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## Metadichol® a Novel Agonist of the Anti-aging Klotho Gene in Cancer Cell Lines

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### Abstract

Klotho is an anti-aging protein that is mostly secreted by the kidneys, the brain, and the thyroid. It plays a significant role in regulating kidney function and vascular health. Klotho gene is named after "the Spinner" (Klotho from Greek mythology), the goddess who spins the thread of life. Klotho is a transmembrane protein known to be a co-receptor for Fibroblast Growth Factor-23. Klotho gene is expressed in a variety of tissues changes in the levels are associated with many diseases. Klotho is a tumor suppressor in breast cancer and its expression is reduced in human pancreatic adenocarcinoma, and treatment with klotho inhibits the growth of pancreatic cancer cells *in vitro* and *in vivo*.

Growing evidence suggests that an increase in KL expression may be beneficial for age-related diseases such as arteriosclerosis and diabetes. It remains a challenge today to induce Klotho expression. Herein we show that treating pancreatic cancer cells PANC1, MIAPACA and COLO-205 with Metadichol® a novel food based lipid emulsion of long chain alcohols at picogram/ml, concentration led to a 4-10 fold increase in Klotho expression as seen quantitative RT-PCR. These results suggest the use of Metadichol® given its constituents that are present in foods we consume every day is a novel therapeutic intervention for pancreatic cancer and other diseases.

**Keywords:** VDR; Metadichol; Klotho; Inverse agonist; Protean agonist; Constitutive receptors; Pancreatic cancer; FGF-23; Diabetes; Anti-aging; PANC1; COLO-205; MIAPACA; Long chain alcohols

### Introduction

The Greek goddess whose name is associated with Klotho protein spins life's thread and is associated with reversing aging in mammals. In Greek Mythology, Klotho has two siblings, Lachesis and Atropos, and one determines the length of the thread of life and the other cuts the thread. Klotho (KL), which was named after one of the three goddesses of fate who controlled aging in Greek mythology, was initially identified in 1997 as the gene responsible for early aging-like symptoms in mice [1], and in several other tissues [2]. It acts as a coreceptor with fibroblast growth factor receptor-1 (FGFR1) to bind fibroblast growth factor 23 (FGF23) and mediate phosphaturia to correct the hyperphosphatemia arising from 1,25-dihydroxy vitamin D (calcitriol or 1,25D) Stimulation of intestinal calcium and phosphate absorption. 1,25D regulates the expression of both membrane and soluble klotho forms in multiple kidney cell types to support FGF23 phosphaturic and vitamin D counter-regulatory actions at the kidney, possibly exerting antiaging effects [3].

The observation that Klotho inhibits insulin/IGF-1 signalling has ramifications for therapeutic intervention in cancer as well. Activation of the IGF receptor has been implicated in the etiology of carcinomas [4]. There is a growing body of evidence implicating Klotho as a tumour suppressor [5]. In particular cervical, colorectal, gastric and lung carcinoma, pancreatic, hepatocellular carcinoma and breast cancer amongst a few [6]. In general, Higher Klotho expression was associated with smaller tumor size and Klotho treatment slowed the progression of cancer. Klotho is significantly down regulated in all cancer types including brain malignancies [7]. Down regulation of Klotho (Table 1) was observed across the different cancer types. The role of Klotho in cancer as a tumor suppressor mentioned by Wolf I [8] showed how Klotho putative tumor suppressor in breast cancer.

The available data indicate that Klotho acts as a universal tumor suppressor and that there may be a role for Klotho cancer treatment. Currently, there are no Klotho-based treatments available, although a

number of commonly used compounds do either directly up-regulate Klotho *in vitro*, like PPAR $\gamma$  agonists [9], vitamin D [10], Testosterone [11] and Resveratrol [12], or otherwise up-regulate or at least inhibit down-regulation of Klotho *in vivo*. Recent data indicate that Klotho has extensive effects over the entire spectrum of human diseases [13] as shown in Table 1.

| Acetylcholine and Nitric Oxide Dysregulation Aging (highly accelerated)   | Bone Loss (such as osteoporosis and low bone mass) |
|---|--|
| All-cause mortality   | Cancer   |
| Anemia  | Cataracts  |
| Anorexia  | Chronic stress                                     |
| Atherosclerosis (as well as calcification of the arteries)                | Depression   |
| Growth hormone deficiency   | Diabetes   |
| Kidney disease (such as CKD and electrolyte imbalances) Kidney transplant | Glaucoma   |
| Hyperphosphatemia   | Multiple system atrophy                            |
| Hyperparathyroidism   | Pseudoexfoliation syndrome                         |
| Hypertension  | Rheumatoid arthritis                               |
| Impaired cognition (such as Alzheimer's Disease)                          | Sarcopenia   |
| Inflammatory bowel disease  | Skin atrophy (such as scleroderma)                 |
| Lung damage   | Vascular disease (such as coronary artery disease) |
| Stroke  | --   |

**Table 1:** Klotho effects over the entire spectrum of human diseases

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Klotho levels are decreased in certain types of human tumor cells/tissues, and reduced levels are associated with decreased survival [14]. Animal studies show that Klotho can improve survival rates [15] reduce metastasis and reduce cancer cell resistance to chemotherapeutic agents [16]. Abramovitz [17] showed in studies on pancreatic adenocarcinoma cell lines that Klotho expression is reduced, and treatment with Klotho effectively slows growth of pancreatic cancer cells *in vitro* and *in vivo*. Biao Xie also have shown that Klotho is a tumor suppressor in gastric cancer [18]. Injection of secreted Klotho protein suppressed metastasis and improved survival in mice transplanted with human lung cancer cells [19]. Long-term administration of KL to mice shows a favorable toxicity profile. As klotho is an endogenous hormone, its administration is potentially feasible and may serve as a novel therapy for pancreatic, as well as other cancers.

Metadichol® nano-emulsion of long chain alcohols is an inverse agonist of VDR (Vitamin D receptor) that is non- toxic. We tested it in the pancreatic cell lines PANC1, COLO-205 and MIAPACA cell lines and the results show that it enhances Klotho expression and thus would pave the way for use a therapeutic in diseases where increased Klotho levels are required.

## Experimental

The experimental work was outsourced and carried out by Skanda Life Sciences Private Limited of Bangalore India. The cell lines were purchased from ATCC, USA and primers from Eurofins India. PCR and qPCR Method Standardization. For each of target gene the PCR conditions viz, Tm, Amplicon specificity & size were optimized using in-house established and validated methods/reagents.

### qPCR

Instruments used CFX96 real time PCR, Bio-Rad. Gene regulation of KLOTHO genes in MiaPaca, Colo-205, Panc-1 cells treated with metadichol.

### Cell lines

COLO 205 (ATCC® CCL-222™), MIA PaCa-2 (ATCC® CRL-1420™), PANC-1 (ATCC® CRL-1469™) (Table 2).

### Sample Preparation and RNA Isolation

Total RNA from the cells was extracted using TRIzol Reagent (Invitrogen) according to manufacturer's instruction. Cells were washed twice with PBS and centrifuged at 2000rpm for 5min. To the cell pellet, 1ml of TRIzol (per p35 dish) was added in 1.5ml Eppendorf

tube and vortexed. Samples were allowed to stand for 5 minutes at room temperature. To the reaction mixture 0.2 ml of chloroform is added and vigorously mixed for 15 seconds. The tube was allowed to stand at room temperature for 5 minutes, centrifuged the resulting mixture at 10,000rpm for 15min at 4° C. Upper aqueous phase is transferred to a new clean Eppendorf tube and treated with 0.5ml of isopropanol. The resultant mixture is mixed gently by inverting the sample 5 times and incubated at room temperature for 5 minutes. Samples were centrifuged at 10,000 rpm for 10 minutes at 4° C. Supernatant liquid was discarded and the RNA pellet was washed by adding 1ml of 70% ethanol. Mix the sample gently by inverting few times. Centrifuged for 5min at 14,000rpm at 4° C. Supernatant was discarded by inverting the tube on a clean tissue paper. Later, the pellet was dried by incubating in a dry bath for 5min at 55° C. The pellet was then resuspended in 25 µl of DEPC treated water.

### RT-PCR

A semi quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using Techno Prime system to determine the levels of Klotho and β-Actin mRNA expressions. The cDNA was synthesized from 2 µg of RNA using the Verso cDNA synthesis kit (Thermo Fischer Scientific) with oligo dT primer according to the manufacturer's instructions. The reaction volume was set to 20µl and cDNA synthesis was performed at 42° C for 60 min, followed by RT inactivation at 85° C for 5 min (Table 3).

### PCR

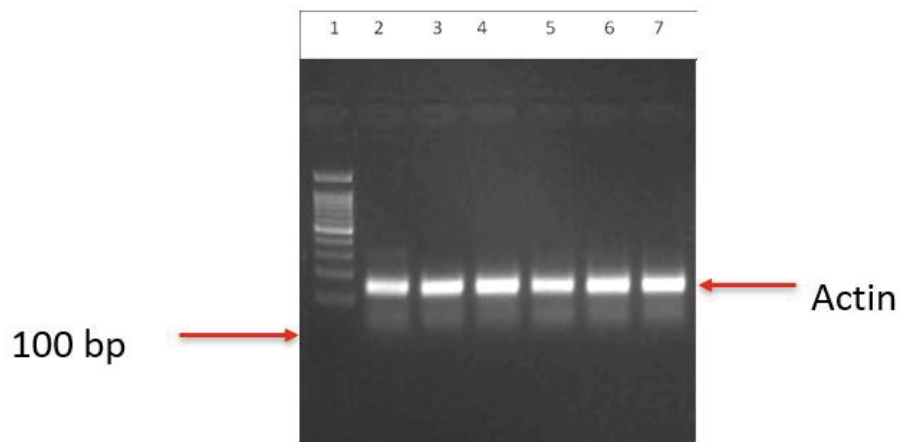
The PCR mixture (final volume of 20 µL) contained 1 µL of cDNA, 10 µL of Red Taq Master Mix 2x (Amplicon) and 1µM of each complementary primer specific for Klotho and β-Actin (internal control) sequence. The samples were denatured at 94° C for 5 minutes and amplified using 35 cycles of 94° C for 30 seconds, 53° C for 30 seconds, and 72° C for 1 minute for KLOTHO renaturation was set to 49° C and for β-Actin the renaturation was set to 55° C for 30 seconds followed by a final elongation at 72° C for 10 minutes. The optimal numbers of cycles have been selected for amplification of this genes experimentally so that amplifications were in the exponential range and had not reached a plateau. Ten microliters of the final amplification product were run on a 2% ethidium-stained agarose gel and photographed. Quantification of the results was accomplished by measuring the optical density of the bands, using the computerized imaging program Image J. The values were normalized to β-Actin intensity levels (Figure 1-10).

| Sample     | Cell culture condition   | Treatment       |
|------------|--|-----------------|
| Metadichol | MIAPACA COLO-205, PANC-1 cells<br>(1 × 10 <sup>6</sup> ) grown in P35 dish were treated with test compound | Control (Media) |
|            |  | 1 pg/ml         |
|            |  | 100 pg/ml       |
|            |  | 1 ng/ml         |
|            |  | 100 ng/ml       |
|            |  | 1 µg/ml         |

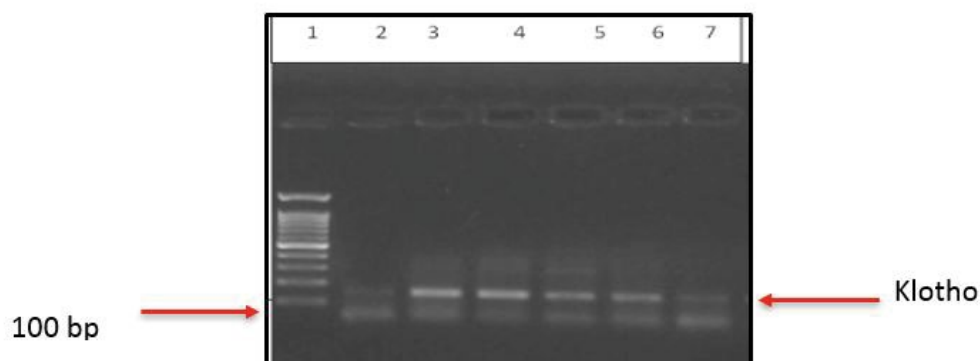
**Table 2:** Treatment protocol.

| Gene    | Primer pair | Sequence                | Tm    | Product size (bp) |
|---------|-------------|-------------------------|-------|-------------------|
| B-Actin | FP          | TCCTCCTGAGCGCAAGTACTCT  | 62.1  | 153               |
|         | RP          | GCTCAGTAACAGTCCGCCTAGAA | 62.4  |                   |
| Klotho  | FP          | GGGAGGTCAGGTGTCCATTG    | 55.88 | 152               |
|         | RP          | TGCTCTCGGGATAGTACCA     | 53.83 |                   |

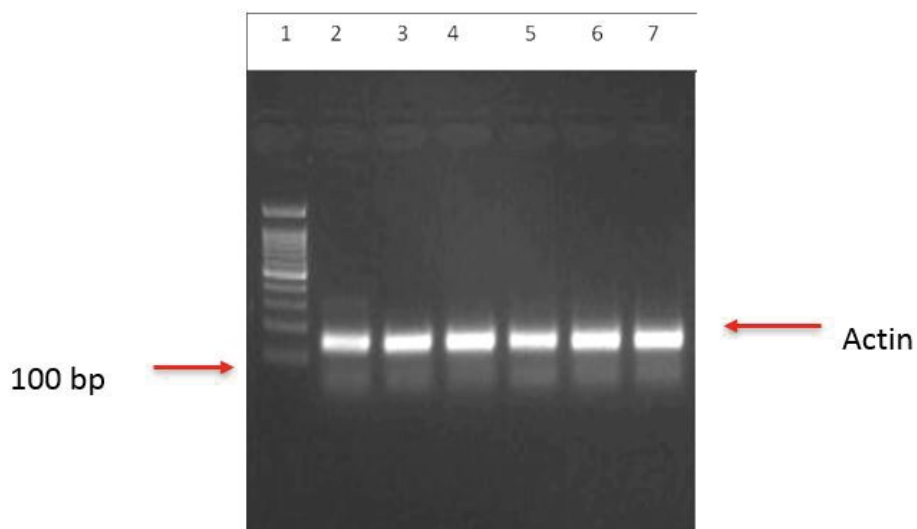
**Table 3:** Primer details.



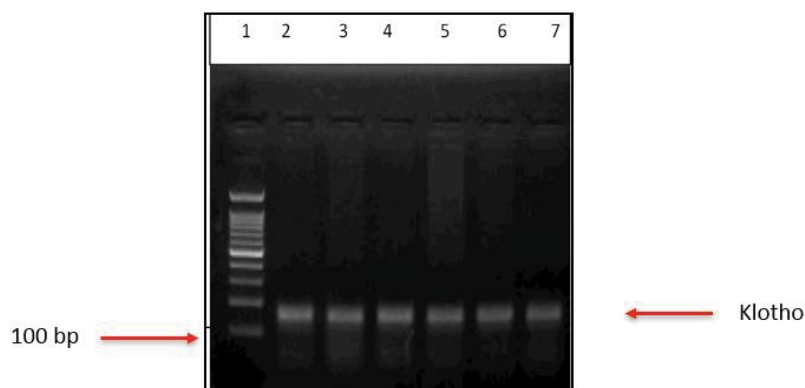
**Figure 1:** Amplification of  $\beta$ -Actin gene in MIAPACA (Lane 1-Ladder; Lane 2-Control; Lane 3-1 pg/mL; Lane 4-100 pg/mL; Lane 5-1 ng/mL; Lane 6-100 ng/mL; Lane 7-1  $\mu$ g/mL).



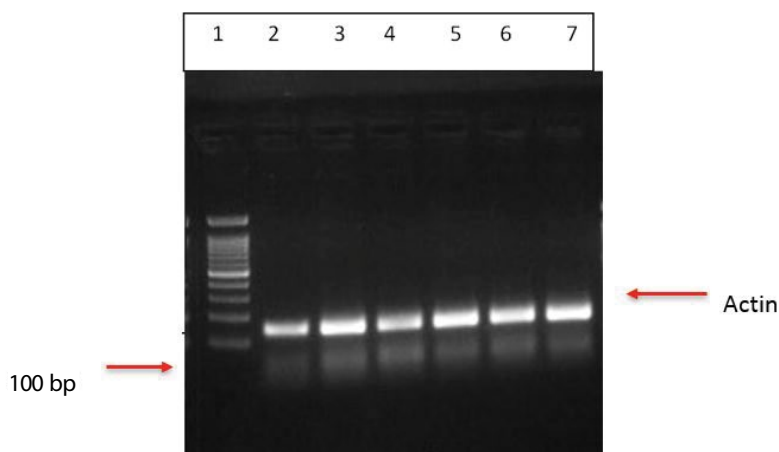
**Figure 2:** Amplification of Klotho gene in MIAPACA cell (Lane 1-Ladder; Lane 2-Control; Lane 3-1 pg/mL; Lane 4-100 pg/mL; Lane 5-1 ng/mL; Lane 6-100 ng/mL; Lane 7-1  $\mu$ g/mL).



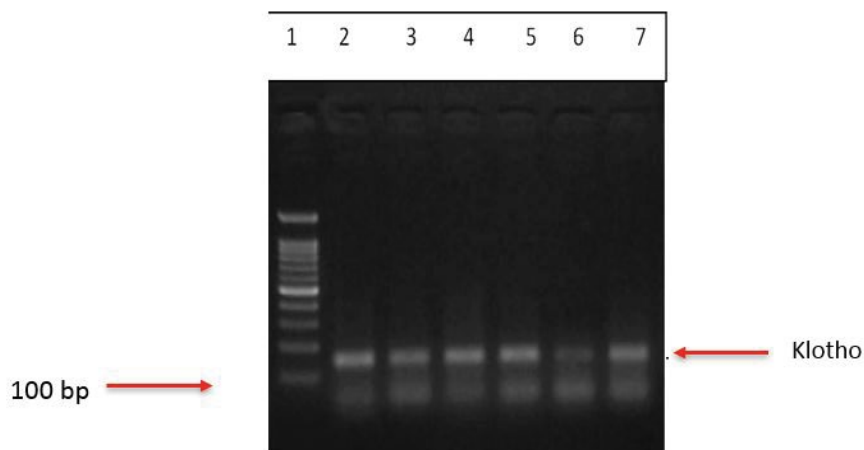
**Figure 3:** Amplification of  $\beta$ -Actin gene in COLO-205 (Lane 1- Ladder; Lane 2-Control; Lane 3-1 ng/mL; Lane 4-100 ng/mL; Lane 5-1 pg/mL; Lane 6-100 pg/mL; Lane 7-1  $\mu$ g/mL).



**Figure 4:** Amplification of Klotho gene in COLO-205 cell (Lane 1- Ladder; Lane 2-Control; Lane 3-1 ng/mL; Lane 4-100 ng/mL; Lane 5-1 pg/ml; Lane 6-100 pg/ml; Lane 7-1 µg/mL).



**Figure 5:** Amplification of  $\beta$ -Actin gene in PANC1 (Lane 1- Ladder; Lane 2-Control; Lane 3-1 ng/mL; Lane 4-100 ng/mL; Lane 5-1 pg/ml; Lane 6-100 pg/ml; Lane 7-1 µg/mL).



**Figure 6:** Amplification of Klotho gene in PANC1 cell (Lane 1- Ladder; Lane 2-Control; Lane 3-1 ng/mL; Lane 4-100 ng/mL ; Lane 5-1 pg/ml; Lane 6-100 pg/ml; Lane 7-1 µg/mL).

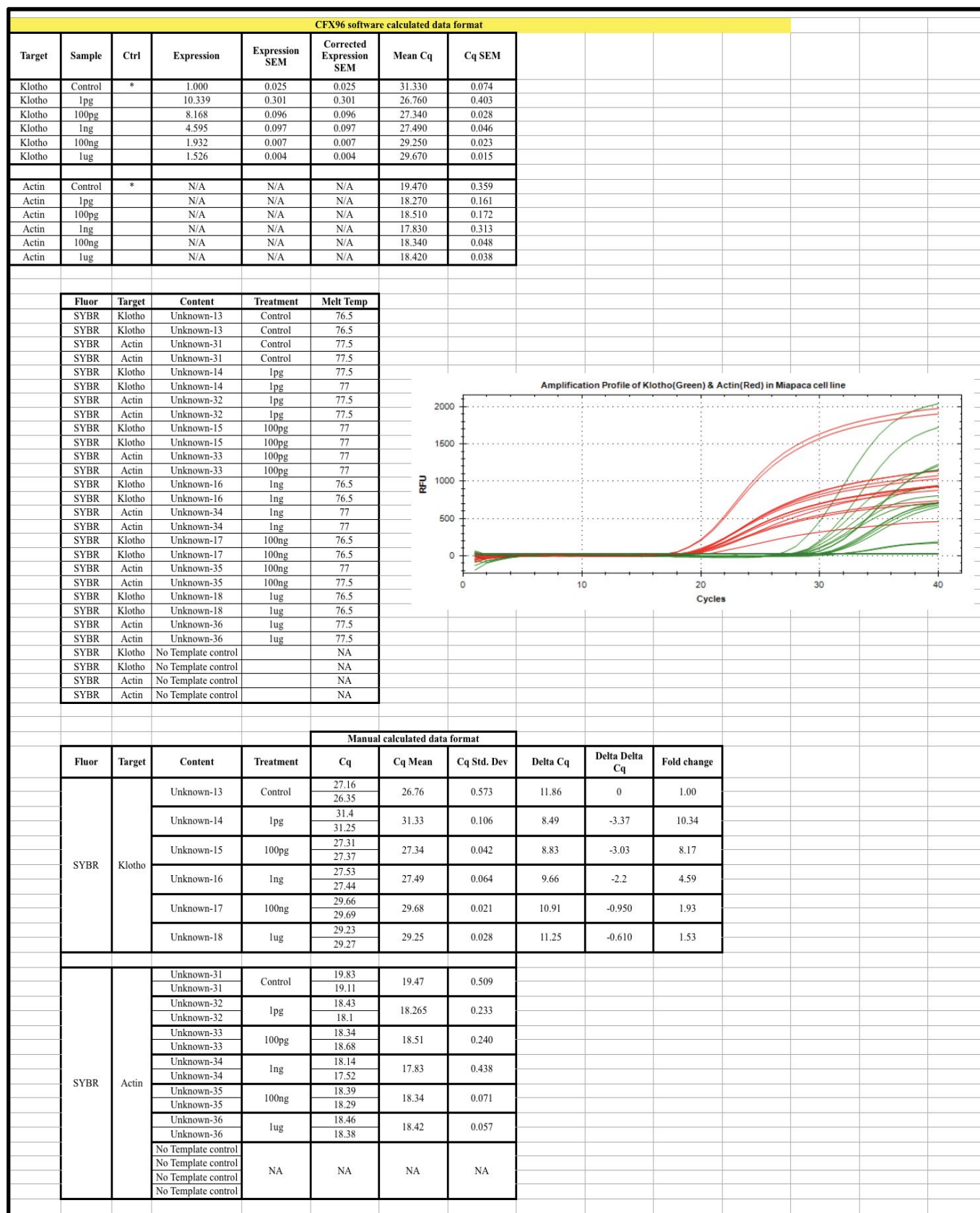


Figure 7: Raw data of MIAPACA cell line.

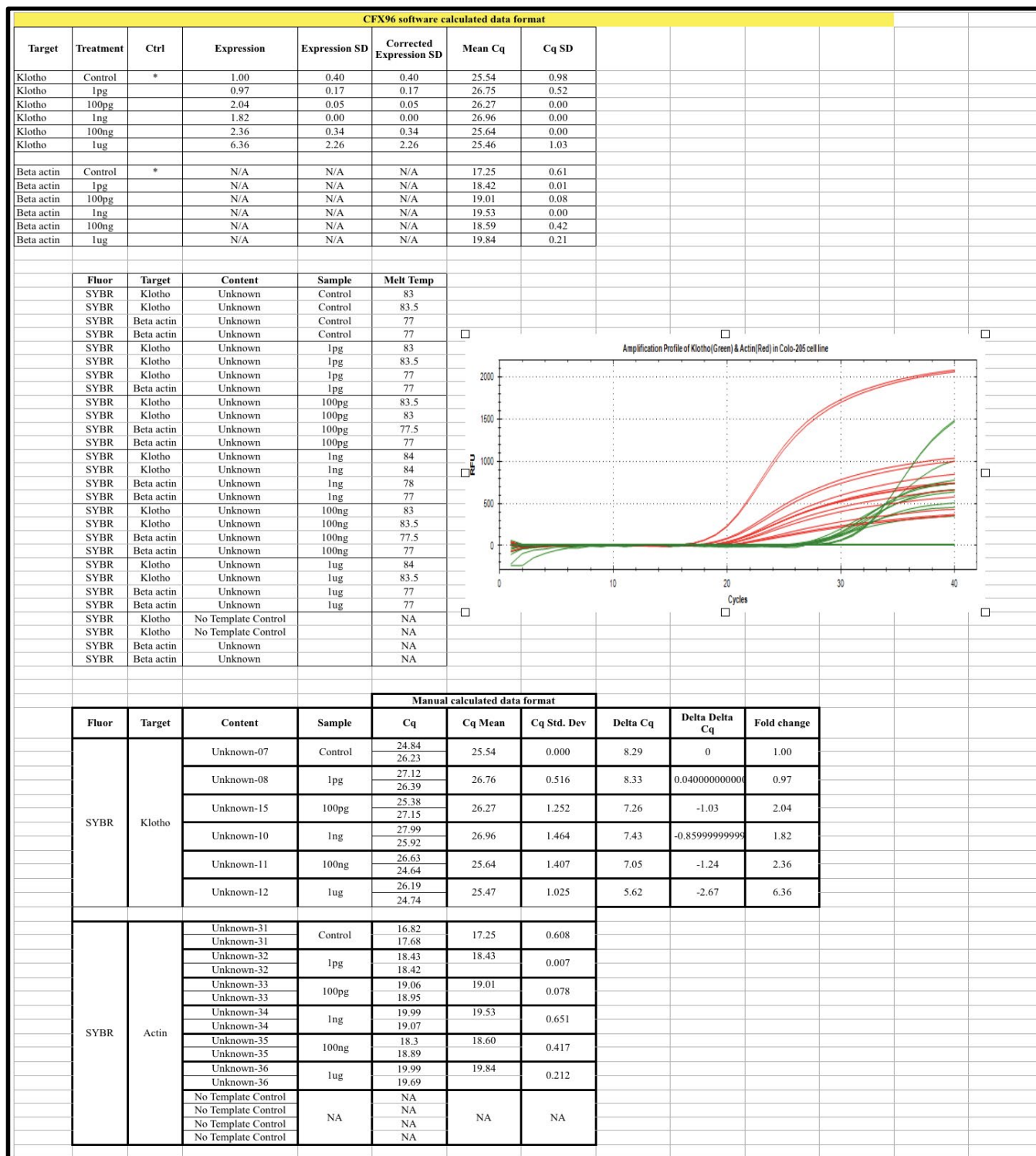


Figure 8: Raw data COLO-205 cell line.

## Results

Three different cell lines were individually treated with Metadichol at various concentrations to assess the expression of Klotho gene. The maximum up regulation of Klotho gene expression is seen at lowest concentration treated, i.e., 1pg/ml in both MIAPACA and PANC-1

cells up 10.34 and 3.66-fold, whereas, in COLO 205 the expression at 1µg/ml was maximum up 6.36-fold compared to control. Overall, the Up regulation of Klotho gene expression level is dose dependent in MIAPACA cells from highest to lowest treatment concentrations from 1µg/ml to 1pg/ml.

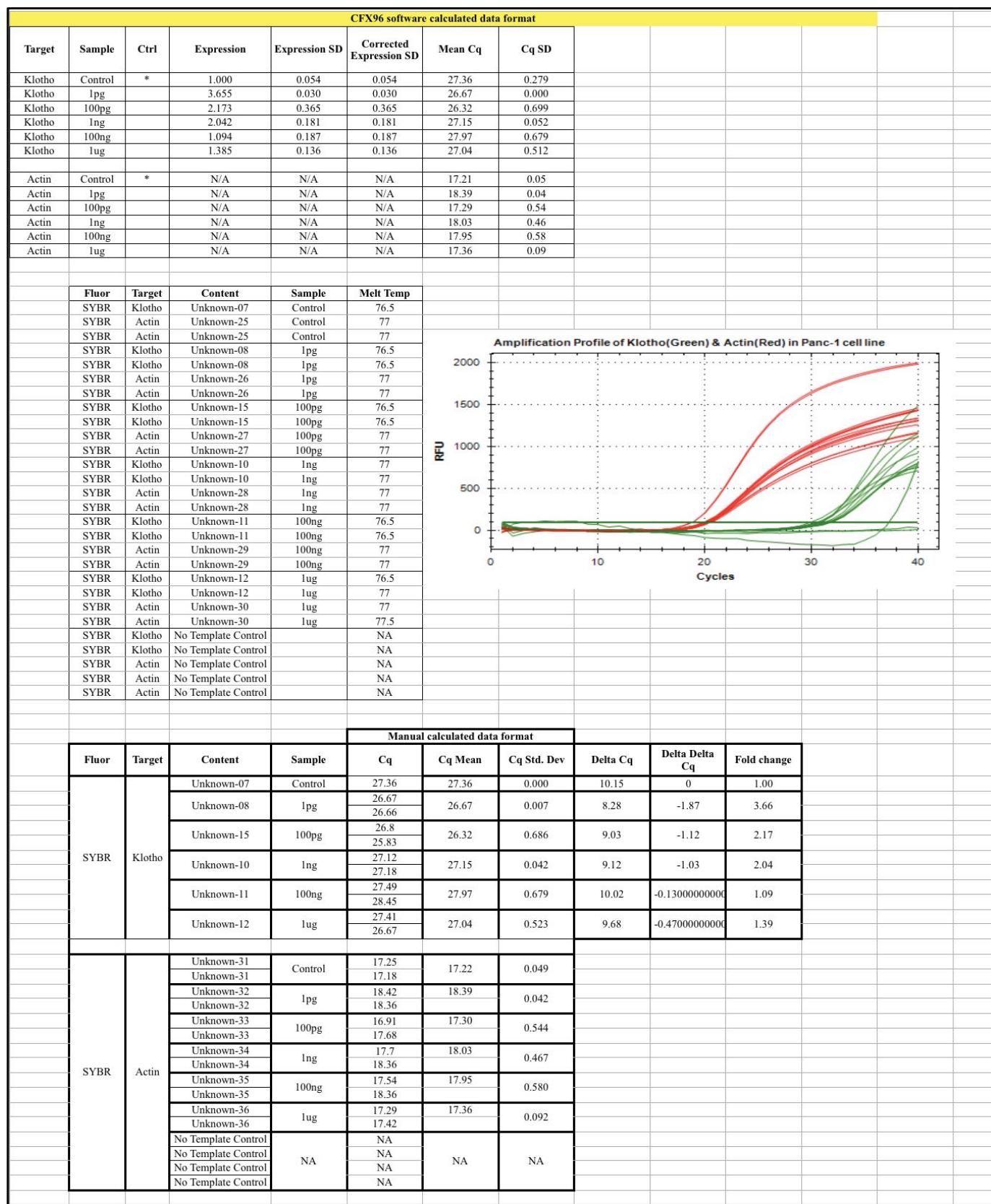
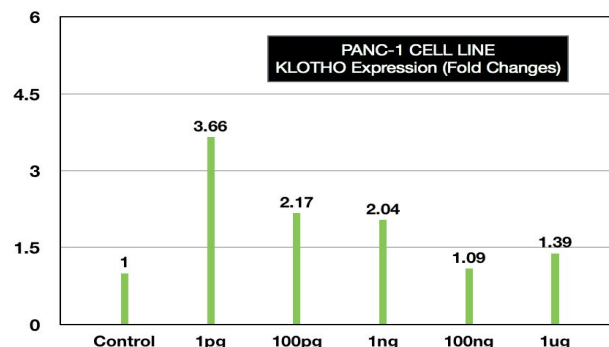
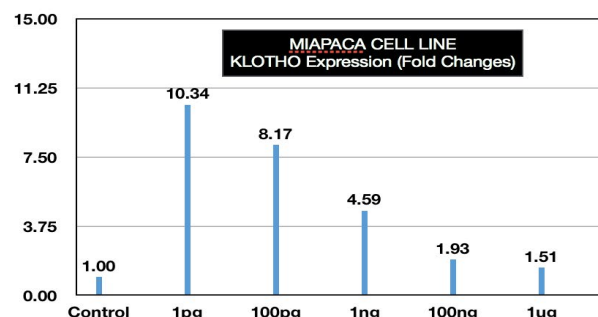
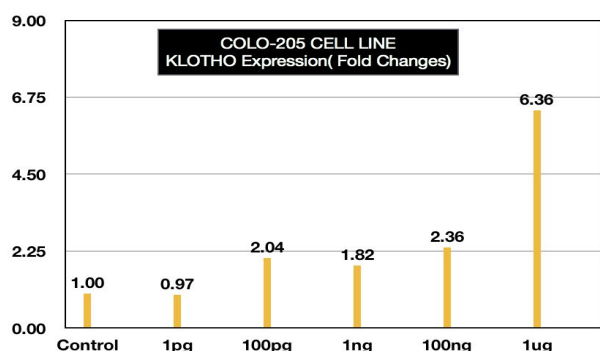


Figure 9: Raw data PANC1 cell line.

|           | MIAPACA     | PANC1       | COLO-205     |
|-----------|-------------|-------------|--------------|
| Treatment | Fold change | Fold Change | FOLD CHANGES |
| Control   | 1.00        | 1.00        | 1.00         |
| 1pg       | 10.34       | 3.66        | 0.97         |
| 100pg     | 8.17        | 2.17        | 2.04         |
| 1ng       | 4.59        | 2.04        | 1.82         |
| 100ng     | 1.93        | 1.09        | 2.36         |
| 1ug       | 1.51        | 1.39        | 6.36         |



**Figure 10:** Summary of results Q-RT-PCR of Meta-dichol treated cells.

## Discussion

King have identified small molecules that elevated Klotho expression, but the increase was only in the range of 20-50% at micro molar (uM) concentrations and with compounds whose toxicity is not known in humans [20].

Marco [21] suggested that Orally-available, transcriptional factors like D-alpha-tocopherol [22], and vitamin D receptors (VDR) agonists such as cholecalciferol [23,24] and lithocholic acid [25] can increase Klotho expression. They suggested that, or a combination of these molecules would result in increased expression of endogenous, human Klotho through transcriptional activation.

R.E. Forster [26] have postulated that the liganded VDR up regulates Klotho gene *via* Vitamin D response elements (VDRE). The actions of 1,25 dihydroxyvitamin D3 (1,25 D3) on phosphorus are opposed *via* the combined effects of FGF23 and Klotho, which is up regulated by the liganded vitamin D receptor.

1,25D3 acting on VDR induces FGF23 in osteocytes to increase circulating FGF23 [27], which protects against hyperphosphatemia [28]. FGF23 also increases 1,25D3 degradation [29].

Metadichol® a nanoemulsion of long-chain binds to VDR Receptor as an inverse agonist, and the formulation contains D-alpha-tocopherol [30]. Inverse agonists bind to the same binding site as the agonists in case of VDR it is 1,25 dihydroxy Vitamin D3. They induce a pharmacological response different and distinct when compared to that of the agonist. Metadichol in our human subjects [31] behaves more likely a Protean agonist as it exhibits dual properties like, e.g. Increasing Insulin Secretion (type 1) and reducing Insulin (type 2). Protean agonists behave as both positive and negative agonists on the same receptor, depending on the degree of constitutive activity. If there is no constitutive activity, the agonist would be a positive agonist [32]. When

constitutive activity is present, the Protean agonist would be an inverse agonist. Metadichol an extract of sugar cane wax exhibits properties that could also be considered as an Adaptogens [33] which are unique in their ability to balance endocrine hormones and the immune system [34-37]. Adaptogens help maintain optimal homeostasis in the body. Adaptogens are proposed to have a normalizing in the body effect on the body and have the ability to toning down the activity of hyper functioning systems in case of constitutive receptors or strengthening the operation of hypo-functioning systems like an agonist. Given the precedence of VDR and its role in regulating Klotho genes it is not surprising that Metadichol® actions on VDR have a similar outcome and as a hormone, it shows activity at picogram levels. Also, Metadichol is nontoxic [38-40] as compared to other solutions in literature to enhance the use of Klotho as a therapeutic target.

## Conclusion

Klotho has been shown to have a wide range of roles in many pathologies. Changes in the levels of Klotho are associated with many diseases. It could be useful as a potential biomarker. However, also has a future as a safe therapeutic in mitigating various diseases where Klotho has a significant role (41).

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