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**Helicases as Antiviral Drug Targets**

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## **Summary**

**BACKGROUND** Helicases is required for proteins to access, read or rearrange genetic information. To identify novel inhibitors of SARS coronavirus, SCV, we developed an in vitro helicase assay, and screened many potential anti-viral drugs already in clinic use for their inhibitory effects on SCV helicase activities. Using this biochemical approach, we identified bismuth containing compounds as potent inhibitors of SCV helicase activity in vitro, and SCV replication in cell cultures.

**OBJECTIVE** To provide a foundation for antiviral drug development, we will expand our screen to identify more helicase inhibitors against SCV. We plan to validate SCV helicase as drug target.

**METHODS** We screened a chemical library for more SCV inhibitors with culture cells infected with SCV. Purified SCV helicase proteins were used to probe whether candidate and newly synthesized compounds can inhibit SCV helicase activities. Promising compounds were further tested in cell cultures for their effect on SCV replication. Furthermore, we generated resistant SCV strains to validate that helicase is indeed the target.

**RESULTS** By expanding our screen, we identified more lead compounds for drug design and development. One of the compounds is bananin. Inhibitory effect of bananin can either interfering directly with SCV helicase or inhibit other viral and host processes. If bananin interact directly with SCV helicase, and SCV helicase mutants resistant to it can be selected, it is a strong indication that SCV helicase is the drug target. We indeed obtained mutations in the SCV helicase that is resistant to the inhibitory compound, bananin.

**SIGNIFICANCE** It is the first time that helicase of a RNA virus is a valid target for drug development. This opens a new venue for the development of antiviral drugs. Several lead compounds targeting SARS coronavirus helicase have been identified. These compounds can potentially be used to target other viruses..

## **Main Body**

### **Introduction**

Severe Acute Respiratory Syndrome (SARS) is a serious type of pneumonia that was first recognized in late 2002 and has been shown to be caused by the SARS coronavirus, SCV<sup>1;2;3</sup>. SCV was rapidly sequenced within weeks of the initial identification<sup>4;5</sup>, and further analysis of the genome and proteome has revealed that SCV is most closely related to group 2 coronaviruses<sup>6</sup>. The recent discovery of SCV-like viruses in Himalayan palm civets and raccoon-dogs<sup>7</sup> and bats<sup>8</sup> suggests there may be a natural animal reservoir of the virus. The ability of SCV to infect cats further warns of possible transmission route of the disease<sup>9</sup>. There are two major approaches to fight against SARS: vaccination or treatment. Although vaccination and prevention is a preferable approach, treatment will be the only possibility should the virus recur before an effective vaccine has been developed. Initial characterization of several SCV potential protein targets has already been carried out such as for the main proteinase<sup>10</sup>, the cysteine proteinases<sup>11</sup>, the helicase<sup>11;12</sup> and the RNA polymerase<sup>13</sup>.

#### Characterization of the SCV helicase

We have recently reported the purification and characterization of the SCV NTPase/helicase, and revealed that the enzyme belongs to a distinct class of 5' to 3' viral helicases<sup>12</sup>, similarly to the helicase of human coronavirus 229E<sup>14</sup>. Helicase has been regarded as an attractive target for antiviral drug design<sup>15</sup>. The Herpes Simplex Virus (HSV) helicase has been successfully targeted by novel compounds in an animal model of the disease<sup>16;17</sup>. It is therefore possible that inhibition of the SCV helicase could be a valid strategy towards a treatment for SARS. The SCV helicase has a predicted metal binding domain in the N-terminal region of the protein, a similar metal binding domain in a closely related arterivirus helicase has been shown to be involved in subgenomic mRNA synthesis, genome replication and virion biogenesis<sup>18</sup>. We therefore initially screened a number of drugs already in clinic use, especially metal-containing complexes that might interact strongly with the metal binding domain of SCV helicase. The strategy that disrupts zinc finger proteins has been proposed for anti-HIV drug design<sup>19;20</sup>.

In the current project, we generated drug-resistant SCV strains. Since a compound can inhibit SCV replication by either interfering directly with the helicase or with a cellular target. If virus mutants resistant to an inhibitory compound can be selected, it is likely that the target of the inhibitor is a viral process. Drug targets can be identified by defining the gene in which the mutation conferring the resistant phenotype has occurred. To determine the molecular target of a SCV inhibitor, we have selected resistant viruses in the presence of increasing concentrations of a SCV inhibitor, bananin that was identified as an effective SCV helicase inhibitor by this study<sup>21</sup>. We further identify the nature of the mutation that confers bananin-resistant. We also expanded the screen for SCV helicase inhibitors<sup>22</sup>. Furthermore, we have synthesized several bananin derivatives with SCV inhibiting effects.

### **Objectives**

- (A) Confirmation of helicase as valid drug target
- (B) Screen for more SCV helicase inhibitors
- (C) Synthesis of bismuth complexes against SCV

### **Methods**

- (A) Confirmation of helicase as valid drug target  
SCV were cultured with FRhK-4 in a 96-well plate in the presence of bananin. The released viruses in the culture medium were collected and used to infect a fresh batch of FRhK-4 cells. After three passages, selected SCV were cultured in the presence of increasing amount of bananin (50, 100, 500  $\mu$ M). Several independently bananin-resistant strains were then isolated. Their growth in the presence of bananin were compared to that of the wild type of SCV to confirm their drug resistance. We then identified the nature of the mutation that confers bananin-resistant. Briefly, viral RNA of the resistant strains were prepared as described

previously<sup>12</sup>. The helicase gene were cloned as RT-PCR products. Clones were verified by restriction enzyme digestion and subjected to sequencing analysis. Sequencing confirmed mutations in the SCV helicase.

(B) Screen for more SCV helicase inhibitors

We acquired a chemical library (ChemBridge Corporation) of 50,240 structurally diverse small molecule compounds that vary in functional groups and charge. As the SARS-CoV replicates effectively in Vero cells (African green monkey kidney cell line) and full cytopathic effects (CPE) of the infected cells can be observed within 96 h post infection, Vero cell CPE was used as a phenotypic indicator of successful viral infection in a cell-based assay to screen for small molecule compounds that perturb the infectivity of the virus. In a primary screening the concentration of 20 µg/ml was used to screen for compounds that protect Vero cells from SARS-CoV induced CPE. The hits were re-arrayed and the concentration of selected compounds was lowered to 10 µg/ml for secondary screening for compounds that retained consistent and significant protective effects against SARS-CoV induced CPE in Vero cells. Further evaluation by quantitative plaque reduction assays (PRA) was also carried out for compounds with EC<sub>50</sub> (median effective concentration) below 10 µg/ml. The TC<sub>50</sub> (median toxic concentration) of selected compounds was determined by MTT

(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Roche).

To identify compounds that inhibit the SCV helicase, we then screened the active compounds against the polynucleotide stimulated ATPase activity of SCV helicase at a concentration of 20 µg/ml.

(C) Synthesis of bismuth complexes against SCV

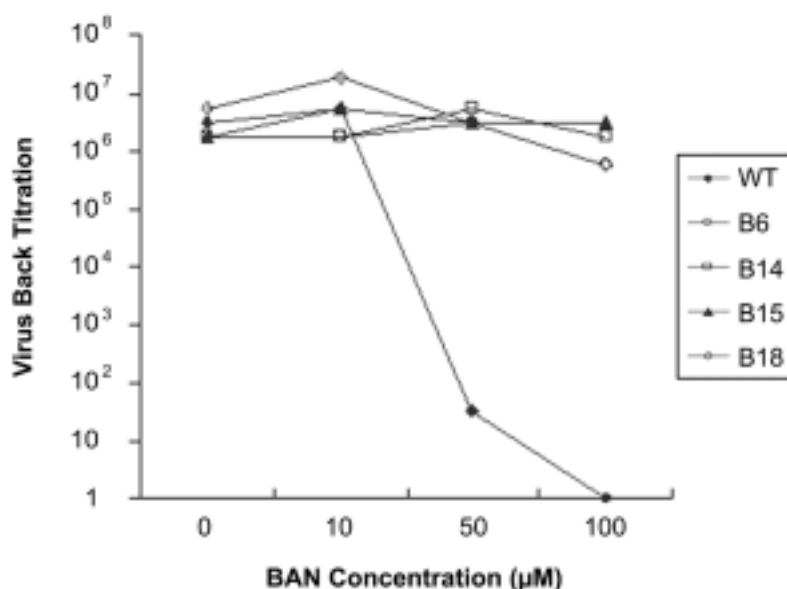
Bismuth complexes were synthesized by mixing appropriate bismuth salts with known ligands and refluxed in an organic solvent (e.g. pyridine) at specific temperature. The resulting complexes were purified and separated by chromatography. Crystals suitable for X-ray analysis, were obtained in solution upon standing at room temperature after several days. The purity of all of the complexes was checked by elemental analysis and <sup>1</sup>H NMR spectroscopy, and the stability of the complexes in solutions was followed by monitoring a specific wavelength in the visible region by UV-vis spectroscopy.

## Results

(A) Confirmation of helicase as valid drug target

We cultured SCV (GZ50 strain) on FRhK-4 cells in the presence of high concentration (100 µM) of bananin (BAN), and after several passages, we obtained 11 resistant virus clones. Back titration (Fig. 1) and Real-Time PCR analysis of FRhK-4 cells showed that mutant clones exhibit 4-6 magnitudes higher resistance than wild type virus in presence of 50 or 100 µM BAN.

**Figure 1**



To investigate if a mutant helicase is responsible for drug resistance, we first sequenced the helicase gene from all drug resistant strains, and found that they all contained a point mutation in the beginning of the helicase domain, causing Ser259, a potent phosphorylation site, changed into leucine (Table 1). In order to find out if there were other targets for

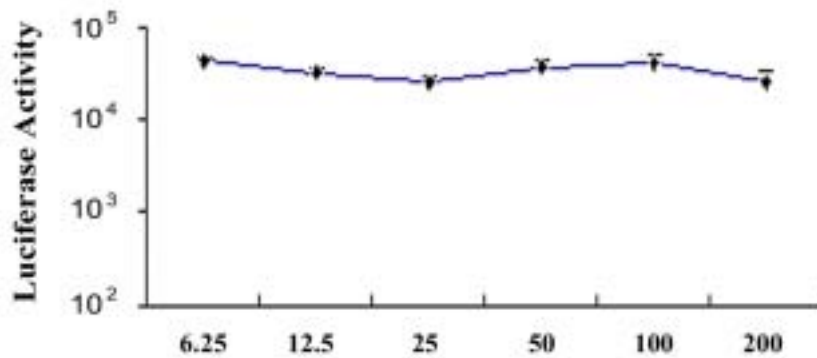
BAN, we sequenced the full length genome of BAN resistant clone 15. We found one mutation in the S protein and two mutations in the M protein (Table 1). Within these mutations, A68V in M protein is identical to two other wild type SCV stains, FRA and HKU39849. In contrast, other mutations are unique when compared with all SCV wild type strains. Furthermore, the wild type virus we started with showed no amino acid change in these proteins.

**Table 1**

Strain	Helicase	S	M	noncoding	other part
B15	S259L	N479I	A 68V, R124W	27692nt A-G	no
B18	S259L, L297aaL/F	NT	A 68V, R124W	NT	NT
B6, B14	S259L	NT	A 68V, R124W	NT	NT
other strains	S259L	NT	NT	NT	NT

\*NT: not tested.

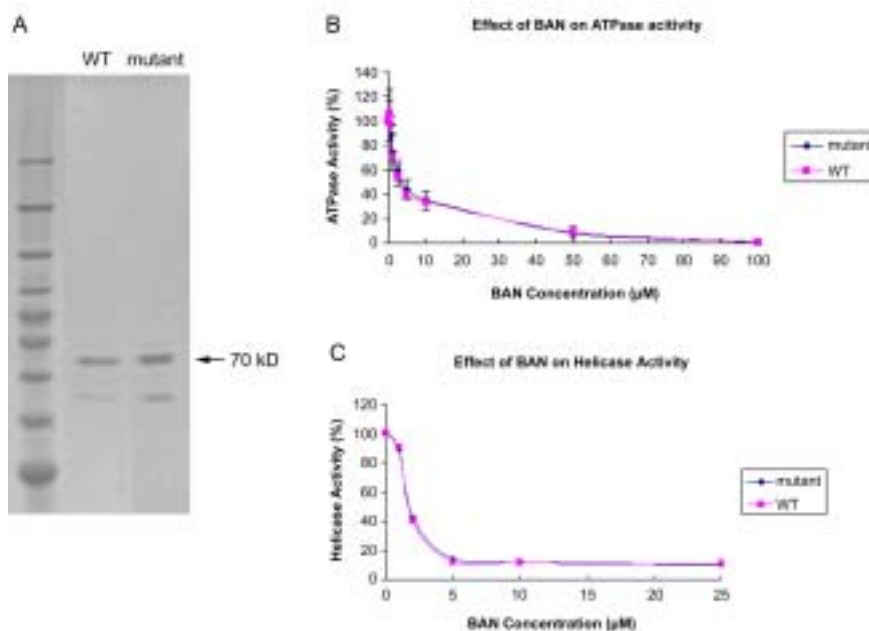
**Figure 2**



S protein (Spike) is the envelope protein major responsible for SCV entry. It exhibits the largest variability among SCV strains. In order to test if S protein could be the target of BAN, we generated HIV/SCV pseudotyped viruses bearing wild type SCV S envelope protein and HIV-luciferase

backbone. The pseudotyped viral entry was mediated by SCV S protein and can be quantified by relative luciferase activity. We used these HIV/SCV pseudotyped particles to infect Huh-7 cells in the presence of different concentrations of BAN, and measured the luciferase activity 48 hours later. As shown in Fig. 2, BAN cannot inhibit HIV/SCV entry into Huh-7 cells, indicating S protein cannot be the target for BAN, and the mutation identified in the sequencing was probably due to spontaneous mutagenesis.

**Figure 3**

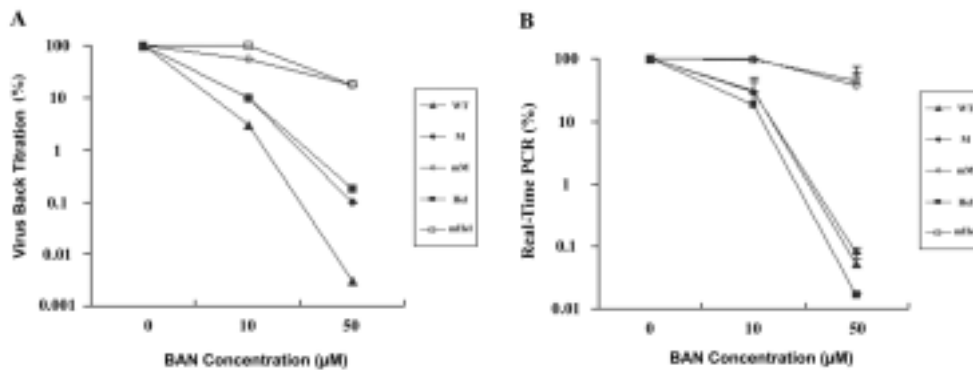


To test if the mutation in helicase gene was responsible for BAN resistance, we cloned wild type and mutant helicase gene into pET-28a vector. We expressed and purified the proteins from *E. coli* (Fig. 3A). Using *in vitro* ATPase assay and FRET-based helicase assay, we demonstrated that the mutant helicase had intact ATPase and helicase activity as wild type protein. Furthermore, similar as

wild type helicase protein, both ATPase (Fig. 3B) and helicase activity (Fig. 3C) of the mutant helicase protein can be inhibited by BAN.

As *in vitro* assay cannot truly represent *in vivo* circumstance, we next explored if the mutant helicase protein expressed as transgene in FRhK-4 cells can rescue wild type SCV replication in the presence of BAN. We also tested the role of mutant M protein by this method. M protein is a transmembrane protein and plays a key role in virion assembly at intracellular membranes. It is possible that mutant helicase or M protein expressed *in vivo* can lead to drug resistance, or the two proteins may interact with each other and resist BAN corporately.

**Figure 4**



We established cell lines expressing wild type or mutant helicase or M protein, which were termed as FRhK-4-Hel

/mHel cells, or FRhK-4-M/mM cells. These cell lines were infected with wild type SCV in presence of different concentrations of BAN. The supernatant were collected 72 hours after infection and back titrated on fresh FRhK-4 cells. Virus RNA was also extracted and Real-Time PCR was performed to monitor viral replication. As shown in Fig. 4, mutant helicase expression can rescue wild type SCV replication in the presence of BAN, indicating helicase is the authentic target for BAN *in vivo*. Similarly, mutant M expression can also lead to BAN resistance of SCV, showing the M protein is another target for BAN. Some of the results have been published<sup>21</sup> (*please see the attachment*). One more manuscript is being prepared for publication.

(B) Screen for more SCV helicase inhibitors.

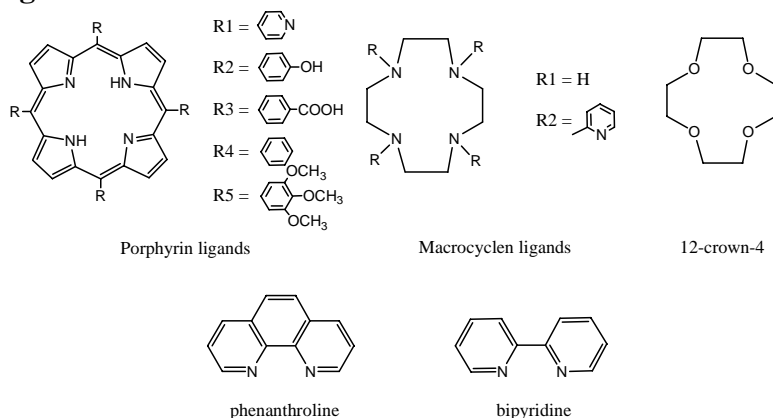
In a primary screening (at 20  $\mu\text{g/ml}$  of each compound) we identified 1,003 “hits” (a hit rate of 2%) that protect Vero cells from SARS-CoV induced CPE. The hits were re-arrayed and the concentration of selected compounds was lowered to 10  $\mu\text{g/ml}$  for secondary screening when 108 compounds retained consistent and significant protective effects against SARS-CoV induced CPE in Vero cells. Further evaluation by quantitative plaque reduction assays (PRA) demonstrated that the  $\text{EC}_{50}$  (median effective concentration) of the selected compounds were below 10  $\mu\text{g/ml}$ , with 78 compounds having an  $\text{EC}_{50}$  below 2  $\mu\text{g/ml}$ . The  $\text{TC}_{50}$  (median toxic concentration) of selected compounds was determined to be  $>50 \mu\text{M}$  by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Roche).<sup>22</sup>

Seven compounds exhibited SCV helicase inhibitory activity. One of the compounds (designated HE602) exhibited substantial inhibitory activity at 2  $\mu\text{g/ml}$ . HE602 was found to strongly inhibit the polynucleotide stimulated ATPase activity of SCV helicase with an  $\text{IC}_{50}$  of 6.9  $\mu\text{M}$ , but barely inhibited the unstimulated ATPase activity even at a concentration of 200  $\mu\text{M}$ . We then confirmed that HE602 inhibited the helicase activity of SCV helicase in a concentration dependent manner, with an  $\text{IC}_{50}$  estimated to be between 2-20  $\mu\text{M}$ . Furthermore, HE602 inhibited the viral plaque formation in Vero cell with an  $\text{EC}_{50}$  of 6  $\mu\text{M}$ . The effective inhibition of only the stimulated ATPase activity together with its ability to inhibit helicase activity indicates that HE602 has distinct parallels with the pharmacological profiles of inhibitors targeted against the herpes simplex virus (HSV) helicase-primase protein. The results have been published<sup>22</sup> (*please see the attachment*).

### (C) Synthesis of bismuth complexes against SCV

Series of bismuth complexes have been designed and synthesized, including the bismuth porphyrin complexes, bismuth macrocyclen complexes, bismuth 12-crown-4 complex, bismuth bipyridine complex, bismuth phenanthroline complex, Bi(NTA), Bi(EDTA) and Bi(AHA)<sub>3</sub>. Bismuth porphyrin complex with novel structures were characterized by X-Ray technique. Other bismuth and antimony compounds have also been synthesized for comparison and their activities against SARS helicase were measured. Among these complexes, we found that the two bismuth porphyrin complexes and RBC exhibit the best inhibition activity in vitro experiment. A manuscript was prepared and is ready for submission (*please see the attachment and Fig. 5*).

**Figure 5**



### Discussion

Helicase protein is conservative among virus proteins due to its vital function during viral replication. In DNA viruses such as herpes simplex virus (HSV), anti-helicase inhibitors were found to be significantly potential for the treatment of HSV disease. It is the first time we demonstrate that helicase protein of a RNA virus can be the target for drug application. In addition, we also showed that M protein is another target for BAN. Thus our study clearly revealed the mechanism of BAN function which is important for the future drug development, and also presented new targets for anti-SCV drug design. Our large scale screen and chemical synthesis also provide new lead compounds for antiviral drug development.

### Conclusions

#### (A) Confirmation of helicase as valid drug target

We used reverse genetic method to identify natural targets for SCV inhibitor BAN. We found functional mutations in helicase proteins in BAN resistant strains. The mutant protein expression in FRhK-4 cells can rescue wild type SCV replication in the presence of BAN

#### (B) Screen for more SCV helicase inhibitors

We have identified 108 compounds that can inhibit SCV growth, seven compounds exhibited inhibitory activity of the helicase.

#### (C) Synthesis of bismuth complexes against SCV

Bismuth complexes showed promising activities against SCV helicase with IC<sub>50</sub> lower than micromole, and importantly, also with extremely low toxicities towards normal cells. It is of interest to further develop this group of complexes.

### **Implications**

The successful completion of this project provides several lead compounds that can target the helicase and inhibit SCV growth in cell culture system. Optimization and further testing of these compounds in animal models is therefore justified. These compounds also have the potentials to be used against other viruses.

### **Dissemination**

The results have been released to the public via publication in peer-reviewed journals, scientific meetings and news papers. Our results are widely publicized by newspapers including Mingpao, South China Morning Post and Web-based media.

#### **Scientific meeting and seminars given:**

1. **Huang, J.D.**, Unraveling the SARS helicase – from function to therapy. (February 23-24, 2004, Tokyo, Japan) The international workshop on SARS. (Invited speaker)
2. Sun, H., **Huang, J.D.**, and Zheng, B., (April 2-5, 2004, Hong Kong, China), Bismuth as an Enzyme Inhibitor: From Antibacterium to Antivirus, *Abstract of The 8th International Symposium on Applied Bioinorganic Chemistry*. IL15.
3. Tanner, J. A., Watt, R. M., Kao, R. Y. and **Huang, J. D.** (April 2-6, 2005, San Diego, California, USA), Targeting the SARS Coronavirus Helicase - Three Approaches to Inhibitor Development. FASEB J 19, Abstract #217.9. Experimental Biology Meeting 2005
4. SARS病毒解旋酶的化学生物学研究 (2004年10月13日), 北京大学深圳研究生院, 深圳
5. Chemical biology studies of SARS coronavirus helicase (May 4, 2005), Florida International University, Miami, Florida, USA
6. Chemical biology studies of SARS coronavirus helicase (February 24, 2006), Institute of Microbiology and Epidemiology, The Academy of Military Medical Science, China
7. Yang, N., Tanner, J.A., Huang J.D., Zheng B.J., Sun, H.Z. Inhibition of SARS Coronavirus by Bismuth Compounds. (October 31-November 3, 2006, Nanjing, China), *The 3<sup>rd</sup> Asian Biological Inorganic Chemistry Conference (AsBIC-3)*.
8. Sun, H.Z., Yang, N, Ge, R.G., Huang J.D., Interactions of bismuth with proteins and enzymes: insight into its mechanism of action (August 13-18, 2006, Cape Town, South Africa), *37<sup>th</sup> International Conference on Coordination Chemistry (ICCCV-37)* (Keynote speaker).

## **Publications**

1. Kao, R. Y., Tsui, W.H.W., Lee, T.S.W., Tanner, J.A., Watt, R.M., **Huang, J.D.**, Hu, L., Chen, G., Chen, Z., Zhang, L., He, T., Chan, K.H., Tse, H., To, A.P.C., Ng, L.W.Y., Wong, B.C.W., Tsoi, H.W., Yang, D., Ho, D.D., and Yuen, K. Y. (September, 2004). Identification of Novel Small-Molecule Inhibitors of Severe Acute Respiratory Syndrome-Associated Coronavirus by Chemical Genetics. [Chemistry & Biology](#), V11 (9): 1293-1299. (IF=5.725, (2004))
2. Tanner, J.A., Zheng, B.J., Zhou, J., Watt, R.M., Jiang, J.Q., Wong, K. L., Lin, Y. P., Lu, L. Y., He, M.L., Kung, H.F., Kesel, A. J., and **Huang, J.D.** (March, 2005). The Adamantane-Derived Bananins are Potent Inhibitors of the Helicase Activities and Replication of SARS Coronavirus, [Chemistry & Biology](#), V12: 303-311 (IF=5.725, (2004))
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## List of Research Workers

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## Appendices

Reprints of papers resulted from the project

1. Kao, R.Y., Tsui, W.H.W., Lee, T.S.W., Tanner, J.A., Watt, R.M., **Huang, J.D.**, Hu, L., Chen, G., Chen, Z., Zhang, L., He, T., Chan, K.H., Tse, H., To, A.P.C., Ng, L.W.Y., Wong, B.C.W., Tsoi, H.W., Yang, D., Ho, D.D., and Yuen, K.Y. (September, 2004). Identification of Novel Small-Molecule Inhibitors of Severe Acute Respiratory Syndrome-Associated Coronavirus by Chemical Genetics. [Chemistry & Biology](#), V11 (9): 1293-1299.
2. Tanner, J.A., Zheng, B.J., Zhou, J., Watt, R.M., Jiang, J.Q., Wong, K. L., Lin, Y. P., Lu, L. Y., He, M.L., Kung, H.F., Kesel, A. J., and **Huang, J.D.** (March, 2005). The Adamantane-Derived Bananins are Potent Inhibitors of the Helicase Activities and Replication of SARS Coronavirus, [Chemistry & Biology](#), V12: 303-311

## Standard Format for Final Reports

1. Version: Microsoft Word
2. Maximum of 5000 words
3. Title Page (see example above)
4. Layout of report
  - a. Page size - A4
  - b. Line Spacing – 1.5 spaces
  - c. Case - Sentence Case
  - d. Single Column
5. Margin
  - a. Top: 2.54 cm
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6. Layout of the Executive Summary
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  - b. Font Type - Times New Roman 12 pt
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7. Layout of Text
  - a. Ragged right margin
  - b. Font type
    - Heading 1 - Times New Roman 12 pt, **bold**, 1 line space before and after
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    - Heading 3 - Times New Roman 12 pt, *italic*, no space before or after
    - References - superscript all reference numbers
8. Layout of Tables
  - a. Font Type: Arial 10 pt
  - b. Title Table x and wording (Table 1 Causes of perinatal death ...)
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