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ARTICLE TYPE

# Synthesis and anti-cancer evaluation of steroidal diglycoside-pyrazoline hybrids

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A new series of steroidal glycoside pyrazoline functional hybrid constructs (SG-pyrazolines **3a-k**) synthesized were evaluated for *in vitro* anti-cancer cytotoxic activity against a panel of human tumor cell lines of lung, breast, CNS, colon and ovarian. These hybrid constructs were also measured at their respective $IC_{50}$ values on normal cell lines of HMEC and CHO for evaluating the biocompatibility. Several of these new hybrid constructs were found to possess highest growth inhibition activity than the standard cisplatin and support the concept to modulate drug receptor interaction. Regarding the synthesis, firstly a new SG molecule, an extract of *Caralluma gracillis*, was converted to the chalcones (**2a-k**) *via* the condensation of $sp^3$ C-H bonds on methyl keto of D-ring of SG with appropriate substituted benzaldehydes. The cyclocondensation of SG-chalcones (**2a-k**) with hydrazine specifically catalyzed by Ag(I) *N*-heterocyclic carbene (Ag(I)-NHC) in ethanol has produced selectively the SG-pyrazoline hybrids (**3a-k**).

## Introduction

Recently the derivatives of natural products (hybrid constructs), have been receiving considerable attention in pharmaceutical applications.[1] Because, these hybrid constructs are designed from carefully selected components, they provide modulated ligational properties and bioactivity. Steroidal glycosides (SGs) are a type of natural products widely occurring in nature with useful structural features that are prone to further derivatization.[2] It has been recognized that the SGs and their hybrids have potential to develop as drugs for the treatment of a large number of cancer diseases including cardiovascular,[3] autoimmune diseases,[4] brain tumors,[5] breast cancer,[6] prostate cancer,[7] osteoarthritis[8] anti-tumor activity.[9,10b]

N, O and S-heterocycles are also important structural units present in many drugs, natural and synthetic products with potent bioactivity profile.[10] In this context, the combination of steroid and heterocycle unit in one structure (hybrid) would provide synergistic effect in drug action and tunable structure-activity relationship. Previously we have reported the synthesis of hybrid structures of isoxazole-mercaptobenzimidazole[11] and isoxazole-indole,[12] and also described the interplay between electronic effects in determining their anti-inflamatory and analgesic activity. Catalyzed (**i**) cycloaddition of dipoles[13] or (**ii**) cyclo-condensation of chalcones are the facile route to obtain these heterocycles.[14]

It is also noticeable that most of the drugs used in medicine behave as weak acid or base in solution. N-heterocycle drugs with a lone pair of electrons on nitrogen available for protonation are recognized as basic drugs. Among them, pyrazolines are one of the distinctive N-heterocycles those are readily available for protonation in solution and provide oppurtunity to design new SG-pyrazoline functional hybrids.

## Results and Discussion

Herein we describe the facile catalyzed synthesis of SG-pyrazoline hybrids from the chalcones of a SG molecule (12*β*-benzoyloxy-8*β*, 14*β*-dihydroxy-pregn-20-one-3-*O*-[ β-D-oleandropyranosyl-(1→4)- *β*-D-cymaropyranoside] (Figure 1), an extract of *Caralluma gracillis* isolated by one of the authors[15a] of this report and others,[15b,c] and their efficacy in *in vitro* anti-cancer activity (cytotoxic) against five human cancer cell lines (lung, breast, CNS, colon, and ovarian), when compared with cisplatin.

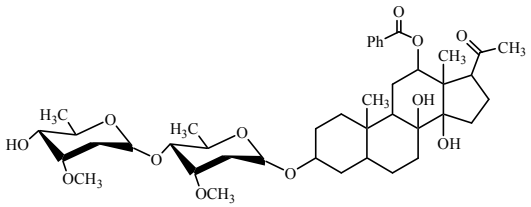
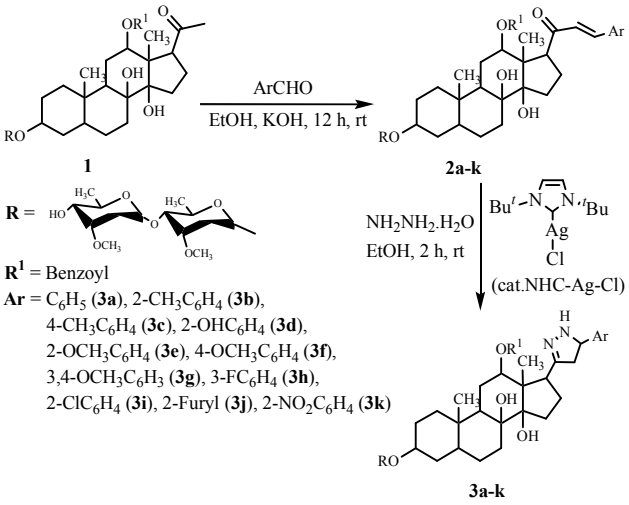


**Figure 1** Structure of Steroidal glycoside (**1**) isolated from *Caralluma gracillis*.

Our exploration began with synthesizing the chalcones (**2a-k**) from the SG molecule (Scheme 1). As shown in Figure 1, the core structure of SG molecule is containing the active $sp^3$ C-H bonds on methyl keto of D-ring, which are useful to form the SG-chalcones. As shown in scheme 1, condensation of the $sp^3$ C-H bond with appropriate substituted benzaldehydes was occurred in the presence of KOH and produced the corresponding chalcones (**2a-k**).



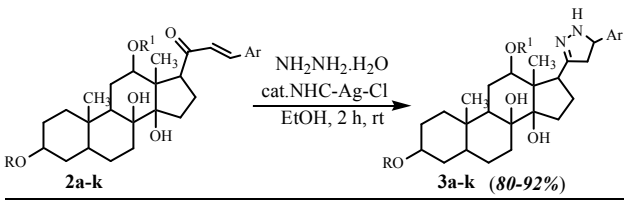

**Scheme 1** Synthesis of D-ring substituted steroidal pyrazolines **(3a-k).**

The synthesized chalcones (**2a-k**) were then subjected for cyclocondensation with hydrazine hydrate in the presence of Ag(I)-NHC as pre-catalyst to obtain SG-pyrazolines **(3a-k)** (Scheme 1). According to previous reports, condensation of chalcones with hydrazine was found to proceed in the presence of variety of catalysts including acetic acid,[16] nucleophilic Lewis base,[17] Ag(I) triflate,[18] $K_2CO_3$, Pd/K-10, tungstophosphoric acid.[19] In view of the significance of Ag(I) catalyst in organic synthesis that driven by specific Lewis acidity, and our experience on Ag(I) complexes of *N*-heterocyclic carbenes (Ag(I)-NHCs) we have investigated the applicability of Ag(I)-NHC catalyst. Instead of using simple Ag(I) salts, the use of Ag(I) complexes as catalysts would be beneficial in terms of catalyst stability, tunable Lewis acidity and efficiency.

Despite of the role Ag(I)-NHCs as carbene transfer agents to synthesize many important transition metal-NHC catalysts as highlighted in many reports,[20] recently Ag(I)-NHCs have also been explored as versatile catalysts in various C-C and C-heteroatom bond forming reactions, ring-opening polymerization and heterocyclization.[21]

In this context, at first we have studied the *in situ* generated Ag(I)-NHC catalyzed condensation between SG-chalcone **2a** and hydrazine hydrate in ethanol. The reaction was accomplished smothly in 2 hrs at room temperature and produced the expected SG-pyrazoline hybrid derivative **3a** as a colorless solid (Table 1, entry 1) without any byproduct. After this, the condensation reactions between SG chalcone **2b-k** and hydrazine were also carried out in ethanol and the details of the synthesized products are given in Table 1.

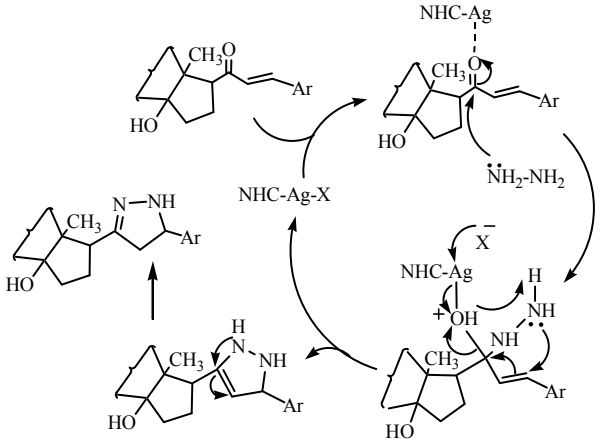
**Table 1** Synthesis of steroidal-diglycoside D-ring substituted pyrazolines (**3a-k**).[a]




| Entry | Ar | Product | yield (%)[b] |
|---|---|---|---|
| 1 | $C_6H_5$ (**2a**) | **3a** | 90 |
| 2 | 2-$MeC_6H_4$ (**2b**) | **3b** | 85 |
| 3 | 4-$MeC_6H_4$ (**2c**) | **3**c | 92 |
| 4 | 2-$OHC_6H_4$ (**2d**) | **3**d | 88 |
| 5 | 2-$OMeC_6H_4$ (**2e**) | **3**e | 80 |
| 6 | 4-$OMeC_6H_4$ (**2f**) | **3**f | 94 |
| 7 | 3,4-di-$OMeC_6H_4$ (**2g**) | **3**g | 92 |
| 8 | 3-$FC_6H_4$ (**2h**) | **3**h | 90 |
| 9 | 3-$ClC_6H_4$ (**2i**) | **3**i | 92 |
| 10 | 2-Furyl (**2j**) | **3**j | 84 |
| 11 | 2-$NO_2C_6H_4$ (**2k**) | **3**k | 90 |

[a]All products were characterized by NMR and mass spectral analysis. [b] Isolated yields after column chromatography.

Structure of the synthesized SG-pyrazoline hybrid compounds **(3a-k)** was established on the basis of elemental analysis and spectral data ($^1$H NMR, $^{13}$C NMR and Mass). The details of the product characterization was presented in the experimental section. A tentative mehanism has proposed in scheme 2 to explain the role of Ag(I)-NHC as a Lewis acid catalyst in the chalcone hyrazine condensation.




**Scheme 2** Possible mechanism for the formation of pyrazolines *via* catalyzed by Ag(I)-NHC.

***In vitro* cytotoxic activity:**

The *in vitro* cytotoxic activity of SG- pyrazoline hybrids **3a-k** and the original SG molecule (**1**) was investigated and compared with standard drug cisplatin. The human cancer cell lines used in the study were (**i**) A-549 (Lung), (**ii**) MCF-7 (Breast), (**iii**) SF-295 (CNS), (**iv**) HCT-15 (Colon) and (**v**) OVCAR-3 (Ovarian). The cytotoxic activity in terms of $IC_{50}$ values i.e. growth inhibition of the cancer cell line in the presence and absence of test material was calcualted uisng the reported methods[22] and the final $IC_{50}$ values are presented in Table 2.

The $IC_{50}$ values presented in Table 2 indicate that as compared to the parent SG molecule all the eleven SG-Pyrazoline hybrids (**3a-k**) posseses strong growth inhibition activity against all the five tested adherent cancer cell lines. However, when compared to the standerd cisplatin, overall these types of hybrid structures were found to exhibit strong or comparable growth inhibition activity specifically against A-549, MCF-7, SF-295 (CNS), HCT-15 (Colon) and OVCAR-3 cell lines. Some of the compounds (**3h** and **3i**) are also highly active against the A-549 (Lung) and MCF-7 (Breast) lines than the standard cisplatin.

**Table 2** *In vitro* cytotoxic activity of steroidal-diglycoside D-ring substituted pyrazolines (**3a-k**) on various human cancer cell lines[a] ($IC_{50}$ µM).[b]

| Entry | Compound | A-549 | MCF-7 | SF-295 | HCT-15 | OVACR-3 |
|---|---|---|---|---|---|---|
| 1 | 3a | 8.38±0.058 | 1.74±0.014 | 0.55±0.013 | 3.15±0.031 | 2.24±0.021 |
| 2 | 3b | 5.92±0.019 | 1.24±0.007 | 0.89±0.017 | 0.35±0.014 | 0.47±0.029 |
| 3 | 3c | 3.76±0.019 | 1.35±0.014 | 0.66±0.011 | 0.36±0.011 | 0.51±0.014 |
| 4 | 3d | 4.56±0.016 | 4.55±0.022 | 1.11±0.013 | 0.48±0.014 | 0.78±0.017 |
| 5 | 3e | 6.85±0.014 | 5.76±0.011 | 1.36±0.008 | 0.78±0.011 | 0.99±0.025 |
| 6 | 3f | 7.19±0.017 | 3.86±0.029 | 0.98±0.005 | 0.96±0.015 | 1.14±0.007 |
| 7 | 3g | 4.95±0.030 | 5.79±0.031 | 1.26±0.007 | 1.44±0.014 | 0.97±0.009 |
| 8 | 3h | 2.50±0.027 | 2.13±0.026 | 0.34±0.014 | 0.26±0.011 | 0.15±0.002 |
| 9 | 3i | 3.14±0.036 | 2.75±0.021 | 0.33±0.008 | 0.14±0.014 | 0.24±0.008 |
| 10 | 3j | 7.42±0.053 | 6.29±0.015 | 2.47±0.021 | 2.18±0.016 | 1.81±0.029 |
| 11 | 3k | 3.71±0.091 | 3.91±0.069 | 0.94±0.034 | 0.35±0.033 | 0.67±0.053 |
| 12 | 1 | 3.77±0.091 | 3.91±0.026 | 0.58±0.047 | 0.35±0.037 | 0.72±0.047 |
| 13 | Cisplatin | 3.55±0.007 | 1.56±0.005 | 0.56±0.006 | 0.18±0.003 | 0.38±0.010 |

[a] Data represent as mean ± SEM values. Cytotoxicity as *$IC_{50}$* for each cell line, is the concentration of compound which reduced by 50% the optical density of treated cell with respect to untreated cells using the MTT assay. [b] Data represent as mean ± SEM values of these independent determinations.

On the other hand, the cytotoxicity of steriodal glycoside hybrid constructs were also measured at their respective $IC_{50}$ values on normal cell line HMEC (Human Mammary Epithelial Cells) and CHO (Chinese Hamster Ovary) for evaluating biocompatibility in which the cells viable more than 80% when compared with control (Figure 2).

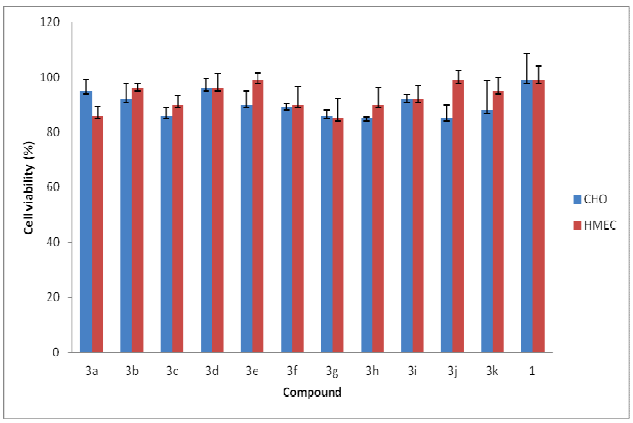



**Figure 2** Cell viability at $IC_{50}$ values in normal cell lines.

Regarding the structure activity relationship, a number of correlations can be made from this data based on steric and electronic properties of the SG-pyrazolines. The improved cytotoxic activity of SG-pyrazoline hybrids (**3a-k**) over parent SG molecule (**1**) specifies that construction of a N-heterocycles on SG molecule could tune the drug-receptor interaction i.e. ligational properties and thereby the cytotoxic activity.

Besides, it is also intersting to note that the type of aryl substituent on pyrazoline ring provide oppurtunity to further amplify the cytotoxic activity. Compound **3h** with 3-F-$C_6H_4$ substituent on pyrazoline ring has shown highest growth inhibition activity (Table 2, entry 8) particularly against the four cancer cell lines (A-549, SF-295, HCT-15 and OVCAR-3) as compared to other SG-pyrazolines (**3a-g** & **3i-k**) and also the standard drug cisplatin. However, in the case of breast cancer cell line MCF-7 the cytotoxic activity **of 3h** was less than cisplatin. The electronic effect by fluoro substituent on cytotoxicity has already been reported.[23] Compound **3i** with substituted 2-Cl-$C_6H_4$ (entry 9) is also more active than cisplatin in all the cell lines except the MCF-7 same as **3h**. The compounds **3d** with 2-OH-$C_6H_4$ (entry 4), and **3k** with 2-$NO_2$-$C_6H_4$ (entry 11) substituent are next to the compound **3h** and **3i**

(entry 8 & 9) has shown relatively better activity in SF-295, HCT-29 and OVACR-3. On the other hand, compounds **3a, 3b** and **3c** (entries 1-3) with simple phenyl/tolyl substituent (i.e. without heteroatom substituent) on pyrazoline ring have shown moderate inhibition activity against all the cell lines. Probably the electron withdrawing nature of these heteroatom substituents present on above SG-pyrazolines also contributes additional control on cell growth inhibition.

In addition, the results obtained from the *in vitro* cytotoxicity towards various cell lines using lactate dehydrogenase (LDH) leakage assay represents that the pyrazoline hybrids are even in accord with MTT by proving the cell death caused by cell membrane rupture which directly relates the cell injury by LDH leakage. These differences among various cell lines and cellular effects towards LDH would suggest the exposure to almost all derivatives of pyrazoline hybrids represents cellular damage had been occurred in cancer cell (Figure 3).

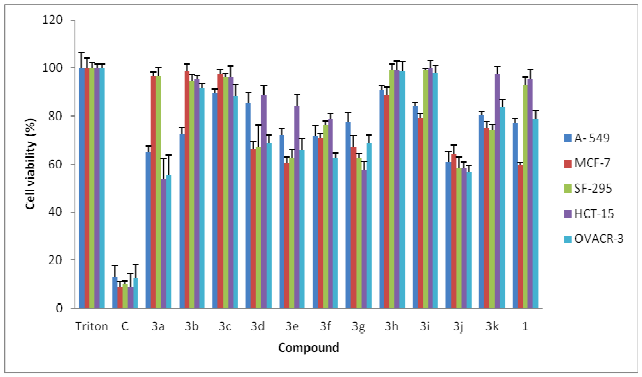



**Figure 3** LDH assay at $IC_{50}$ values of pyrazoline hybrid constructs in various cell lines.

## Conclusions

We have developed a facile catalyzed synthesis of SG-pyrazoline hybrid constructs and their application as promising anti-cancer cytotoxic agents for a panel of human cancer cell lines. Specifically, some of these hybrids worked well for the growth inhibition in Colon, Ovarian and CNS human cancer cell lines and were found to be superior than standard cisplatin in terms of $IC_{50}$ values.

Regarding the synthesis of these molecules, in our work while the chalcone and hydrazine condensation catalyzed by Ag(I)-NHCs has produced selectively the desired SG-pyrazoline hybrids, the acetic acid catalyzed condensation produced N-acetyl pyrazoline by-products. Moreover, ethanol, a green solvent, was used for the synthetic work.

The broad spectrum of anti-cancer activity displayed by these hybrids may be of interest for further derivatization as well as to extend these studies to *in vivo* and clinical studies in the hope for finding more active and selective anti-cancer agents.

## Acknowledgments

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## Experimental Section

### General

All commercially available reagents were used without further purification. Reaction solvents were dried by standard methods before use. Purity of the compounds was checked by TLC using Merck 60F254 silica gel plates. Elemental analyses were obtained with an Elemental Analyser Perkin-Elmer 240C apparatus. $^{1}H$ and $^{13}C$ NMR spectra were recorded with a Mercuryplus 400 spectrometer (operating at 400 MHz for $^{1}H$ and 100.58 MHz for $^{13}C$); chemical shifts were referenced to TMS. EI (electron impact) mass spectra (at an ionising voltage of 70 eV) were obtained using a Shimadzu QP5050A quadrupole-based mass spectrometer.

***In vitro* cytotoxic activity.** The human cancer cell lines used in the study were A-549 (Lung), MCF-7 (human Breast cancer cell line), SF-295 (CNS), HCT-15 (Colon), and OVCAR-3 (Human ovarian cancer cell line) along with normall cell line HMEC (Human Mammary Epithelial Cells) and CHO (Chinese Hamster Ovary) purchased from National Centre for Cell Sciences (NCCS), Pune, India were cultured aseptically using Dulbecco's modified eagles medium (DMEM), RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and penicillin (100 units $mL^{-1}$)/streptomycin (100 mg $mL^{-1}$), pH-7.2 and 5% $CO_2$ humidified atmosphere at 37 ºC. After attaining 80% confluence, the cells were trypsinized with 0.25 Trypsin-EDTA and diluted with media to a fixed number of cells.

**Cell viability Assay for $IC_{50}$.** The newly synthesized compounds (**3a-k**) and SG (**1**) were evaluated through *in vitro* cytotoxicity study for the calculation of $IC_{50}$ value i.e., cellular viability in the presence and absence of the test material was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay which was previously reported.[22] 100µl of medium containing cells at a density of 10,000 cells/well were inoculated into each well of 96 well plate and incubated overnight in a $CO_2$ incubator maintained 5% at humidified atmosphere for 24 hours maintained at 37 ºC. Cell cultures were treated with varying concentrations (0.1-10 µM) of each compound made with 1:10 serial dilutions and left in contact for 24 hours in which four replicate wells were set up for each experiment condition. After incubation at the end, 100 µl of MTT reagent (5 mg of MTT/1 ml of PBS) was added. Finally after incubation for 3 hours with MTT reagent, the media was pippeted out and removed, violet colored formazen crystals were dissolved using 150 µl DMSO to each well, and the reduction of MTT by mitochondrial dehydrogenase was measured at 560 nm using an ELISA reader. The percentage of viable cells in each well were calculated from absorbance of purple colored formazan crystals. All experiments were carried out quadrapulate in addition to maintaining a control (with solvent only) and a reference standard drug cisplatin (DDP) is used.

The activity in sense of percent growth and inhibition is calculated by considering the growth of the cancer cell line in absence (100%) and presence of test material respectively.

The *in vitro* anti-cancer activity of the newly synthesized D-

ring substituted steroidal diglycoside pyrazoline hybrids (**3a-k**) was evaluated against five human cancer cell lines according to MTT assay method[22] using cisplatin (DDP) as a reference drug. The results are presented in Table 2. The following table gives the cancer cell inhibitory data obtained after treating different cancer cell lines with test doses of the different steroidal pyrazoline derivatives and the values are reported in terms of $IC_{50}$.

**LDH Assay for $IC_{50}$.** Cytotoxicity induced by various newly synthesized D-ring substituted steroidal diglycoside pyrazoline hybrid constucts at their $IC_{50}$ values were assessed by lactate dehydrogenase (LDH) leakage into the culture medium.[24] The treatment of various pyrazoline hybridss along with the positive control (Triton) in culture medium was aspirated and centrifuged at 3000 rpm for 5 min to obtain a cell debris free supernatant. The cytotoxicity for LDH assay was determined using a commercially available kit from Sigma Diagnostics. The assay is primarily based on the conversion of lactate to pyruvate in the presence of Lactate dehydrogenase with parallel reduction of coenzyme Nicotinamide adenine dinucleotide (NAD) to NADH resulted in change of absorbance at 340 nm. Aliquots of media along with the warm reagent at 37 ºC were mixed and absorbance was recorded using a microplate spectrophotometer system (Perkin Elmer's EnSpire Multi-label Plate Reader). Results were analyzed and are presented as percentage of positive control values.

**General procedure for the synthesis of Steroidal Pyrazolines (3a-k).** Steroidal chalcones (**2a-k**) (1 mmol) and hydrazine hydrate (99%, 1 mmol) was dissolved in EtOH (10 ml) and added dropwise to a solution of Ag(I)-NHC (5 mol%), generated *in situ* by the treatment of $Ag_2O$ (1 mmol) with the corresponding NHC-precursor (1,3-di-*tert*-butylimidazolium chloride) (0.5 mmol) in EtOH (5 ml) over a period of 2 minutes at room temperature and stirring was continued for 2 hours. Complete consumption of starting material as judged by TLC and GC analysis. The reaction mass was evaporated under reduced pressure to afford a crude product which was subjected to column chromatography (silica gel, 60-120 mesh, eluent; n-hexane/EtOAc gradient) to afford pure products (**3a-k**), **Compound** (**3a**). $^1$H NMR (400 MHz, $CDCl_3$) δ = 1.01 (s, 3H, Me-19), 1.04 (m, 1H, H-1α), 1.15 (m, 1H, H-5α), 1.20 (m, 1H, H-6α), 1.23 (d, 6H, *J* = 6.4 Hz, Me-6` & Me-6``), 1.30 (m, 1H, H-4β), 1.32 (s, 3H, Me-18), 1.37 (m, 1H, H-9α), 1.41 (m, 1H, H-15α), 1.52 (m, 1H, H-2β), 1.60 (m, 2H, H-2`a & H-2``a), 1.65 (m, 1H, H-4α), 1.68 (m, 1H, H-6β), 1.75 (m, 1H, H-1β), 1.78 (m, 1H, H-11α), 1.85 (m, 1H, H-2α), 1.88 (m, 1H, H-7α), 1.90 (m, 1H, H-15β), 2.04 (m, 1H, H-11β), 2.05 (m, 1H, H-16α), 2.08 (m, 1H, H-7β), 2.10 (m, 2H, H-2`b & H-2``b), 2.16 (m, 1H, H-16β), 2.79 (m, 2H, H-21), 3.10 (q, 1H, H-17α), 3.20 (dd, 2H, *J* = 9.5, 3.0 Hz, H-4` & H-4``), 3.45 (s, 6H, OMe), 3.68 (m, 1H, H-3α), 3.84 (m, 2H, H-5` & H-5``), 3.88 (q, 2H, H-3` & H-3``), 4.81 (dd, 1H, *J* = 9.5, 2.0 Hz, H-1``), 4.88 (t, 1H, H-22), 4.91 (dd, 1H, *J* = 9.5, 2.0 Hz, H-1`), 4.95 (dd, 1H, H-12α), 6.52 (s, 1H, NH), 7.15 (d, 2H, Ar-H), 7.22-7.32 (m, 3H, Ar-H), 7.54-7.65 (m, 3H, Ar-H), 8.10 (d, 2H, Ar-H) ppm. $^{13}$C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 13.05, 18.10, 25.06, 25.90, 29.94, 32.15, 35.20, 36.22, 36.40, 36.52, 37.32, 39.05, 41.42, 46.35, 48.72, 52.60, 55.21, 58.05, 59.72, 69.10, 78.02, 78.41, 79.46, 79.62, 83.20, 87.12, 97.22, 101.32, 126.22, 126.81, 129.42, 129.62, 130.42, 131.04, 134.42, 141.82, 155.75, 167.72 ppm. MS (EI, 70 eV): *m/z* (%) = 861 $[M + H]^+$. EA calcd (%) for $C_{49}H_{68}N_2O_{11}$ (860.42): calcd. C 68.35, H 7.96, N 3.25; found C 68.33, H 7.95, N 3.32.

## Notes and references

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† Electronic Supplementary Information (ESI) available: The spectral data of steroidal diglycoside pyrazoline hybrids **3b-k**. See DOI: 10.1039/b000000x/

‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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## Graphical Abstract

**Synthesis and anti-cancer evaluation of steroidal diglycoside-pyrazoline hybrids**

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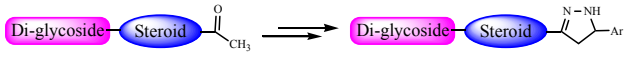
A new series of pyrazoline derived steroidal diglycoside hybrids (**3a-k**), were synthesized in good yields from the corresponding chalcones (**2a-k**), was described. The original form of **1** and its modified forms **3a-k** were characterized and screened for *in vitro* cytotoxic activity and deduced the structure-activity-relationship (SAR).




**Ar** = $C_6H_5$, 2-$CH_3C_6H_4$, 4-$CH_3C_6H_4$, 2-$OHC_6H_4$, 2-$OCH_3C_6H_4$, 4-$OCH_3C_6H_4$, 3,4-di-$OCH_3C_6H_3$, 3-$FC_6H_4$, 2-$ClC_6H_4$, 2-Furyl, 2-$NO_2C_6H_4$