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Rapid Identification of a Putative Interaction between β_2-Adrenoreceptor Agonists and ATF4 using a Chemical Genomics Approach

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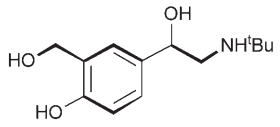
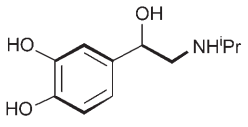
β_2-Adrenoreceptor agonists, such as salbutamol **1** and isoprenaline **2**, are widely used to treat obstructive lung disease.[1]



salbutamol **1**



isoprenaline **2**

However, the use of β_2-agonists is controversial, as it appears likely that their mechanisms of action are complex and far from fully understood.[2]

We have developed new technology that allows us to uncover unknown interactions between small, biologically active molecules and polypeptide "targets", in a chemical genomics approach. As we communicated recently,[3] our method combines the benefits of 1) phage display of a set of polypeptides representing a proteome, 2) a range of five different photochemical reactions that immobilise the bioactive molecule in different orientations to maximise the chance of specific binding to polypeptides in the library, and 3) the use of a "protein-resistant" surface[4] to minimise nonspecific binding of phage-displayed polypeptides.

As our Magic Tag® chemical genomics approach has shown considerable promise in elucidating biochemical pathways in plants, we wished to extend it to the biomedical area. The complex biochemical responses initiated by inhalation of the drug molecule salbutamol **1** provide an attractive field for chemical genomics studies and we therefore elected to immobilise salbutamol using the Magic Tag® method, screening it against a phage-displayed library of polypeptides representing the proteome of the human lung.

Salbutamol was photoimmobilised from aqueous solution by irradiation with 254 nm light. The surface-bound drug was then exposed to a library of polypeptides derived from human lung mRNA displayed on T7Select® bacteriophage in three rounds of biopanning, amplifying the phage in *E. coli* BLT5615 between rounds. In each round, members of the library that bound to the immobilised drug were retained in the wells, whilst nonbinders were washed away. After three rounds, and once controls also observed in wells without immobilised drug had been accounted for, 42 phage clones of interest emerged.

It is known that photoimmobilisation coupled with biopanning can lead to false positives,[5] but we are quickly able to eliminate these by a combination of a basic bioinformatic screen and a simple selective elution experiment as follows. Translation of the nucleotide sequences for the 42 selected clones in all six reading frames, to allow for the possibility of frame slippage,[6] revealed five polypeptide sequences of reasonable length (greater than 100 amino acids) that had significant similarity with the human proteome as judged by NCBI BLASTP.

The five clones that proceeded from the bioinformatics screen were again placed in contact with salbutamol that had been immobilised by the Magic Tag® chemistry from which that clone had been isolated. For example, clone 9 was exposed to salbutamol immobilised using the benzophenone Magic Tag® that had led to its original isolation.

Figure 1 shows the relative number of phage eluted by a solution containing salbutamol compared with a control wash. Of the five, one clone clearly displayed significant selectivity in its binding of salbutamol.

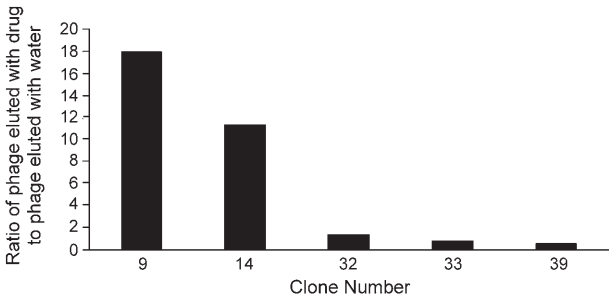


Figure 1. Selective elution of clones from the surfaces using salbutamol in solution.

The polypeptide displayed on this clone is demonstrated to show identity with the DNA-binding domain of the nuclear hormone activating transcription factor 4 (ATF4, previously known as CREB2),[2] which includes the so-called basic region, (see Figure 2).[7]

Specifically, the nucleotide sequence coding for amino acids between 193 and 298 is identical (Figure 3). The X-ray crystallographic structure of ATF4 in its native coiled-coil dimer with C/EPβ has been solved[8] and it can be seen (Figure 4) that the known DNA binding residues are exposed and sit within the

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                    160                                 180
                     |                                   |
Clone   ---------- ---------- ---------- -
ATF4    PDQVAPFTFL QPLPLSPGVL SSTPDHSFSL 180
Consens PDQVAPFTFL QPLPLSPGVL SSTPDHSFSL
                                  200
                                   |
Clone   ---------- --RKPDYTAY VAMIPQCIKE 18
ATF4    ELGSEVDITE GDRKPDYTAY VAMIPQCIKE 210
Consens ELGSEVDITE GDRKPDYTAY VAMIPQCIKE
                    220                                 240
                     |                                   |
Clone   EDTPSDNDXG ICMSPXSYLG SPQHSPSTXG 48
ATF4    EDTPSDNDSG ICMSPESYLG SPQHSPSTRG 240
Consens EDTPSDNDSG ICMSPESYLG SPQHSPSTRG
                                  260
                                   |
Clone   SPNRSLPSPG VLCGSARPKP YDPPGEKMVA 78
ATF4    SPNRSLPSPG VLCGSARPKP YDPPGEKMVA 270
Consens SPNRSLPSPG VLCGSARPKP YDPPGEKMVA
                    280                                 300
                     |                                   |
Clone   AKVKGEKLDK KLKKMEQNKT AATRYRQKLA 108
ATF4    AKVKGEKLDK KLKKMEQNKT AATRYRQKKR 300
Consens AKVKGEKLDK KLKKMEQNKT AATRYRQKXX
```

Figure 2. Translated sequence for clone 9 aligned versus ATF4 protein sequence.

```
                                  820                                 840
                                   |                                   |
Clone   ---------- ---------- ----------A GGAAGCCNGA 11
ATF4Human GCAGTGAAGT GGATATCACT GAAGGAGATA GGAAGCCAGA 840
Consensus GCAGTGAAGT GGATATCACT GAAGGAGATA GGAAGCCAGA
                                  860                                 880
                                   |                                   |
Clone   CTACACTGCT TACGTTGCCA TGATCCCTCA GTGCATAAAG 51
ATF4Human CTACACTGCT TACGTTGCCA TGATCCCTCA GTGCATAAAG 880
Consensus CTACACTGCT TACGTTGCCA TGATCCCTCA GTGCATAAAG
                                  900                                 920
                                   |                                   |
Clone   GAGGAAGACA CCCCTTCAGA TAATGATAGN GGCATCTGTA 91
ATF4Human GAGGAAGACA CCCCTTCAGA TAATGATAGT GGCATCTGTA 920
Consensus GAGGAAGACA CCCCTTCAGA TAATGATAGT GGCATCTGTA
                                  940                                 960
                                   |                                   |
Clone   TGAGCCCANA GTCCTATCTG GGGTCTCCTC AGCACAGCCC 131
ATF4Human TGAGCCCAGA GTCCTATCTG GGGTCTCCTC AGCACAGCCC 960
Consensus TGAGCCCAGA GTCCTATCTG GGGTCTCCTC AGCACAGCCC
                                  980                                1000
                                   |                                   |
Clone   CTCTACCANG GGCTCTCCAA ATAGGAGCCT CCCATCTCCA 171
ATF4Human CTCTACCAGG GGCTCTCCAA ATAGGAGCCT CCCATCTCCA 1000
Consensus CTCTACCAGG GGCTCTCCAA ATAGGAGCCT CCCATCTCCA
                                 1020                                1040
                                   |                                   |
Clone   GGTGTTCTCT GTGGGTCTGC CCGTCCCAAA CCTTACGATC 211
ATF4Human GGTGTTCTCT GTGGGTCTGC CCGTCCCAAA CCTTACGATC 1040
Consensus GGTGTTCTCT GTGGGTCTGC CCGTCCCAAA CCTTACGATC
                                 1060                                1080
                                   |                                   |
Clone   CTCCTGGAGA GAAGATGGTA GCAGCAAAAG TAAAGGGTGA 251
ATF4Human CTCCTGGAGA GAAGATGGTA GCAGCAAAAG TAAAGGGTGA 1080
Consensus CTCCTGGAGA GAAGATGGTA GCAGCAAAAG TAAAGGGTGA
                                 1100                                1120
                                   |                                   |
Clone   GAAACTGGAT AAGAAGCTGA AAAAAATGGA GCAAAACAAG 291
ATF4Human GAAACTGGAT AAGAAGCTGA AAAAAATGGA GCAAAACAAG 1120
Consensus GAAACTGGAT AAGAAGCTGA AAAAAATGGA GCAAAACAAG
                                 1140                                1160
                                   |                                   |
Clone   ACAGCAGCCA CTAGGTACCG CCAGAAG--- ---------- 318
ATF4Human ACAGCAGCCA CTAGGTACCG CCAGAAGAAG AGGGCGGAGC 1160
Consensus ACAGCAGCCA CTAGGTACCG CCAGAAGAAG AGGGCGGAGC
```

Figure 3. Nucleotide sequence for clone 9 aligned versus ATF4 sequence.

discovered region (amino acid residues 285 to 306). Salbutamol, presented on a surface would offer an attractive interfacial region onto which this polypeptide might bind.

Intriguingly, a very recent study from Panebra et al.[2] has shown in vitro, that the β_2-agonist isoprenaline **2**, which is an analogue of salbutamol, affects the expression of ATF4 in airway smooth muscle cells that have been stressed to simulate asthmatic conditions. In that work, when interleukin 13 (IL-13), interleukin 4 (IL-4), transforming growth factor β (TGFβ), and leukotriene D_4 (LTD_4) were added to the cells to simulate asthma, production of ATF4 ceased. Addition of isoprenaline **2** restored ATF expression to normal levels, as monitored by mRNA quantitation. It should be noted that **2** had no effect on ATF4 production under basal conditions.

Clearly, neither our study nor the recent work of Panebra et al. lead to the conclusion that β_2-agonists affect ATF4 levels

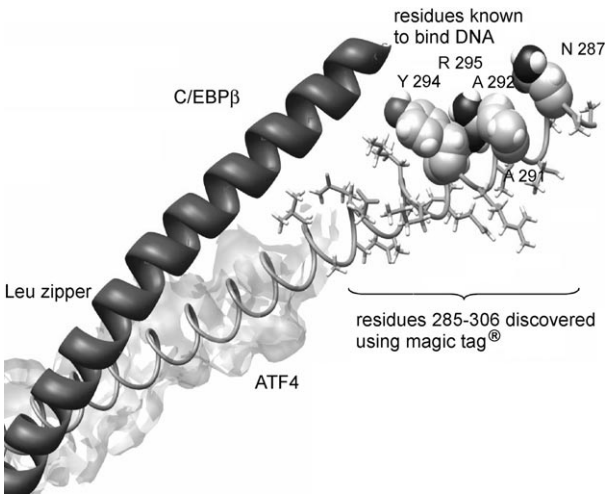

Figure 4. PDB structure 1CI6 [7] highlighting the discovered sequence EQNKTAATRYRQK from clone 9 (side chains illustrated) and known DNA-binding motif (NxxAAxxxYR, shown as C-P-K spacefill model) that putatively interact with salbutamol. The co-crystallised coiled-coil motif also shows the CCAAT box/enhancer-binding protein β (C/EBPβ) as a helix forming the known leucine zipper with ATF4. Figure 4 prepared using Chimera.[8]

in vivo. Nevertheless, our study shows how a chemical genomics approach using simultaneous display of small molecule multiple isomers can lead very rapidly to information about drug–protein interactions and the combined results from ourselves and Panebra et al. suggest that the interaction of β_2-agonists with ATF4 is ripe for further investigation.

Experimental Section

Salbutamol **1**, 2-(hydroxymethyl)-4-[1-hydroxy-2-(tert-butylamino)ethyl] phenol was purchased from Sigma–Aldrich. All biological materials were purchased from Novagen. A solution of salbutamol in tris-buffered saline (10 nmol/well; 200 μL) was immoblised photochemically at 254 nm (handheld thin layer chromatography lamp, Camag Universal UV lamp) onto five Corning Stripwells which have been previously derivatised with one of the Tags 1–5 for 10 min. Salbutamol solution was added to four wells of each strip and TBS was added to the other four wells to act as a control. Excess drug solution was discarded and the wells were washed with 1 M TBS (2×200 μL per well). The library used was a premade T7Select® lung library with a diversity of 1×10^7 pfu mL^{-1}, which was amplified in *E. coli* BLT5615 to give a titre of 1×10^{10} pfu mL^{-1}. A number of clones from the titred library were picked and subjected to PCR, then gel electrophoresis to verify that a range of inserts was present. The immobilised drug was subjected to three biopanning rounds as follows.

For each round of biopanning, two wells from each Magic Tag® strip was used, one well immobilised with drug and one without to act as a control. The freshly titred T7Select® lung library (200 μL per well) was added into the ten wells. The strips were shaken at RT for 40 mins. The phage solution was then removed from each well using a pipette. Each well was then washed with 6×200 μL 1 M TBS/0.5% Tween® 20, shaking for two minutes between each wash. Logarithmically growing *E. coli* BLT5615 (200 μL for each well) was then added to each well and wells were left to shake at RT for 10 mins. Each of the wells contents were transferred to individual tubes of logarithmically growing *E. coli* BLT5615 (5 mL) and

the phage from each well was amplified in the *E. coli* for 2 h. After this time, the suspension was centrifuged and the supernatant containing the amplified phage was transferred to new Eppendorf tubes, ready for use in round two.

Round two was conducted in the same way but this time the phage used were from the previous round, phage being pipetted into corresponding wells from the previous rounds. Round three was carried out in a similar fashion. After round three the phage retrieved were titred with *E. coli* at a dilution of 10^{-5}. After 3–4 h, 24 phage plaques were picked from each plate and subjected to PCR. Electrophoresis gels were run to check the PCR reactions had worked and then sequencing to identify the polypeptide sequences from successful PCR reactions. Bioinformatics was then carried out to identify clones for selective elution screening.

Selective Elution

From the 200 clones sequenced from the first round of screening, five clones were identified to be put into the selective elution screening process, carried out as follows:

For each clone, two wells of the corresponding Magic Tag® from which the clone was isolated were exposed to salbutamol in TBS (10 nmol/well) by adding the drug solution (200 μL) into each well and exposing the Magic Tag® to UV light (254 nm) for 10 min. Excess drug solution was discarded and the wells were washed with 1 M TBS (2×200 μL per well).

For each clone, a suspension of amplified phage (200 μL) was added to its two corresponding Magic Tag® wells. The strips were then shaken at RT for 40 mins. The phage solution was removed from each well using a pipette. Each well was then washed (6× 200 μL 1 M TBS/0.5% Tween® 20), shaking for two minutes for each wash.

For each clone, one of the wells was eluted with salbutamol (200 μL of a 30 nM solution in TBS). To the other wells was added water (200 μL). The Magic Tag® wells were shaken at RT for 40 min. The contents of each well were then pipetted into individual 1.5 mL Eppendorf tubes.

The phage retrieved were titred with *E. coli* BLT5615 at a dilution of 10^{-2}. After 3–4 h, phage plaques grown were counted. For each clone, the number of phage eluted with drug were divided by number of phage eluted with water to obtain a ratio presented in Figure 1.

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Keywords: ATF4 · chemical genomics · phage display · photoimmobilisation · salbutamol

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