and *in vivo* may be able to displace or replace the nucleotide completely. Many compounds provide several different fits of one minimum energy structure to the cGMP nucleotide template. Extension of this observation to cell nucleotide

receptors may explain how such compounds induce alternative biochemical and bi-functional changes, via activation of different cell pathways. The additive and allosteric effects of compounds on apoptosis and autophagy processes through binding to NBD sites is of relevance to the study of drug resistance. Finally, this study places cholesterol oxides on an extensive list of tumour cell growth modulators that demonstrate relative molecular similarity to the cGMP structure.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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